

# The effect of solid-state forms on the topical delivery of roxithromycin

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*Success is no accident. It is hard work, perseverance, learning, studying, sacrifice and most of all, love for what you are doing – Pele*

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The skin is a very accessible and convenient route of administration for topical and systemic drugs (Williams, 2003:1). The only problem most formulators face is overcoming the barrier function of the stratum corneum, which has proved to be quite a challenge (Varun *et al.*, 2012:632). This being said, the topical/transdermal route still holds many advantages over other routes of administration, with the most obvious being no first-pass effect from the liver and being a non-invasive, painless route of administration (Washington *et al.*, 2001:187). The skin itself is affected by many diseases and one of the most common, from which a large number of the population suffers, is acne (Bershad, 2001:279). Acne vulgaris is a chronic inflammatory disease which affects the pilosebaceous units found in the dermis layer of the skin and the micro-organism which accumulates in these sebaceous glands and causes the inflammation, is known as *Propionibacterium acnes*. Topical antibiotics have a direct effect against *P. acnes* found in the sebum glands and in this way reduce the acne inflammation (Williams *et al.*, 2012:361, 364). The antibiotics used today for the treatment of acne have been reported to be up to 60% resistant to the acne causing bacteria (*P. acnes*) (Scheinfeld *et al.*, 2003:43). In the recent past, trials have been conducted on newer antibiotics for acne treatment, one in particular is roxithromycin (Oschendorf, 2006:830).

Roxithromycin is a macrolide antibiotic which has a bacteriostatic effect on *P. acnes* which accumulates in the dermis, but its poor solubility has been a major drawback for topical drug formulation (Gollnick, 2003:1585; Medsafe, 2014). For optimal skin penetration, a compound must preferably have an aqueous solubility above 1 mg/ml (Williams, 2003:37) and roxithromycin was reported to have a solubility of only 0.0335 mg/ml at 25 °C, which is below the optimal solubility for topical penetration (Aucamp *et al.*, 2013:26; Williams, 2003:37). It has previously been proved that by using amorphous forms of a compound, along with its changed crystal lattice, can result in improved drug properties including increased solubility (Biradar *et al.*, 2006:22; Purohit & Venugopalan, 2009:883). Patents from Liebenberg *et al.* (2013) and Liebenberg & Aucamp (2013) proved the glassy amorphous form of roxithromycin and the chloroform desolvated amorphous form had improved solubilities in comparison to the crystalline monohydrate form.

Another area of research that has shown much growth is that of vesicle carrier systems, which have the ability to improve therapeutic activity of drugs by increasing the topical delivery of especially poorly soluble drugs such as roxithromycin (Bansal *et al.*, 2012:704). Niosomes are used as an alternative to liposomes in current years as it overcomes the chemical instability, high cost and lack of purity of phospholipids (Jadon *et al.*, 2009:1186). Niosomes are liposomes which are prepared using non-ionic surfactants instead of phospholipids and

ufosomes are liposomes made from fatty acids (Bansal *et al.*, 2012:710; Williams, 2003:128-129). Provesicular systems, such as proniosomes and pro-ufosomes, are prepared in order to overcome the stability problems that vesicular carriers face (Bansal *et al.*, 2012:706, 709).

The aim of this study was to determine if the two amorphous forms of roxithromycin, namely the glassy form and the chloroform desolvate, coupled with better solubility would have better topical diffusion. These three solid-state forms were each encapsulated into four chosen vesicle systems namely, niosomes, proniosomes, ufosomes and pro-ufosomes and the delivery of the two amorphous forms were compared to that of the crystalline monohydrate form to determine if an increase in topical delivery took place. The target area for the active pharmaceutical ingredient (API) was the dermis, as this is the area where *P. acnes* accumulates (Gollnick, 2003:1585).

The optimisation and characterisation of amorphous forms entrapped in vesicles proved that all carrier systems were well formed and had optimal properties for topical delivery. An accurate and reliable high performance liquid chromatography (HPLC) method of analysis was developed and validated for the analysis of roxithromycin samples during experiments. The release studies showed that the API was successfully released from all carrier systems, with niosomes and proniosomes having superior release over the ufosomes and pro-ufosomes. The reason for this was that the API had higher affinity (and therefore less release) for the ingredients used to make ufosomes and pro-ufosomes (Agarwal *et al.*, 2001:49; Dayan, 2005:74).

The topical diffusion studies showed that there was no API concentration detected in the stratum corneum, which meant the API successfully penetrated the barrier. There was practically no API found in the receptor phase of the Franz cells which indicated that there was no systemic absorption and that the vesicle systems aided in drug targeting. An API concentration was found in the epidermis-dermis of all vesicle systems, which proved the intended target area for roxithromycin was successfully reached. The vesicle systems which assisted in the delivery of roxithromycin and its amorphous forms, from highest to lowest diffused concentration, were niosomes, ufosomes, proniosomes and pro-ufosomes. The total concentration was more dependent on the carrier type than the solid-state form, as there was no obvious leading roxithromycin form. Nevertheless, when the solid-state forms were grouped together, regardless of what carrier systems they were delivered in, the amorphous forms had higher epidermis-dermis concentrations than the roxithromycin monohydrate. This suggests the amorphous forms retained their increased solubilities while entrapped and resulted in improved topical delivery.

**Keywords:** Roxithromycin, Topical delivery, Amorphous, Vesicle systems, Pro-vesicle systems

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Die vel is 'n uiters toeganklike en geskikte toedieningsroete vir topikale en sistemiese geneesmiddels (Williams, 2003:1). 'n Algemene probleem wat deur formuleerders ondervind word en 'n groot uitdaging blyk te wees, is die oorkoming van skansfunksie van die stratum korneum (Varun *et al.*, 2012:632). Ten spyte hiervan, hou die topikale/transdermale toedieningsroete steeds baie voordele bo ander toedieningsroetes in. Die mees voor-die-hand-liggendste voordeel is dat geen eerste-deurgangseffek in die lewer voorkom nie. Voorts is dit ook 'n nie-ingrypende en pynvrye toedieningsroete (Washington *et al.*, 2001:187). Die vel as sulks word deur vele veltoestande aangetas. Een van die mees algemene velaandoenings, waaraan 'n groot getal van die bevolking ly, is aknee (Bershad, 2001:279). Aknee vulgaris is 'n kroniese inflammatoriese toestand. Dit tas die haar-talgklier-kompleks, wat in die dermislaag van die vel voorkom, aan. Die mikro-organisme wat in hierdie talgkliere akkumuleer, veroorsaak inflammasie en dit staan as *Propionibacterium acnes* bekend. Topikale antibiotika het 'n direkte invloed op die *P. acnes* wat in die talgklier voorkom en verminder sodoende aknee inflammasie (Williams *et al.*, 2012:361, 364). Daar is bewys dat hedendaagse antibiotika wat vir akneebehandeling gebruik word tot 60% teen die aknee bakterieë (*P. acnes*) weerstandig is (Scheinfeld *et al.*, 2003:43). Onlangse proewe op nuwe antibiotika vir akneebehandeling, in besonder roksitromisien, is uitgevoer (Oschendorf, 2006:830).

Roksitromisien is 'n makrolied-antibiotika met 'n bakteriostatiese uitwerking op *P. acnes* wat in die dermis akkumuleer. 'n Groot probleem wat ervaar word met die insluiting van roksitromisien in 'n topikale formulering, is die swak oplosbaarheid daarvan (Gollnick, 2003:1585; Medsafe, 2014). Om optimale deurdringbaarheid deur die vel te verkry, moet 'n geneesmiddel verkieslik oor 'n wateroplosbaarheid van meer as 1 mg/ml beskik (Williams, 2003:37). Studies het bewys dat roksitromisien 'n oplosbaarheid van slegs 0.0335 mg/ml by 25 °C het; dit is dus laer as die optimale oplosbaarheid vir topikale deurdringbaarheid (Aucamp *et al.*, 2013:26; Williams, 2003:37). Vroeëre studies het bewys dat die gebruik van amorge vastestofvorme van geneesmiddels, tesame met hul veranderde kristalrooster, tot verbeterde geneesmiddeleienskappe kan lei, insluitend dié van verhoogde oplosbaarheid (Biradar *et al.*, 2006:22; Purohit & Venugopalan, 2009:883). Patente van Liebenberg *et al.* (2013) en Liebenberg & Aucamp (2013) het bewys dat die glasagtige amorge vorm van roksitromisien, sowel as die chloroform gedesolveerde amorge vorm, verbeterde oplosbaarheid in vergelyking met dié van die kristallyne monohidraat vorm toon.

'n Ander navorsingsveld wat aansienlike groei getoon het, is die vesikeldraerstelsels. Hierdie vesikeldraerstelsels beskik oor die vermoë, om die terapeutiese aktiwiteit van geneesmiddels te verbeter deur die topikale aflewering van swak wateroplosbare geneesmiddels, soos

roksitromisien, te verhoog (Bansal *et al.*, 2012:704). Niosome word tans as alternatief vir liposome gebruik, aangesien dit die chemiese onstabiliteit, hoë formuleringskoste en gebrek aan die suiwerheid van fosfolipiede, te bowe kan kom (Jadon *et al.*, 2009:1186). Niosome is liposome wat geformuleer word deur gebruik te maak van nie-ioniese oppervlakaktiewe stowwe in plaas van fosfolipiede. Ufosome is liposome wat geformuleer word deur gebruik te maak van vetsure (Bansal *et al.*, 2012:710; Williams, 2003:128-129). Pro-vesikulêre stelsels, soos proniosome en pro-ufosome, word gebruik om moontlike stabiliteitsprobleme met vesikeldraerstelsels te voorkom (Bansal *et al.*, 2012:706, 709).

Die doel van hierdie studie was, om te bepaal of die twee amorfe vorme van roksitromisien, naamlik die glasagtige vorm en die chloroform gedesolweerde vorm, gekoppel met verbeterde oplosbaarheid, effektiewer topikale diffusie tot gevolg sal hê. Hierdie drie vastetoestandvorme is elk geënkapsuleer in vier verkose vesikeldraerstelsels, naamlik niosome, proniosome, ufosome en pro-ufosome. Die aflewering van die twee amorfe vorme was vergelyk met dié van die kristallyne monohidraat vorm, om te bepaal of verhoogde topikale aflewering plaasgevind het. Die teikengebied vir die geneesmiddel was die dermis, aangesien *P. acnes* in hierdie area akkumuleer (Gollnick, 2003:585).

Die optimalisering en karakterisering van amorfe vorme, wat in die vesikels geënkapsuleer was, het bewys dat alle draerstelsels goed gevorm het, en dat die vesikels oor die optimale eienskappe vir topikale aflewering beskik. 'n Akkurate en betroubare hoë drukvloeistof-chromatografie (HDVK) metode, om roksitromisienmonsters gedurende eksperimente te analiseer, is ontwikkel en gevalideer. Die vrystellingstudies het getoon dat die geneesmiddel suksesvol vanuit al die draerstelsels vrygestel was. Niosome en proniosome het egter hoër vrystelling getoon as die ufosome en pro-ufosome. Die rede hiervoor is dat die geneesmiddel 'n hoër affiniteit (en dus verminderde vrystelling) het, ten opsigte van die bestanddele wat gebruik is om ufosome en pro-ufosome te formuleer (Agarwal *et al.*, 2001:49; Dayan, 2005:74).

Die topikale diffusiestudies het aangetoon dat daar geen geneesmiddelkonsentrasie in die stratum korneum te vinde was nie, wat dus beteken dat die geneesmiddel die skans suksesvol deurgedring het. Daar was feitlik geen geneesmiddel in die reseptorfase van die Franz selle gevind nie, wat 'n indikasie is dat daar geen sistemiese absorpsie was nie en dat die vesikeldraerstelsels meegehelp het om die geneesmiddel in die teikenarea af te lewer. Geneesmiddelkonsentrasies vir al die vesikelstelsels, was in die epidermis-dermis te bespeur, wat 'n aanduiding is dat roksitromisien die beoogde teikenarea suksesvol bereik het. Die vesikelstelsel wat die hoogste diffusiekonsentrasie roksitromisien en sy amorfe vorme afgelewer het, was die niosome, gevolg deur die ufosome, proniosome en laastens die pro-ufosome. Die totale afgelewerde geneesmiddelkonsentrasies was meer afhanklik van die tipe draerstelsel as die vastetoestand vorm, aangesien geen roksitromisien vorm bo die ander uitgestaan het nie.

Desnieteenstaande het dit geblyk dat wanneer die vastetoestand vorme saam gegroepeer word, ondanks die tipe draerstelsel wat vir aflewering gebruik was, die amorfe vorme in vergelyking met roksitromisien monohidraat, hoër epidermis-dermis konsentrasies tot gevolg gehad het. Hierdie is dus 'n aanduiding dat die amorfe vorme hul verhoogde oplosbaarheid ten tye van hulle enkapsulasie behou het, wat tot verbeterde topikale geneesmiddelaflewering gelei het.

**Slutelwoorde:** Roksitromisien, Topikale toedieningsroete, Amorfe, Vesikeldraerstelsels, Pro-vesikulêre stelsels

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Equation 3.1

## CHAPTER 5

Log D = concentration in *n*-octanol / concentration in PBS (pH 7)

Equation 1

$$EE\% = C_t - C_f / C_t \times 100$$

Equation 2

## ANNEXURE A

$$y = mx + c$$

Equation A.1

## ANNEXURE B

$$EE\% = C_t - C_f / C_t \times 100$$

Equation B.1

## ANNEXURE D

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

Equation D.1

The skin is a very large, convenient and accessible site for active pharmaceutical ingredient (API) administration (Williams, 2003:1), which shows great potential for future topical delivery systems. The only drawback of topical/transdermal delivery is surpassing the great limiting barrier of the skin, the stratum corneum, which proves to be problematic for the absorption of many drugs (Foldvari, 2000:418). Roxithromycin is a macrolide antibiotic which has an effect against *Propionibacterium acnes*. This organism is found in the pilosebaceous glands in the dermis layer of the skin and is known as the organism responsible for inflammatory acne (Gollnick, 2003:1585; Menon, 2002:4). Roxithromycin has the potential to be used in a topical formulation, but its poor solubility of 0.0335 mg/ml in water at 25 °C is below the solubility for optimal topical penetration (1 mg/ml), which serves as a huge drawback (Aucamp *et al.*, 2013:26, Medsafe, 2014, Williams, 2003:37). Amorphous solid-state forms have different crystal lattices and as a result, their properties, such as solubility, also differ (Grant, 1999:1-33). This increased solubility, using amorphous forms of roxithromycin, was proved with experiments performed by Liebenberg & Aucamp (2013) and Liebenberg *et al.* (2013). Vesicles are also an innovative delivery technique, which is known to increase topical delivery of APIs as well as prevent systemic absorption of them (Varun *et al.*, 2012:632). They have specifically been used in the past for improving the topical and/or transdermal delivery of poorly soluble drugs (Bansal *et al.*, 2012:704).

The research problem of this study involved determining if the amorphous forms (with improved solubility) increased the API diffusion through the skin. In order to investigate this, three different solid-state forms of the API were used. The first form was the 'glassy' amorphous form of roxithromycin, the second form was the chloroform desolvated amorphous form of roxithromycin and the third was the crystalline monohydrate (raw material commercially available) form of roxithromycin.

The aim of this study was to compare the topical and/or transdermal delivery of crystalline roxithromycin with the two amorphous solid-state forms. The three forms were encapsulated into four different vesicle systems, namely, niosomes, ufosomes, proniosomes and pro-ufosomes. The topical delivery of the two amorphous forms encapsulated in the vesicles were compared to the crystalline roxithromycin encapsulated in the vesicles in order to conclude if any improvement in skin permeation existed. The intended target area for the API was the epidermis-dermis.

The distribution and penetration of the three different forms of roxithromycin in four various encapsulations were compared by means of membrane release studies and transdermal diffusion studies.

The objectives of the study include the following:

- Development and validation of a high performance liquid chromatography (HPLC) method in order to quantitatively determine the concentration of roxithromycin in each vesicle system.
- Preparation of the glassy form of roxithromycin and the chloroform desolvated amorphous form of roxithromycin.
- Determination of the aqueous solubility and the octanol-buffer distribution coefficient (log D) of roxithromycin and its two amorphous forms.
- Optimisation of the vesicle formulations and encapsulation of the three solid-state forms into the four optimised formulas, namely, niosomes, proniosomes, ufosomes and pro-ufosomes to prepare twelve systems.
- Characterisation of the vesicle systems in terms of droplet size and distribution, zeta-potential, pH, entrapment efficiency percentage (EE%), transmission electron microscopy (TEM) and light microscopy.
- Performing membrane studies to determine if roxithromycin and its two amorphous forms were released from the different vesicle systems.
- Performing transdermal diffusion studies followed by tape stripping to determine whether roxithromycin and its two amorphous forms were delivered systemically and/or topically, respectively.

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# **Article for publishing in Archives of Dermatological Research**

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## *Chapter 2*

Chapter 2 is written in a review article format for the purpose of publishing in the Archives of Dermatological Research journal. The master's student contributed to the article by providing researched information and figures, and also assisted in the writing process. The article was written in US English. The complete author guidelines for the publishing of articles in this journal is found in Annexure E.

## TREATMENT MODALITIES FOR ACNE

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## TREATMENT MODALITIES FOR ACNE

### ABSTRACT

Acne is a common inflammatory skin disease which affects the pilosebaceous units of the skin. It can have severe psychological effects and can leave the patient with severe skin scarring. There are four well-recognized pathological factors responsible for acne which is also the target for acne therapy. In this review different treatment options were discussed. This included topical (i.e. retinoids, antibiotics) and systemic (i.e. retinoids, antibiotics, hormonal) treatments. Since the general public has been showing an increasing interest in more natural and generally safer treatment options, the use of complementary and alternative medicines (CAM) for treating acne was also discussed. The use of physical therapies such as comedone extraction, cryoslush therapy, cryotherapy, electrocauterization, intralesional corticosteroids and optical treatments were also mentioned. Acne has been extensively researched with regards to the disease mechanism as well as treatment options. However; due to the increasing resistance of *Propionibacterium acnes* towards the available antibiotics there is a need for new treatment methods. Additionally the lack of necessary evidence on the efficacy of CAM therapies makes it necessary for researchers to investigate these treatment options further.

### Keywords

*Acne vulgaris*, Acne treatment, Topical, Systemic, Physical therapies, Natural

### 1 Introduction

*Acne vulgaris* is a common chronic inflammatory disease of the skin. It is found in about 80% of young adults and adolescents. It is a disease that affects the pilosebaceous units of the skin and may result in inflammatory or non-inflammatory lesions [6, 16, 50]. Strauss *et al.* [87] defined acne as a chronic inflammatory dermatosis which consists of open comedones (blackheads), closed comedones (whiteheads) and inflammatory lesions such as nodules, pustules and papules. Thiboutot *et al.* [88] suggested that acne should be recognized as a chronic disease which may also affect the patient psychologically.

In recent years acne has been observed in younger patients due to the earlier onset of puberty [52]. Adebamowo *et al.* [1] stated that acne is more common in girls in the age range of 12 years and younger, but it presents more in boys in the age range of 15 years or older. In most cases, acne disappears within the patient's early twenties; however, acne may persist into adulthood which usually occurs more often in females [6].

Acne has many negative effects on young adolescents. It causes discomfort, emotional stress, disfigurement and even permanent scarring to the skin. It may also cause anxiety and

embarrassment in patients and may diminish the patient's physiological and social wellbeing [4, 27]. Several factors may induce acne production or increase its severity. Some of these factors include genetics, the male sex, youth, stress and smoking as well as comedogenic medications such as androgens, halogens, corticosteroids and pore clogging cosmetics. Past research suggests that genetic influence combined with comedogenic hormones (especially androgens) produce abnormal volumes of sebum which contribute to acne lesions [6, 50, 67].

At present there is a widespread interest in the relationship between diet and *Acne vulgaris* [43]. This relationship will, however, not be discussed in the current article as there is a great deal of information available and forms a subject on its own. Diagnosing acne is simple and straightforward. Differential diagnosis that exists is rosacea (lacks comedones), folliculitis, dermatitis and drug-induced eruptions [67].

## **2 Pathogenesis of acne**

Acne affects the pilosebaceous units of the skin which presents with a variety of lesions at various inflammatory stages, including acne scars and hyperpigmentation [67,101]. According to Olutunmbi *et al.* [67], acne lesions are most commonly present on the face, chest, upper back and upper arms which is known to have a high density of sebaceous glands [52].

The four main pathological factors involved in the development of acne are the increased sebum production, irregular follicular desquamation, *Propionibacterium acnes* proliferation and inflammation of area [34]. These four factors are illustrated in Figure 1.

### **2.1 Excess sebum production**

Gollnick [33] stated that androgen hormones (especially testosterone) stimulate increased production and secretion of sebum. Increased sebum production directly correlates with the severity and occurrence of acne lesions and for this reason it is an important factor that should be taken into consideration when dealing with patients suffering from *Acne vulgaris* [33, 103].

### **2.2 Epidermal hyper-proliferation and formation of comedones**

The keratinocytes in normal follicles are usually shed to the lumen as single cells which are then excreted. In patients with acne, hyper-proliferation of the keratinocytes occur and they are not shed as they should be, which results in the gathering of the abnormal desquamated corneocytes in the sebaceous follicle along with other lipids and monofilaments. This phenomenon results in comedogenesis [34].

Webster [99] refers to a microcomedone as the first microscopic lesion that forms from occlusion of the follicle, and it is the precursor of the other acnes lesions. The microcomedone

gradually fills up with more lipids and monofilaments and develops into visible non-inflammatory comedones and inflammatory acne lesions [33, 34, 38]. Comedones are referred to as blackheads (open comedones) when they are dilated at the skin surface. They appear blackish on the skin and are filled with sebum and desquamated keratinocytes. They can also be termed as whiteheads (closed comedones) which appear as a white bump underneath the skin surface with no open pores. If sebum continues to accumulate, the closed comedone will continue to expand and may rupture into the surrounding tissue [33]. Figure 2 indicates the different lesions that originate from microcomedones.

### **2.3 *Propionibacterium acnes* infiltration**

The microflora present in a normal sebaceous follicle is qualitatively similar to that found in comedones. This includes three coexisting groups of bacteria, namely (1) coagulase-negative staphylococci (*Staphylococcus epidermidis*), (2) anaerobic diphtheroids (*P. acnes* and *Propionibacterium granulosum*) and (3) lipophilic yeasts (*Pityrosporum* species) [10].

*P. acnes* and *S. epidermidis* differ in their potential to provoke local skin inflammation and to generate pro-inflammatory mediators. It was however determined that *S. epidermidis* is not likely to partake in the pathogenesis of inflammatory *Acne vulgaris* skin lesions as the antibody response to *S. epidermidis* was somewhat harmless compared to the antibodies generated by *P. acnes* [8]. As *S. epidermidis* is an aerobe organism and their growth site is superficial, it is incapable of residing in the anaerobe environment of the infra-infundibulum where the inflammatory process occurs [10]. The lipophilic yeasts present in the pilosebaceous unit do not seem to play a noteworthy aetiological part in any disease conditions [10].

*P. acnes* is an anaerobic, gram positive pathogen that colonizes in sebaceous follicles. It is generally more prevalent in areas of the skin that are densely packed with sebaceous follicles because these follicles produce large volumes of sebum that provide a lipid-rich, anaerobic environment that is optimal for *P. acnes* [33]. It is evident that all individuals have *P. acnes* present on the surface of the skin which can contribute to follicular clogging, but not all individuals present with acne due to the differences in individual immune response to the pathogen [100]. According to McInturff & Kim [63], *P. acnes* produces a lipase enzyme that metabolizes the triglycerides of sebum into glycerol and fatty acids, which may in turn assist in the formation of comedones and the inflammation that follows. *P. acnes* appears to be the most probable organism to cause *Acne vulgaris* and is therefore the target of oral and topical antibiotic treatments [10].

## **2.4 Inflammation process**

The inflammatory process begins when *P. acnes* is detected by the immune system. *P. acnes* has a highly inflammatory effect which may trigger the release of chemostatic factors such as lymphocytes, neutrophils and macrophages. These factors may cause follicular damage, rupture and leakage of bacteria, fatty acids and lipids into the surrounding dermis. This process will give rise to inflammatory lesions (pustules, nodules, cysts and papules). Inflammatory lesions are filled with pus and are larger than non-inflammatory lesions [27, 33, 34, 101]. Additionally it was found that neutrophils generate reactive oxygen species (ROS) which partially contributes to acne inflammation by damaging the follicular epithelium. This leads to the expulsion of the follicular content into the dermis which consequently causes various inflammatory processes [3].

## **3 Current treatment of acne**

The main goal of acne treatment is to control and treat existing acne lesions, prevent permanent scarring as far as possible, limit the duration of the disorder and to minimize morbidity. The patient should be informed on the aims involved in preventing new acne lesions while allowing the existing ones to heal. Patients should also be made aware that it may take 3-6 weeks until an improvement can be observed [27, 52, 100].

Individual patient factors must be taken into account when determining a regimen for the treatment of acne. Some of these factors are the current medical condition, disease state, severity of the lesions, endocrine history and the preferred treatment of the patient (oral or topical). Acne may be treated topically or systemically (with oral drugs) as seen in Table 1. Other treatment options include the use of natural products or the use of non-drug treatments, such as for example optical therapy. However; a combination treatment that targets more than one of the mechanisms of acne pathogenesis is often successful. The response of the patient is recorded and the regimen can be adjusted as the clinical condition improves [67].

### **3.1 Topical treatment**

Topical products have the advantage of being applied to the affected area directly; thus decreasing systemic absorption and increasing the exposure of the pilosebaceous units to the treatment. However, a major side effect of topically applied anti-acne products is skin irritation. Preparations for topical application are available as various formulations, including creams, gels, lotions, solutions and washes [9].

Topical therapy is based on the type and severity of acne. Mild acne is often treated with topical retinoids, or a variety of diverse treatments such as azelaic acid, salicylic acid and benzoyl peroxide. Mild to moderate inflammatory acne can be treated with topical anti-

inflammatory agents as well as topical antibiotics [27]. The different topical anti-acne drugs target different pathophysiological factors [36] and a few of the common topical treatments will be discussed below.

### **3.1.1 Retinoids**

Topical retinoids can be used as monotherapy for inflammatory acne, in combination with more severe forms of acne or as a maintenance treatment. They generally control the formation of microcomedones, reduce the formation of lesions and existing comedones, decrease sebum production and normalize desquamation of the epithelium. They target the microcomedones and suppress comedone formation. They may also show anti-inflammatory properties [34].

Gollnick & Krautheim [36] gave the following suggestions on the use of topical retinoids: (1) the use of topical retinoids is vital for maintenance treatment, (2) retinoids can repair the scarring and hyperpigmentation of the skin, (3) these class of drugs should be first choice of treatment for most of the acne types and (4) when combined with topical antimicrobials it is more effective in inflammatory acne. A common side effect during the first few weeks of topical retinoid treatment is a flare up of acne. This should, however, clear as the patient continues with the treatment [106]. Only some of the most common topical retinoids (i.e. tretinoin, adapalene and tazarotene) used in acne treatment will be discussed.

#### *A. Tretinoin*

Tretinoin is a form of vitamin A [4]. It is a standard comedolytic agent used in acne treatment to regularize desquamation of the epithelium, which prevents blockage of pilosebaceous units. It also seems to have anti-inflammatory properties. It has been a topical treatment for acne for over three decades [27, 49].

#### *B. Adapalene*

Adapalene is a synthetic retinoid analogue which is most commonly used as a first line topical retinoid treatment for *Acne vulgaris*. It normalizes the cell differentiation of the follicular epithelium and prevents comedone formation. It also shows anti-inflammatory action on the acne lesions [4, 6, 27].

#### *C. Tazarotene*

Tazarotene is a synthetic acetylenic pro-drug which is converted to tazarotenic acid in keratinocytes [4]. It is one of the newer retinoids used for acne treatment. It affects the keratinocyte differentiation and proliferation in the epithelial tissue and may also show anti-inflammatory properties [33]. It is regarded as a second line treatment after no response was observed after previous tretinoin or adapalene treatment, as it may cause skin irritation in acne patients [27].

#### *D. Other retinoids*

Other retinoids used for topical treatment of acne include isotretinoin, retinoyl  $\beta$ -glucuronide and motretinide [36, 106]. However; according to Zaenglein [106] these topical retinoid formulations are unavailable in the USA, although they are commonly used in the European Union. Of these three retinoids, only isotretinoin is available as a topical formulation in South Africa.

#### **3.1.2 Antibiotics**

Topical antibiotics are generally used for mild to moderate inflammatory acne. They have activity against *P. acnes*, and therefore act on the surface of the skin to reduce the stimulus for inflammation of the lesions [99]. Due to certain side effects and lesser effectiveness of topical chloramphenicol and tetracyclines, these drugs are less frequently used [36]. The most popular topical antibiotics used in acne treatment are erythromycin and clindamycin, but in recent years, the continuous use of these antibiotics has led to the increasing development of resistance against *P. acnes* strains [81].

Therefore, it is recommended that monotherapy with topical antibiotics are used for only a short time period (12 weeks) and that the antibiotics should be combined with benzoyl peroxide, zinc or retinoids to prevent bacterial resistance [36, 52]. The use of oral and topical antibiotics in combination to treat acne should be avoided [52].

#### *A. Erythromycin*

Erythromycin is a macrolide antibiotic that attaches to the 50S ribosomal unit of bacterium and prevents translocation; which is necessary for protein synthesis of the bacteria [4]. It is active against *P. acnes* and reduces the colony on the surface of the skin and within follicles. It has been regarded as a very effective topical antibiotic in acne therapy, but recently it was discovered that erythromycin is up to 60% resistant to *P. acnes* which makes it less desirable. This has led to interest in the future development of other topical antibiotics [49, 75].

#### *B. Clindamycin*

Clindamycin is classified as a lincosamide antibiotic. It is a semi-synthetic derivative of the antimicrobial agent, lincomycin. Clindamycin attaches to the 50S ribosomal subunit and inhibits protein synthesis of the bacteria and as with erythromycin; it also inhibits *P. acnes* on the surface of the skin [4, 49].

#### **3.1.3 Diverse treatments**

Other topical treatments used for acne, such as for example chemical peels; benzoyl peroxide, dapsone, etc. will be discussed in the following section.

### *A. Salicylic acid*

Salicylic acid is known as a keratolytic agent whose mechanism of action is to dissolve the intercellular cement which holds the cells of the epithelium together [4]. It has a minor anti-inflammatory effect, enhances penetration of certain substances and at low concentrations it is fungistatic and bacteriostatic. Salicylic acid is found in a number of over-the-counter products for acne treatment [4, 49].

### *B. Chemical peeling with hydroxy acids*

Chemical peels involve facial resurfacing whereby removal of the epidermis stimulates re-epithelization and skin rejuvenation [47]. Chemical peeling also appears to reduce hyperpigmentation and superficial scarring of the skin [36]. This therapy can be divided into different groups according to its penetration depth and destruction. Alpha-hydroxy acids (i.e. glycolic acid and lactic acid) and beta-hydroxy acids (i.e. salicylic acid) are the most common chemicals used in chemical peels [9, 45, 47]. A much higher concentration of salicylic acid (20-30%) is present in chemical peels than found in daily acne cleansers [47]. Although there exist little evidence/data, peels are relatively safe to use [7]; however, it should be regarded as a complementary treatment rather than a first-line treatment [45].

### *C. Benzoyl peroxide*

Benzoyl peroxide is a topical disinfectant, originally employed as a peeling agent for treating acne [102]. It possesses diverse properties, making it both a comedolytic and an antibacterial agent, with no effect on sebum production. Benzoyl peroxide has proven bactericidal activity against *P. acnes* by releasing free radical oxygen, which degrades the bacterial proteins [4, 49]. Bershad [6] stated that in addition to its successful treatment of inflammatory acne, benzoyl peroxide also decreases the number of comedones on the skin.

Benzoyl peroxide is a golden treatment for mild to moderate acne and, although it can be used as monotherapy for a period of 6-8 weeks, is often combined with topical antibiotics in order to reduce the resistance of the *P. acnes* species and to increase the efficacy of treatment [27, 33, 36]. Gollnick & Krautheim [36] suggested that benzoyl peroxide is best combined with topical retinoids. However, it has been found that all retinoids (except for adapalene), are unstable when combined with benzoyl peroxide and should therefore be applied separately [52]. The main side effects of benzoyl peroxide include burning, dryness, erythema, peeling or stinging [9].

### *D. Azelaic acid*

Azelaic acid is a natural dicarboxylic acid that inhibits protein synthesis of the *P. acnes* species [82, 89]. It is an effective agent because it has bacteriostatic, anti-inflammatory, antioxidant and

anti-keratinizing properties [21]. So far, no bacterial resistance of *P. acnes* exists with azelaic acid [98]. It has also been suggested that when azelaic acid is used in conjunction with clindamycin, benzoyl peroxide or  $\alpha$ -hydroxy acids it will be more effective [36].

#### *E. Sulfur*

In the past, sulfur was frequently used in preparations for acne. This active has, however, become unpopular due to its bad odor [45]. Sulfur is a chemical that has demonstrated to have mild keratolytic and bacteriostatic properties. Sulfur is reduced to hydrogen sulfide inside the keratinocytes which is said to break down keratin in the skin [4]. According to Akhavan & Bershad [4], sulfur also has activity against *P. acnes*.

#### *F. Hydrogen peroxide*

A study by Tung *et al.* [93] have shown that a regimen based on hydrogen peroxide for treating mild-to-moderate acne compared well with a regimen based on benzoyl peroxide in terms of cosmetic acceptability, efficacy and safety.

#### *G. Niacinamide*

Niacinamide is an active amide of vitamin B3 and is composed of niacin (also known as nicotinic acid) and its amide. It may also be referred to as nicotinamide [5, 65]. Its mechanism of action can be explained as the inhibition of sebocyte secretions, resulting in less sebum production which reduces the oiliness of the skin [22, 65]. It also has anti-inflammatory properties which have proved to be beneficial in pustular as well as papular acne [20, 32]. Topical application of a 4% niacinamide has led to drastic improvements to acne all over the world [65].

#### *H. Topical corticosteroids*

Topical corticosteroids can be used in certain conditions, as for example to treat very inflammatory acne. The treatment period should, however, be short [36] and proof of their efficiency should still be determined [45].

#### *I. Triclosan*

Triclosan is an antibacterial agent (antiseptic) which can be used to treat acne [54]. It was determined that bacterial populations did not develop resistance to triclosan under clinical conditions [42]. No adverse effects are anticipated when triclosan containing products are used as recommended [73].

### *J. Sodium sulfacetamide*

This agent belongs to the sulfonamide antibacterial group. It is bacteriostatic by inhibiting deoxyribonucleic acid (DNA)-synthesis through competitive antagonism of para-aminobenzoic acid (PABA). Sodium sulfacetamide has activity against a number of gram-positive and gram-negative agents, but is generally only used when other topical agents cannot be tolerated by patients [4, 27].

### *K. Dapsone*

Dapsone possesses antibacterial and anti-inflammatory activity, although its precise mechanism of action against acne is still unknown [14, 45]. Dapsone gel (5%) can be used to reduce inflammatory as well as non-inflammatory acne lesions [84]. This agent's lower cost makes it more favorable for use in developing countries [45]; however, it is not recommended as first-line therapy [14].

## **3.2 Systemic treatment**

Oral systemic treatment is required when acne is resistant to topical treatment or if it manifests as nodular lesions or leaves scarring. It is the preferred choice in the treatment of inflammatory lesions. Systemic treatment may also be required to prevent social embarrassment and psychological impairment in people suffering from acne. The most common systemic treatment includes isotretinoin, oral antibiotics and hormonal agents [46, 101].

### **3.2.1 Retinoids**

Isotretinoin is a systemic retinoid and derivative of vitamin A. It is currently being used as a first line treatment for severe nodular or inflammatory acne and is the only known medication which has the potential for the suppression of acne in the long term [27, 46]. It can also benefit patients with mild to moderate cases of acne that have proved resistant to topical or other oral agents in the past. It is also considered as a first line treatment in severe acne of the face and trunk, acne that causes scarring and acne that causes psychological complications [46].

Currently, isotretinoin is the only drug available which has an effect on all four pathogenic factors of acne [52, 108]. Isotretinoin causes de-differentiation of the sebaceous gland, decreasing the sebum production which will lead to a change in the ecosystem of the cutaneous bacterial flora, ultimately reducing *P. acnes* colonization in the follicles. It also causes the shedding of the keratinocytes [6, 27, 56, 108]. Isotretinoin treatment is normally over a course of 16-24 weeks [52]. It is necessary to closely monitor patients who use isotretinoin because of its harmful side-effects [101].

### 3.2.2 Antibiotics

Oral antibiotics are generally indicated for moderate to severe inflammatory acne, acne that shows resistance to previous topical treatment or for acne that covers a large surface area of the body [46, 102]. Acne is often treated with oral antibiotics such as macrolides (erythromycin, clindamycin, azithromycin and roxithromycin), fluoroquinolones (levofloxacin), tetracyclines (doxycycline, minocycline and lymecycline) and co-trimoxazole [34, 85, 108].

These antimicrobial agents inhibit the growth of *P. acnes* and the synthesis of inflammatory mediators released from *P. acnes*. The success of the antibiotic treatment is based on the ability of the agent to reach the lipid environment of the pilosebaceous follicles in the dermis, which is the area where *P. acnes* colonize. Tetracyclines are very popular because they are effective and inexpensive. Doxycycline and minocycline are preferred because they cause less gastrointestinal irritation, and they are more lipid soluble, penetrating the pilosebaceous follicle more efficiently [34, 46]. The tetracycline family exhibits both anti-inflammatory as well as antibacterial properties. Additionally, less resistance in *P. acnes* have been reported with the tetracyclines than the macrolides [102].

Not many studies have been performed to determine the efficacy of azithromycin in the treatment of acne, whereas clindamycin (topical) and erythromycin (topical and oral) have been well recognized as acne treatments [102]. Erythromycin and clindamycin have little anti-inflammatory activity and mainly works by reducing the levels of *P. acnes* [102].

Since these antibiotics are used repetitively at low doses for extended periods of time [34] during acne treatment, increasing resistance has developed overtime which has resulted in limited use of these agents [34, 46]. To reduce resistance and improve the efficacy, oral antibiotics should be combined with topical benzoyl peroxide or retinoids. Additionally, the duration of treatment should not exceed 12 weeks when feasible [52]. It has also been suggested that if a patient is a good candidate for treatment with isotretinoin, long-term antibiotic treatment is unfeasible [102].

### 3.2.3 Hormonal

Sebaceous glands are androgen dependent and therefore the effect of androgen on sebaceous glands can be treated with hormone therapy [34]. Hormonal treatment can be used as an alternative for adolescent and adult females. These hormones are most commonly given in the form of oral contraceptive pills. The contraceptive hormones reduce the sebum production that is initially induced by androgen. It increases the synthesis of sex hormone-binding globulin which in turn decreases biologically active free testosterone in the female body. Although all

contraceptives can be used to treat hormone related acne, progestins are usually preferred because they possess no androgen activity [46].

Oral contraceptives can be used alone or in combination with other therapies to treat acne in women [23]. The treatment period of acne with hormonal anti-androgens must be at least for 12 months and oftentimes even longer [108] as the favorable effect of hormonal agents will only be visible after 3-6 months of treatment [52]. Spironolactone is an alternative drug which can also be combined with oral contraceptives in the treatment of hormone related acne. Its mechanism is based on the fact that it is an androgen receptor blocker. It is especially effective for patients with inflammatory acne [6, 46].

### **3.2.4 Diverse treatments**

Other oral treatments that can possibly be used as adjunctive acne therapy include zinc sulfate, ibuprofen (due to its anti-inflammatory effect) and clofazimine [9, 45]. Systemic corticosteroids can be used for initial treatment of inflammatory manifestations (*Acne fulminans*). It can also be used to manage aggravation of acne when treating with systemic isotretinoin [35]. It has been recommended that severe inflammatory *Acne vulgaris*, *Acne fulminans* and *Pyoderma faciale* be treated with oral prednisone (0.5-1.0 mg/kg daily) for a period of 4-6 weeks, after which the dosage can be decreased gradually [45].

### **3.3 Complementary and alternative medicines (CAM)**

More efficient and safer treatment options are needed for the treatment of acne [2]. Numerous CAM therapies have been noted and/or promoted for use as acne treatment [58] and is generally regarded as safe. Botanical therapies have the added benefit of possessing several modes of action due their composition consisting out of a range of possible active components [28]. It has been proposed that CAM therapies influence the androgenicity, increased sebum activity, infection, inflammation and hyperkeratinization associated with acne [58]. However, in most cases evidence for their use is inadequate [58] and one should still be wary of the possible harm and side effects these plant-derived products can lead to [28]. Some researchers are of the opinion that botanicals may lessen antibiotic resistance when used as alternatives to or in combination with antibiotics. This should, however, still be verified with clinical studies [28].

Various articles [28, 51, 58] list all the possible plant/herbal remedies for acne. Some of these ingredients do however have some anti-inflammatory, moisturizing and soothing properties. Therefore, theoretically, these ingredients should be able to help relieve some of the drying effects caused by the more vigorous acne therapies and the erythema associated with inflammatory acne [9]. The absence of clinical data on the efficacy of these complementary remedies is of big concern and needs to be addressed by future research. For the purpose of

this article, only a few of the major CAM therapies will be discussed in detail. There is, however, a strong possibility that the range of CAM therapies being used by acne patients is much larger than the series of treatments mentioned in this review [58].

### **3.3.1 Basil oil**

Advocated topically applied basil essential oils for the treatment of acne include *Ocimum sanctum*, *Ocimum basilicum* and *Ocimum gratissimum* [58]. Since ancient time Thai basil oils such as *O. basilicum* L. (sweet basil) and *O. sanctum* L. (holy basil) have been used as traditional medicine to treat ringworm and insect bites [95].

Studies showed that topical application of a preparation containing *O. gratissimum* oil in a cetomacrogol blend base were more efficient and reduced lesion counts faster than a 10% benzoyl peroxide lotion [68]. A study on Thai basil oils showed that *O. basilicum* and *O. sanctum* showed promise to be used for acne treatment as they exhibited antimicrobial activity against *P. acnes*. The formulations containing *O. basilicum* showed a higher anti-*P. acnes* activity than the *O. sanctum* containing formulation [95].

### **3.3.2 Copaiba oil**

Copaiba oil-resin has traditionally been used as an antiseptic, anti-inflammatory and healing agent. Da Silva *et al.* [15] conducted a double-blind placebo controlled clinical trial in which the copaiba oil was prepared into a topical gel to determine its activity against *Acne vulgaris*. After 21 days of treatment the copaiba oil gel stopped the outbreak of new pustules, healed pre-existent pustules and reduced the area of erythema. The authors [15] concluded that copaiba oil may be used in the treatment of mild acne, although larger studies are necessary to confirm.

### **3.3.3 Green tea**

Green tea possesses anti-inflammatory, anti-oxidant, antimicrobial and antimutagenic properties which can be ascribed to its high content of polyphenols, including catechins (flavan-3-ols). The main catechins found in green tea include epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC) [31], of which EGCG is the most abundant polyphenol found in green tea [107].

Polyphenon-60 from green tea is a mixture of polyphenolic compounds [83]. Topical application of polyphenon-60 in patients with mild-to-moderate acne (*in vivo* testing) decreased the average amount of open-comedos and pustules. However, polyphenon-60 showed no improvement on closed-comedos. *In vitro* studies to determine the underlying mechanism by which polyphenon-60 has this therapeutic effect on acne showed that this compound suppresses the inflammation process [44].

Yoon *et al.* [105] conducted *in vitro* studies in which it was determined that EGCG directly targets three pathological processes of acne as it has sebo-suppressive effects, it inhibits the growth of *P. acnes* and it has anti-inflammatory effects. They also found that EGCG may reverse the modified keratinization of follicular keratinocytes associated with acne. These results were followed by a double-blinded, split-face clinical trial which showed that the mean inflammatory and non-inflammatory lesion counts significantly decreased after eight weeks of treatment with an EGCG solution when compared to the baseline values.

Results obtained from a double-blind, placebo-controlled, randomized clinical trial showed that when green tea extract was given orally it was found to be effective against acne lesions in mild-to-moderate acne cases. Compared to the control, the green tea extract significantly decreased the inflamed and total lesion counts, although no significant effect was observed on the non-inflamed lesion count [29]. A topical 3% green tea emulsion was found to decrease the sebum production of the cheeks of healthy male volunteers over a 60 day period [59].

### **3.3.4 Minerals**

Minerals have been used for healing purposes since prehistoric times. The minerals being used for therapeutic intents are mostly clay minerals such as kaolinite, palygorskite, smectites and talc. Clay minerals can be used to treat acne, blackheads and spots. Generally it is applied as a face mask consisting of a warm mixture of water and clay which will open pilosebaceous orifices, stimulate perspiration and sebaceous secretions [12]. A mix of minerals (consisting primarily of halloysite, sericite and talc) obtained from ores indigenous to Korea showed to inhibit the growth of *S. epidermidis* and *P. acnes* [71].

Dead Sea black mud showed marked antimicrobial action when test microorganisms (*P. acnes*) were added to the mud where after they lost their viability. Additionally, when Dead Sea mud was placed on *P. acnes* inoculated agar plates, a growth inhibition zone was observed [57]. Another mineral commonly used both systemically as well as topically for the treatment of *Acne vulgaris* includes zinc [37] which was mentioned earlier.

### **3.3.5 Antimicrobial peptides**

Natural antimicrobial peptides represent promising therapies for treating acne [84] as they are unlikely to provoke drug resistance in microorganisms [97]. However, some authors have stated that development of peptide-based drug resistance has been proven experimentally, although, when compared to conventional antibiotics it is considered to arise at a much slower pace [94].

A synthetic peptide, derived from epinecidin-1 (from the marine organism *Epinephelus coioides*), have shown bactericidal properties against *P. acnes* by means of destroying its

membrane [69]. In another study, antimicrobial peptides derived from ranid frog skins showed a high potency against *P. acnes* [94].

Wang and co-workers [97] purified a snake cathelicidin-derived antimicrobial peptide, cathelicidin-BF, from the venoms of *Bungarus fasciatus*. When tested *in vitro* it was observed that cathelicidin-BF possessed potential antimicrobial activity against *P. acnes*, comparable to that of the antibiotic, clindamycin. Additionally, this antimicrobial peptide showed some anti-inflammatory effects and inhibited *P. acnes*-induced O<sub>2</sub> production. All these properties suggested the potential use of cathelicidin-BF for treating *Acne vulgaris*.

### **3.3.6 Resveratrol**

Considering the pathophysiology of acne, the ideal drug should be capable of reducing the inflammatory response as well as inhibiting *P. acnes* [18]. As a result resveratrol is emerging as a new approach in treating acne [84] as it possesses anti-proliferative, anti-inflammatory and *P. acnes* inhibiting properties [18, 19, 26, 30].

Resveratrol is a natural phytoalexin which is produced by certain spermatophytes, such as for example grapes [26]. A single-blind, vehicle-controlled pilot study was performed in which resveratrol (*trans*-isomer) was formulated into a gel with a carboxymethylcellulose base. This formulation was applied on the right side of the face of volunteers with inflammatory *Acne vulgaris* in the facial area for 60 days and compared to the left side of the face on which the control (hydrogel vehicle) was applied. All the volunteers had a noteworthy reduction in pustular lesions and inflammation with an overall noticeable clinical improvement on the side of the face treated with resveratrol. The resveratrol-treated side of the face also showed a significant decrease of macrocomedones and microcomedones when compared to the vehicle-treated side of the face. It seemed as though resveratrol inhibited the keratinocyte hyperproliferation process [26].

Resveratrol was found to inhibit *P. acnes* growth when tested *in vitro*. It was bacteriostatic at lower concentrations (50 mg/l and 100 mg/l) and bactericidal at the highest concentration tested (200 mg/l). The inhibiting effect of resveratrol compared well with the activity of frequently used acne treatments benzoyl peroxide and erythromycin [18].

### **3.3.7 Rosa damascena**

Rose water and essential oils are produced from the damask rose plants (*R. damascena* Mill.) in hydro-distillation industries [17]. Rose water can be used for numerous skin problems and due to its pleasing fragrance and beneficial properties, it is a vital ingredient in several cosmetics and body creams [91]. Some authors have suggested the use of *R. damascena* for

the treatment of skin disorders such as acne [91]. Rose oil can be utilized as an astringent to tone and clean the skin [41].

*R. damascena* extract has shown antioxidant activity and inhibits lipid peroxidation, similar to  $\alpha$ -tocopherol [78]. The hydroalcoholic *R. damascena* extract showed analgesic and anti-inflammatory activity, although the oil of this plant failed to show any such activities [39].

An extract of *R. damascena* petals showed antibacterial activity against *Pseudomonas aeruginosa*, *S. epidermidis* and *Bacillus cereus* [91]. A noticeable antimicrobial activity was observed against *P. acnes* in a study by Tsai *et al.*[92]. However, the authors suggested that further studies are required to evaluate the beneficial effect of this extract in *P. acnes* treatment.

### **3.3.8 Seaweed**

A double-blind, vehicle-controlled trial showed that mild acne was significantly improved when treated topically with a complex of seaweed-derived oligosaccharide (*Laminaria digitata* or kelp) and 0.1% zinc pyrrolidone. Even though both the treatments reduced the amount of comedones and papules/pustules on the facial area of the volunteers, the active containing treatment was significantly more effective. The sebum production was also reduced by both treatments, although no significant differences were observed between them. Subsequently, the investigators suggested that the complex works by decreasing comedone formation and inflammation in preference to affecting the sebaceous glands pharmacologically. Some preliminary *in vitro* data indicated that the active suppresses *P. acnes* growth. This in combination with its anti-inflammatory effects may be the reason for the improvement of comedones, papules and pustules in mild acne [11].

Choi *et al.* [13] evaluated the potential antimicrobial activity of 57 seaweed species commonly found around the coast of South Korea. The methanol extracts of three species, i.e. *Ecklonia kurome*, *Ecklonia cava* and *Ishige sinicola*, showed to be the most promising possible therapeutic agents for *Acne vulgaris* due to their strong anti-*P. acnes* and anti-inflammatory activity. At the moderate doses these extracts were investigated, none of them showed to have any severe toxic effects.

### **3.3.9 Taurine bromamine (TauBr)**

Taurine bromamine (TauBr) and taurine chloramine (TauCl) are the main haloamines produced by neutrophils and eosinophils at an inflammation site. Both of these haloamines have shown anti-oxidant and anti-inflammatory properties [60]. A study by Marcinkiewicz *et al.* [61] concluded that TauBr (synthesised) in particular is a promising candidate for treating *Acne vulgaris* topically. At non-cytotoxic concentrations TauBr showed significantly stronger bactericidal activity *in vitro* compared to TauCl. Additionally, TauBr exhibited selective topical

disinfectant properties as *P. acnes* was more susceptible to TauBr than *S. epidermidis* [61]. A double blind pilot study showed that TauBr cream reduced acne lesions of patients with mild to moderate inflammatory facial *Acne vulgaris* in a similar manner than clindamycin gel [60, 62].

### 3.3.10 Tea tree oil

Tea tree oil is obtained from the Australian tree *Melaleuca alternifolia* and has been shown to have some antimicrobial activity [9]. Tea tree oil products are commonly used by patients self-treating their acne. It has been suggested that the antibacterial and anti-inflammatory activity of the oil adds to its promising clinical performance. Numerous studies have shown that tree tea oil products decrease lesion numbers in patients with mild-to-moderate acne [40].

A double-blind placebo-controlled study by Enshaieh *et al.* [25] showed that a topical gel containing 5% tea tree oil was effective in treating mild-to-moderate *Acne vulgaris* when compared to the placebo, i.e. the vehicle gel alone. Both inflammatory as well as non-inflammatory lesions were reduced by this topical gel.

A study by Lee *et al.* [53] showed that tea tree oil isolated from the leaves of the plant and its components (terpinen-4-ol,  $\alpha$ -terpineol, terpinolene and  $\alpha$ -terpinene) exhibited anti-*P. acnes* activity. They also determined that the major active component present in tea tree oil, namely terpinen-4-ol, was mostly responsible for this essential oil's antibacterial activity. However, minor components in tea tree oil also added to its efficiency.

### 3.3.11 Other complementary and alternative medicines

There are numerous other herbal remedies which are commonly used for the treatment of acne, such as amaranth, arnica, asparagus, birch, calendula, celandine, chaste tree, coriander, jojoba oil, labrador tea, neem, orange peel, pine, poplar, rhubarb, soapwort, stinging nettle and turmeric [51].

Yet, the list of CAM therapies goes on as other authors [58] list even more acne therapies, such as topically applied essential oils of *A. millefolium*, bay, benzoin, black cumin, chamomile, *Eucalyptus dives*, geranium, juniper twig, lemon, lemon grass, orange, patchouli, petitgrain, rosemary, safflower oil, sandalwood, sunflower oil, *T. officinale* and thyme. They also list a range of other topical plants/herbs such as: bittersweet nightshade, black walnut, borage, cucumber, duckweed, English walnut, fresh lemon, garlic, grapefruit seeds, oak bark, onion, Oregon grape root, pea, pumpkin, rue, vinegar, vitex and witch hazel. Other ingestible plants/herbs include Brewer's yeast, burdock root, *C. mukul*, *S. flavescens* and *W. somnifera*. Numerous homeopathic, Indian Ayurvedic therapies and Asian topical therapies were also noted to be used in acne treatment [58].

A lotion containing 2% tea effectively cleared papules and pustules in mild to moderate *Acne vulgaris* [80]. A study on Taiwanese herbal extracts revealed that Du Zhong and yerba mate extracts may possibly be used to treat acne due to their anti-inflammatory and antimicrobial activity against *P. acnes* [92]. Standardised pomegranate rind extract has shown bacteriostatic activity against *P. acnes*, *Staphylococcus aureus* and *S. epidermidis*. This extract also showed anti-inflammatory and anti-allergic properties [70].

Another potential anti-acne treatment is the ethanol extract of *Rhodomyrtus tomentosa* (Aiton) Hassk. leaves (also known as rose myrtle) and its principle compound, rhodomyrtone. Both substances showed antibacterial activity against *P. acnes*. However, the rhodomyrtone showed more noteworthy activity closer to that of erythromycin than the extract [74].

Selvan *et al.* [77] stated that due to restricted antibacterial drug options and a shortage of vaccines against *Acne vulgaris*, concomitant therapeutic strategies, such as the use of specific antibodies, are needed. Specific polyclonal chicken egg yolk antibodies against *P. acnes* were developed by Revathy *et al.* [72]. Due to their effectiveness, it was suggested that they can be used in the treatment of acne, although their efficiency should still be confirmed *in vivo*.

A new concept in the treatment of *Acne vulgaris* and other skin diseases were discussed by Wang *et al.* [96]. This involved the development of probiotics against *Acne vulgaris* by harnessing the bacterial interference (through fermentation) between *P. acnes* and *S. epidermidis*. Their results indicated that the fermentation of naturally occurring glycerol in the skin, as mediated by skin microorganisms (mostly *S. epidermis*), improved their *P. acnes* inhibitory properties [96].

### **3.4 Physical treatment**

There are several physical treatments available which can be used as adjunctive acne treatment. Henceforth, these therapies can play a major role in the treatment of acne as the pathogenesis of acne becomes more understood and technology improves [47].

#### **3.4.1 Comedone extraction**

Some authors have suggested that this technique can be used concurrently with isotretinoin treatment to treat macrocomedones (comedones larger than 1 mm). No residual scarring should be left if this technique is performed correctly [104]. This mechanical method of extraction involves the following: the lesion should be prepped with alcohol and the epidermis lightly pierced with a large-bore needle or surgical blade. Thereafter, a comedone extractor is used to apply light to medium pressure on top of the lesion until all the contents are forced out [45, 104]. Prior to the manual removal of the comedone, enzymatic or mechanical exfoliation

can be used to decrease hyperkeratosis. After treatment, the skin should be treated with an anti-inflammatory or antimicrobial agent [86].

### **3.4.2 Cryoslush therapy**

A slush-like mixture consisting of solid carbon dioxide and acetone can be brushed lightly over the infected skin. This will produce desquamation and erythema [45].

### **3.4.3 Cryotherapy**

Cryotherapy involves the regulated and targeted destruction of diseased skin tissue by applying a substance with a very low temperature. Although liquid nitrogen is the most common cryogen used, there are several other cryogens also available, such as carbon dioxide and nitrous oxide. Different techniques can be used to apply the cryogen, including the cryoprobe, dipstick method or the spot freeze technique. Cryotherapy is generally performed without local anaesthesia under aseptic conditions and if performed correctly it should result in extremely good cosmetic results [79].

### **3.4.4 Electrocauterization**

Electrocauterization eradicates comedones by means of generating very low-grade thermal damage [45]. The exact mechanism by which it helps to resolve comedones is, however, unknown [90]. It is thought that electrocauterization works by stimulating the defence mechanism (inflammatory) of the body or it could be that cautery provides a route for the contents of the macrocomedone to be discharged to the skin surface [45, 90].

### **3.4.5 Intralesional corticosteroids**

Intralesional corticosteroid injections reduce the formation of keloid scars and prevent reappearance after surgical removal [55]. This procedure is especially efficient for the treatment of inflammatory nodules. However; it can be painful and possibly cause cutaneous atrophy [66]. The most frequently used corticosteroid is triamcinolone acetonide [55].

### **3.4.6 Optical treatments**

Optical treatments for acne include laser therapy, light sources and photodynamic therapy [84]. Lasers and light-based therapy is commonly used for the treatment of mild to moderate inflammatory *Acne vulgaris*. It has been found that this type of therapy resolves acne faster, is effective, and has fewer side effects, thereby enhancing patient satisfaction. Numerous light sources are available to treat/improve acne by targeting *P. acnes*, including fluorescent lamps, full spectrum light, green light, violet light, blue metal halide lamps and xenon flash lamps.

Another light source is lasers which can target the sebaceous glands (alters structure of glands thermally) or oxyhemoglobin (to improve erythema) [24].

Photodynamic therapy (PDT) functions similar to laser/light-based therapy in that the light energy kills *P. acnes*. Whereas other devices need costly, high-power equipment to produce these lights, PDT makes use of a lower power light source [24]. PDTs efficacy is, however, enhanced by the use of topical agents such as aminolevulinic acid (ALA), methyl-aminolevulinic acid (MAL) or alternative photosensitizing agents [47].

### **3.5 Combination therapy**

Due to the various pathological factors responsible for acne development, the use of multimodal therapy which targets different processes simultaneously has been receiving considerable attention [47, 48]. Combination products have been found to be more effective in treating acne than monotherapy [7]. Additionally, the availability of existing and introduction of new fixed combination treatments can increase patient adherence as the treatment for patients can be more personalized [48].

Fixed topical combinations such as benzoyl peroxide/topical antibiotic or retinoid/topical antibiotic can be used [48]. It has also been recommended to combine oral antibiotics (doxycycline > minocycline, tetracycline) with topical drugs (benzoyl peroxide, azelaic acid, retinoids) to treat moderate to severe acne and less severe inflammatory acne which did not respond to sole topical therapy [35].

By combining systemic treatments in this way, it can bring on a prompt reduction in dose and a sooner discontinuation of oral antibiotics. These combinations can also improve patient compliance, increase the effectiveness of the treatment and decrease the development of bacterial resistance [36, 108]. However; it is important to remember that this last named beneficial outcome is only relevant for the benzoyl peroxide contact areas, and not for the gut and other areas [102].

One study did, however, determine that a combination of clindamycin and benzoyl peroxide performed only slightly better than benzoyl peroxide alone. In this case it is important for prescribers to take into account availability (obtainable over-the-counter versus prescription required), additional costs and risks (i.e. antibiotic resistance) before prescribing these actives simultaneously [76].

Isotretinoin often causes a flare up of acne (known as 'pseudo' acne fulminans) and therefore it can be combined with corticosteroids for the most severe inflammatory acne (i.e. abscesses, cysts and nodules) [45].

Physical removal of microcysts, macrocomedones or closed comedones will enhance the therapeutic efficacy of topically applied comedolytic agents [36]. It has also been suggested that benzoyl peroxide and salicylic acid, which have different mechanisms of action, be combined to treat acne due to their complementary effect when used together [9, 76].

#### **4 Conclusion**

Acne is a common inflammatory skin disease which causes much distress to patients constantly suffering from it. It has been researched extensively with regards to the disease itself as well as available and potential treatment options. The target for acne therapy is the four well-known pathogenic factors responsible for this disease state. This current review discussed the different options for treating acne such as topical therapies, systemic therapies, CAM and physical treatments. However; due to the increasing resistance of *P. acnes* towards the available antibiotics [81] and inter-patient differences, further research in this field will always be required. Furthermore the general public has been opting for more natural products for treating various skin diseases making it necessary to further investigate CAM as possible adjunctive acne therapy.

#### **Conflict of interest**

The authors declare that they have no conflict of interest.

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On Title page, as requested by author guidelines.

#### **Disclaimer**

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## Figure legends

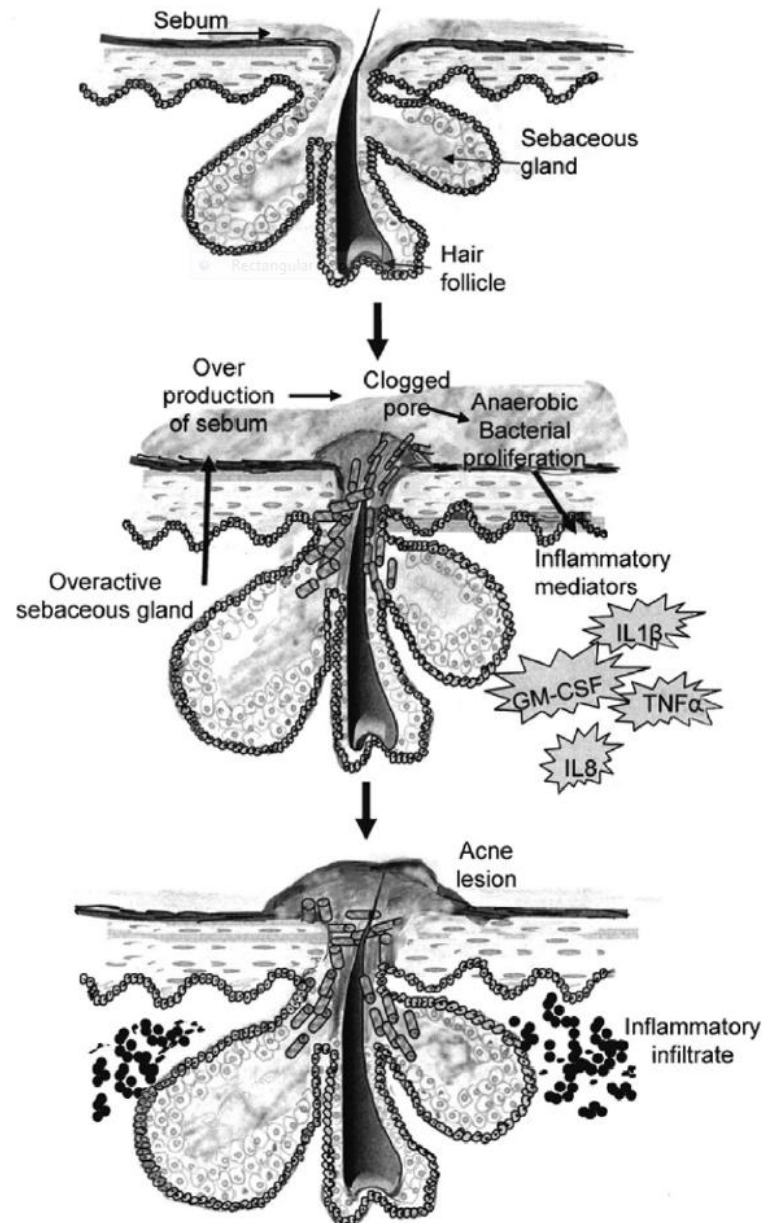
**Figure 1:** Pathogenic factors contributing to the development of acne. (1) The normal pilosebaceous unit. (2) The clogging of the pore is aggravated by hyperkeratinization and excess sebum production whilst anaerobic bacteria (mainly *P. acnes*) proliferate and inflammatory mediators are released. (3) Inflammatory infiltrates cause the development of increasing degrees of severity in inflammatory acne forms (Reprinted from Drug Discovery Today: Disease Mechanisms, 5, Muizzuddin *et al.* [64], Acne – a multifaceted problem, e184-e188, Copyright (2008), with permission from Elsevier.)

**Figure 2:** Various lesion formations originating from microcomedones (Reprinted from Journal of the American Academy of Dermatology, 49, Gollnick *et al.* [34], Management of acne: a Report from a Global Alliance to improve outcomes in acne, S1-S37, Copyright (2003), with permission from Elsevier).

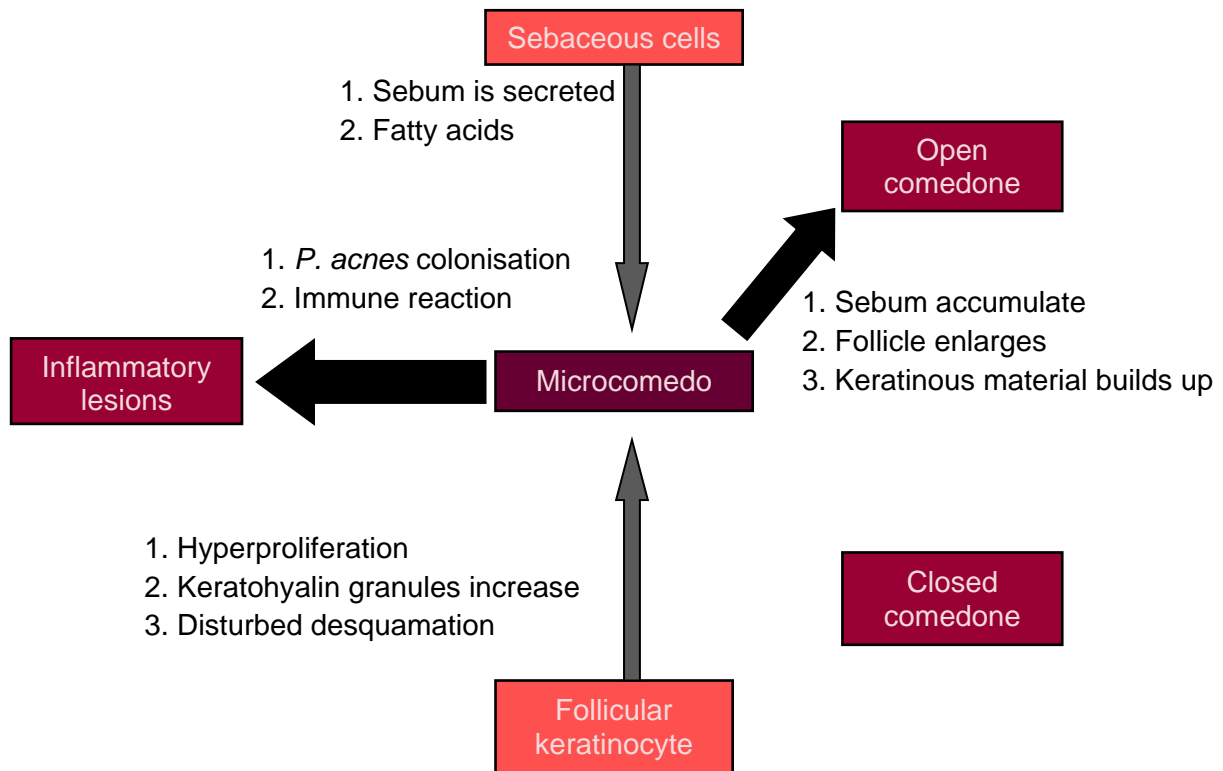
## Table legends

**Table 1:** Different treatment options for acne

## Figures



**Figure 1:** Pathogenic factors contributing to the development of acne. (1) The normal pilosebaceous unit. (2) The clogging of the pore is aggravated by hyperkeratinization and excess sebum production whilst anaerobic bacteria (mainly *P. acnes*) proliferate and inflammatory mediators are released. (3) Inflammatory infiltrates cause the development of increasing degrees of severity in inflammatory acne forms (Reprinted from Drug Discovery Today: Disease Mechanisms, 5, Muizzuddin *et al.* [64], Acne – a multifaceted problem, e184-e188, Copyright (2008), with permission from Elsevier.)



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## Tables

**Table 1:** Different treatment options for acne

| Treatment methods                             | Examples   |
|---|--|
| Topical                                       | <b>Retinoids:</b> adapalene, isotretinoin, motretinide, retinoyl- $\beta$ -glucuronide, tazarotene, tretinoin  |
|   | <b>Antibiotics:</b> clindamycin, erythromycin  |
|   | <b>Diverse:</b> azelaic acid, benzoyl peroxide, chemical peels, corticosteroids, dapson, hydrogen peroxide, niacinamide, salicylic acid, sodium sulfacetamide, sulfur, triclosan   |
| Systemic                                      | <b>Retinoids:</b> isotretinoin   |
|   | <b>Antibiotics:</b> azithromycin, clindamycin, co-trimoxazole, doxycycline, erythromycin, levofloxacin, lymecycline, minocycline, roxithromycin  |
|   | <b>Hormonal:</b> contraceptives  |
|   | <b>Diverse:</b> clofazimine, corticosteroids, ibuprofen, zinc sulfate  |
| Complementary and Alternative Medicines (CAM) | <i>Achillea millefolium</i> , amaranth, antimicrobial peptides, arnica, asparagus, basil oil, bay, benzoin, birch, bittersweet nightshade, black cumin, black walnut, borage, Brewer's yeast, burdock root, calendula, celandine, chamomile, chaste tree, <i>Commiphora mukul</i> , copaiba oil, coriander, cucumber, duckweed, Du Zhong extract, English walnut, <i>Eucalyptus dives</i> , fresh lemon, garlic, geranium, grapefruit seeds, green tea, jojoba oil, juniper twig, labrador tea, lemon grass, lemon, minerals, neem, oak bark, onion, orange peel, orange, Oregon grape root, patchouli, pea, petitgrain, pine, pomegranate rind extract, poplar, probiotics, pumpkin, resveratrol, rose myrtle, rhubarb, <i>Rosa damascena</i> , rosemary, rue, safflower oil, sandalwood, seaweed, soapwort, <i>Sophora flavescens</i> , specific antibodies, stinging nettle, sunflower oil, <i>Taraxacum officinale</i> , taurine bromamine, tea tree oil, thyme, turmeric, vinegar, vitex, witch hazel, <i>Withania somnifera</i> and yerba mate extract |
| Physical treatment                            | Comedone extraction, cryoslush therapy, cryotherapy, electrocauterization, intralesional corticosteroids and optical treatments  |

### 3.1 Introduction

The skin is ideal for the topical delivery of APIs. This being said, researchers still face many problems related to topically delivery, with the stratum corneum being the greatest challenge (Varun *et al.*, 2012:632). A synthetic antibiotic, roxithromycin, has the potential to be added to the list of topical dosage forms used in acne treatment. It has previously been proved that altering the solid state properties of a compound (by preparation of its amorphous form) can manipulate the physicochemical factors and as a result improve certain properties of an API (Liebenberg & Aucamp, 2013; Liebenberg *et al.*, 2013; Threlfall, 1995:2452). Another factor previously explored is that drug carriers (vesicle systems), such as liposomes and niosomes, have considerable penetration advantages when compared to conventional dosage forms applied topically to the skin (Ammar, *et al.*, 2011:142-143). With increased drug delivery into the skin by altering solid-state properties and by using vesicle systems, roxithromycin may be a very effective topical application for the treatment of acne in the future.

### 3.2 Skin and topical drug delivery

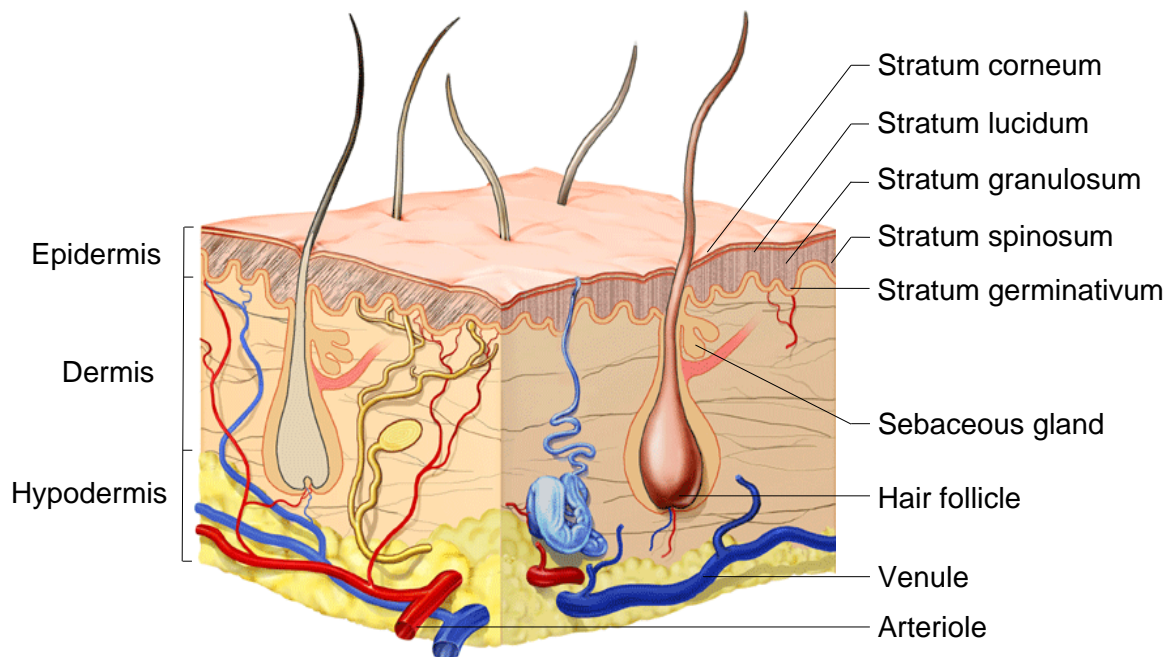
The skin is the largest and most readily accessible organ of the human body, accounting for approximately 16% of total body mass of an adult and spanning an average surface area of 2 m<sup>2</sup> (Washington *et al.*, 2001:182; Wickett & Visscher, 2006:98). This large area of skin offers many convenient sites for administration of topical as well as systemic drugs (Williams, 2003:1).

Transdermal drug delivery presents many advantages over other routes of administration. APIs applied topically avoid hepatic first-pass metabolism. Better patient compliance exists due to non-invasive and painless aspects of the formula which can be applied repeatedly without the complications that exist with oral or parental daily dosing (Washington *et al.*, 2001:187). The major pharmacokinetic advantages include elimination of dose-dumping, sustained API delivery, steady plasma levels and reduction in dosage frequency and systemic toxicity (Thomas & Finnin, 2004:697-698; Washington *et al.*, 2001:187). Although a number of advantages exist, formulators are still challenged to overcome the natural barrier function of the skin for optimal transdermal drug delivery.

The skin forms a unique interface between the human host and the external environment, where its most important function is to act as a barrier and protect our bodies from the outside world (Dayan, 2005:67; Hadgraft, 2004:291). This protection refers to the chemical, physical, immune, pathogen and ultraviolet (UV) radiation defences. Other functions of the skin include

sensory reactions to temperature, pain, pressure and touch, thermoregulatory mechanisms such as insulation, sweating and control of blood flow and it also performs endocrine functions such as vitamin D synthesis and peripheral conversion of prohormones (McLafferty *et al.*, 2012:39-40; Menon, 2002:4).

### 3.2.1 Anatomy and function of skin



**Figure 3.1:** Anatomy of the human skin layers (Adapted from Dermnet NZ, 2008).

When viewed microscopically, the skin is seen as a multi-layered organ consisting of three main layers. The outermost layer is the epidermis, followed by the dermis and the inner hypodermis layer (EL Maghraby *et al.*, 2008:204). The epidermis can further be divided into the outer stratum corneum and the underlying viable-epidermis (Abraham *et al.*, 1995:8). A simplified diagram of the anatomy of the skin with its layers is demonstrated in Figure 3.1.

#### 3.2.1.1 Stratum corneum

The stratum corneum is the outermost layer of the epidermis and is often termed the horny layer because it has similar structures to that of nails and hair (Barry, 1983:6; Franz & Lehman, 2000:16). The stratum corneum consists of dead keratinised cells (corneocytes) surrounded by a lipid matrix. The arrangement of this layer can be described as the “brick and mortar” array, where the corneocytes represent the bricks which are embedded into the mortar (lipid bilayers) (Williams, 2003:9). The corneocytes are continuously being shed and replaced by younger keratinocytes generated from inner layers of the epidermis (Flynn, 2002:189).

The structure of the stratum corneum provides for an excellent rate-limiting barrier which proves problematic in ensuring the API flux through the lipid bilayers (Foldvari, 2000:418). The stratum corneum has high hydrophobic properties and has much lower water permeability than a certain number of other biological membranes (Marjukka Suhonen *et al.*, 1999:150). For this reason, the stratum corneum is selectively permeable and only lipophilic substances, or APIs with a low molecular weight are able to penetrate through it, while polar or hydrophilic molecules are impermeable (Naik *et al.*, 2000:319; Venus *et al.*, 2010). The stratum corneum is the most challenging barrier APIs need to overcome in order to achieve successful topical delivery.

### **3.2.1.2 Viable epidermis**

The viable epidermis is an average of 150 µm thick and consists of three layers from bottom to top, namely, the stratum germinativum (basal layer), the stratum spinosum (spinous layer) and the stratum granulosum (granular layer). A fourth layer, the stratum lucidum, is often mentioned, but it is mostly considered as the lower part of the stratum corneum (Abraham *et al.*, 1995:8; Flynn, 2002:191).

According to Wickett & Visscher (2006:98), the epidermis primarily comprises of keratinocytes, which are responsible for cell division, but also consists of melanocytes for melanin pigment synthesis, Langerhans cells for immunological responses and Merkel cells which contribute to the touch sensation (Williams, 2003:7-8).

The viable epidermis is avascular (lack blood vessels) and has to obtain its nutrients from the highly perfused dermis located beneath it (Potts *et al.*, 1992:14). The main function of the viable epidermal layer is to form the stratum corneum through continuous proliferation and differentiation of epidermal cells (Williams, 2003:1). The keratinocytes (epidermal cells) are in different progressive stages as they migrate slowly through each layer toward the surface of the skin (Marjukka Suhonen *et al.*, 1999:150).

### **3.2.1.3 Dermis**

The dermis layer is situated between the epidermis and the inner hypodermis and consists of collagen needed for skin support as well as elastin for skin flexibility (Barry, 2002:502). The dermis consists mainly of water and is therefore not a barrier to polar drugs during topical delivery (Williams, 2003:2). It is evident then, that the dermis layer will act as a barrier for highly lipophilic substances.

The dermis is a vascular layer consisting of many capillaries which are needed for oxygen, nutrient and toxin delivery of the epidermis and dermis, regulation of temperature and absorption of substances that have passed through the stratum corneum and viable epidermis (Barry, 1983:7; Williams, 2003:2). Menon (2002:4) stated that the dermis also contains sweat

glands, nerve endings, hair follicles and sebaceous glands. Hair follicles are essential for hair growth and an oily substance, known as sebum, is secreted by sebaceous glands into hair follicles to ensure skin moisture and to form a barrier of protection on the skin (Mac Neal, 2006; Williams, 2003:4). It is in these pilosebaceous follicles where increased sebum production and colonisation of *P. acnes* in the sebum contributes to acne formation (Gollnick, 2003:1581, 1585).

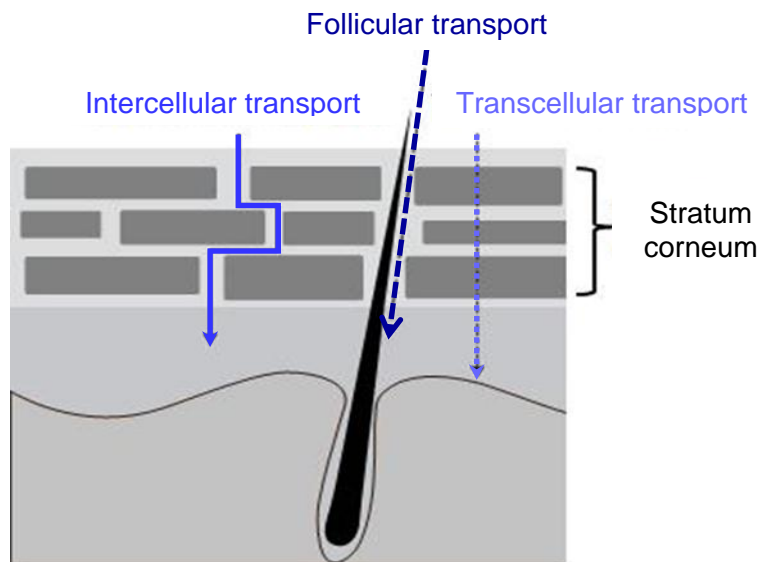
#### **3.2.1.4 Hypodermis**

The deepest layer of the skin adjacent to the body constituents is the hypodermis, also known as the subcutaneous fat layer. It consists of adipocytes (fat cells) which are arranged in lobules and linked to the epidermis by collagen (Franz & Lehman, 2000:16). Williams (2003:2) stated the primary function of the hypodermis is to act as a thermal insulator, to protect the body against physical forces and to hold the skin's principal nerves and blood vessels.

#### **3.2.2 Mechanisms of skin penetration**

The process involved in the permeation of a molecule transdermally includes the partitioning of the API to the stratum corneum from its carrier vehicle, followed by diffusion of the stratum corneum and each skin layer to reach capillaries at the interface of the epidermis and dermis. Finally, the API is transported via the blood stream to the target area for a therapeutic effect to take place (Fang & Leu, 2006). It is important not to confuse the terms permeation and penetration. *Permeation* in transdermal delivery is the passage of a compound through the skin into the bloodstream. Topical *penetration* is complete when the API has reached its targeted skin layer and therefore does not enter the systemic blood circulation (Dayan, 2005:67). The transport mechanism focused on in this study is the topical penetration of the API into the targetted skin layer.

Penetration of APIs may occur via three mechanisms, namely transcellular, intercellular and transappendageal routes, as illustrated in Figure 3.2. According to Williams (2003:35), when choosing a suitable penetration path the physicochemical properties of the API should be taken into consideration. All three routes of penetration are used in combination for the successful delivery of topical APIs (EL Maghraby *et al.*, 2008:205).



**Figure 3.2:** Penetration pathways (Rahimpour & Hamishehkar, 2012:142).

### 3.2.2.1 Intercellular route

The intercellular lipid route is the favourable pathway for small, uncharged molecules across the stratum corneum (Williams, 2003:34). The molecules move through a continuous route within the lipid bilayer between the corneocytes of the stratum corneum (Marjukka Suhonen *et al.*, 1999:151). This pathway is tortuous and about 150 - 500  $\mu\text{m}$  in length which is longer than the transcellular diffusional pathway (Williams, 2003:34).

### 3.2.2.2 Transcellular route

Transcellular transport of an API follows a diffusion pathway directly through the stratum corneum, crossing the corneocytes as well as the lipid matrix (Marjukka Suhonen *et al.*, 1999:151). This provides a swift polar route for hydrophilic molecules due to the aqueous environment provided by cellular components, but the rate-limiting factor is the API penetration through the lipophilic bilayers surrounding the corneocytes in the stratum corneum. For this reason, it would be fitting for hydrophilic compounds crossing the skin to be partly hydrophobic as well, but most APIs do not possess this character. The length of the diffusion pathway is approximately 20  $\mu\text{m}$  which is equal to the thickness of the stratum corneum (Williams, 2003:32-34).

### 3.2.2.3 Transappendageal (follicular) route

The transappendageal pathway is one where substances can penetrate through skin appendages, i.e. sweat glands and hair follicles (EL Maghraby *et al.*, 2008:204). This route provides pores for the API to penetrate through in avoidance of the stratum corneum (Abraham *et al.*, 1995:9). The limitations which exist for this pathway are as follows: the area available for

transappendageal permeation only covers 0.1% of the skins total surface area, sweat hinders the absorption of molecules as they secrete in opposite direction to absorption and lastly, lipophilic sebum in hair follicles will impede absorption of hydrophilic molecules. For these reasons, this route contributes insignificantly to steady state flux, but it may, nonetheless, be of value for large polar molecules and ions that resist penetration through the stratum corneum (Barry, 2001:101; Morrow *et al.*, 2007:38; Williams, 2003:31-32).

### **3.2.3 Physicochemical properties**

Physicochemical properties of APIs have an effect on the success of topical delivery. The optimal properties as well as those of the API, roxithromycin, will be discussed in the following section.

#### **3.2.3.1 Solubility**

Solubility is important in determining topical drug delivery. It is ideal for a drug to possess both aqueous as well as lipid solubility in order to permeate the hydrophilic and the lipophilic domains of the layered skin. Ideally, the aqueous solubility of the drug should be more than 1 mg/ml in order to be successfully delivered as a transdermal formulation with optimal bioavailability (Naik *et al.*, 2000:319; Williams, 2003:37). According to Aucamp *et al.* (2013:26), roxithromycin's experimental equilibrium aqueous solubility is 0.0335 mg/ml at 25 °C, which is below the required solubility for topical delivery.

The effect of solid-state polymorphism on solubility is of vital importance. The solubility of drug compounds may vary due to the different lattice energies of the different solid state forms (polymorphs, amorphs or solvates). The attractive and repulsive forces of the crystal-solvent interface are known to affect the dissolution rate of the compound (Brittain & Grant, 1999:281). According to Liebenberg *et al.*, 2013, the solubility of roxithromycin is increased when preparing the amorphous form of roxithromycin which could, as a result, lead to successful topical skin penetration.

#### **3.2.3.2 Partition coefficient**

The partition coefficient (P) is the redistribution of molecules between an oil and water phase. It indicates the lipophilicity of a drug and its likelihood of being transported across lipophilic stratum corneum barrier and the hydrophilic viable epidermis (Aulton, 2002:243; Farahmand & Maibach, 2009:2). Octanol, an organic solvent with similar properties to that of biological membranes, is frequently used as the lipophilic phase in determining log D of an API. This value is then used to predict the drug distribution between the stratum corneum lipid barrier and water in transdermal delivery (Aulton, 2002:243; Barry, 2002:512).

According to Hadgraft (2004:292), the optimal permeation of an API takes place if the compound has a log P of between 1 and 3, which indicates that it dissolves in both the oil and aqueous phases (Williams, 2003:36). If molecules have a very low log P (hydrophilic) they will not pass through the lipophilic stratum corneum and molecules that have a high log P (lipophilic), will remain in the stratum corneum layer. Both the above instances will result in poor skin permeation (Barry, 2007:578; Thomas & Finnin, 2004:699). Roxithromycin has a log P of 2.9 and an experimental log P value of 1.7 (Drugbank, 2013). Therefore it is eligible for optimal permeation through the stratum corneum as well as the inner layers of the skin.

### 3.2.3.3 Diffusion coefficient (D)

Williams (2003:27,223) defined the diffusion coefficient as the amount of API which diffuses across a membrane, the stratum corneum, in an area per unit time and determines the ease of diffusion through membranes. The diffusion coefficient is influenced by the temperature and viscosity of the vehicle and the API's affinity for the vehicle. The diffusion coefficient increases with an increase in temperature, but decreases with an increased vehicle viscosity. Conversely, an increase in diffusion coefficient also takes place when there is a decrease in the vehicles viscosity (Williams, 2003:18).

Passive diffusion is the mechanism by which APIs are transported across the outermost skin layers (Begoña Delgado-Charro & Guy, 2001:213). Crank (1999:1) states that diffusion is known as the random movement of molecules from an area of high concentration (in the formulation) to a low concentrated region (in the stratum corneum). Passive diffusion across the stratum corneum obeys Fick's first law of diffusion, which states that the rate of diffusion of substances across a unit area of the skin is proportional to the measured concentration gradient (Benson, 2005:24; Williams, 2003:41-42). The diffusion coefficient is one of the variables present in Fick's first law.

Fick's first law of diffusion can be presented as the following equation:

$$J = \frac{K \times D}{h} \Delta C \quad \text{Equation 3.1}$$

Where: J = the API flux across the stratum corneum ( $\mu\text{g}/\text{cm}^2.\text{h}$ )  
K = partition coefficient of the API  
D = diffusion coefficient of the API ( $\text{cm}^2/\text{h}$ )  
 $\Delta C$  = difference in concentration on either side of membrane ( $\mu\text{g}/\text{cm}^2$ )  
h = thickness of the membrane or diffusional path length (cm) (Hillery, 2001:13).

It is evident from Equation 3.1 that along with the diffusion coefficient, there are two other important factors which influence the rate of the API flux during transdermal delivery. As

discussed earlier, the partition coefficient will influence the rate of permeation as will the concentration of the API in the vehicle, which will be discussed later in the chapter (Walters, 1990:86).

#### **3.2.3.4 pK<sub>a</sub> and ionisation**

The skin can tolerate a pH range of 3 - 9 because a pH above or below this range can cause damage to the stratum corneum, which will in turn increase skin permeability. The ideal pH of a saturated aqueous solution is in the range of 5 - 9 (Barry, 1983:159; Barry 2002:513; Naik *et al.*, 2000:319). The pH partition hypothesis suggests that unionised molecules (lipophilic) readily pass through lipid membranes (stratum corneum) and thus determine the concentration gradient of the API, while ionised molecules (hydrophilic) pass through lipid membranes to a very limited extent due to their increased aqueous solubility. An ionised drug can be combined to an oppositely charge molecule to form a neutrally charged ion pair which will improve the aqueous solubility limitation in transdermal delivery (Shargel *et al.*, 2005:382).

Drugs are mostly weak acids, or bases, where their dissociation and degree of ionisation is dependent on the pH of solution or body and on the pK<sub>a</sub> or pK<sub>b</sub> values of the APIs (Barry, 2007:576; Hillery, 2001:23-24). If an API requires a certain degree of ionisation, a common rule can be applied to create a desired degree of ionisation. This is done by selecting a buffer which is 2 - 3 pH units less than the acidic API's pK<sub>a</sub> value, or in the case of a basic API, 2 - 3 pH units above its pK<sub>b</sub> value (Williams, 2003:70). Roxithromycin has a pKa value of 9.08 (Drugbank, 2013). For this study, roxithromycin was calculated as having a percentage ionisation of 99.17%, at the chosen pH value of 7.4 which suggests that the API will have difficulty passing into the skin when applied topically.

#### **3.2.3.5 Molecular size and weight**

It is well documented, by Williams (2003:36), that the size of the molecule plays an important role in determining the API permeation of a compound across the skin. Small molecules diffuse rapidly and easily across the skin, whilst larger molecules diffuse much slower with less efficiency (Hadgraft, 2004:292; Williams, 2003:36). Naik *et al.* (2000:319) stated that the ideal molecular weight for a transdermal API should be less than 500 Dalton (500 g/mol); this will increase the diffusion coefficient of the API (Barry, 2002:513). The molecular weight of roxithromycin is 837.04 g/mol (O'Neil, 2001:1486) and with this being fairly larger than that of the optimal, it may give rise to limitations in transdermal delivery.

The effect of molecular weight size on permeation can only be determined if it is isolated from the drugs solubility properties and this is challenging to do without changing the partition coefficient which plays a vital role in topical absorption (Barry, 2002:513).

Molecules with a diameter of 10  $\mu\text{m}$  or more usually remain on the surface of the skin, while those between 3 - 7  $\mu\text{m}$  penetrate through the follicular duct. Particles that are smaller than 3  $\mu\text{m}$  penetrate the stratum corneum and the follicular ducts (Allec *et al.*, 1997, Barry, 2001a:970). By the production of vesicles in the nanometre particle size range (10 - 1000 nm), the molecules should penetrate the stratum corneum more easily and enhance topical delivery (Escobar-Chávez *et al.*, 2012:217; Kumar & Rajeshwarrao, 2011:209).

### **3.2.3.6 Drug concentration**

As mentioned above in Fick's first law of diffusion, the flux of the drug is proportional to the drug concentration gradient across the skin layers. A steeper diffusion gradient exists with an increased drug concentration on the surface of the stratum corneum of the skin. It is required that the donor solution be fully saturated in order to obtain maximum flux in a thermodynamically stable situation (Barry, 2002:512).

### **3.2.3.7 Melting point**

Organic compounds with low melting points will show better solubility and increased skin permeation than those of high melting points. A direct relationship exists between the melting point of a substance and its solubility, in that the melting point data can be used in the prediction of the drug's solubility (Williams, 2003:37). It is ideal for a drug to have a melting point of less than 200  $^{\circ}\text{C}$  for optimal transdermal absorption (Naik *et al.*, 2000:319). Roxithromycin has a melting point of 116 - 122  $^{\circ}\text{C}$ , which makes it viable for topical penetration (Santa Cruz Biotechnology, 2007).

## **3.3 Roxithromycin**

Roxithromycin is a 14-membered ring, ether oxime derivative of the natural occurring erythromycin A. Roxithromycin is classified as a semi-synthetic macrolide antibiotic (Jarukamjorn *et al.*, 1998:515). It is very similar to other macrolides such as erythromycin, clarithromycin and azithromycin in chemical structure, composition and mechanism of action.

### **3.3.1 Mechanism of action of roxithromycin**

Roxithromycin inhibits bacterial growth by interference with the protein synthesis of bacteria. More specifically, it binds to the 50S ribosomal subunits of the bacterial micro-organisms and inhibits the translocation of peptides, which in turn terminates protein synthesis and growth (Drugbank, 2013; MacDougall & Chambers, 2011). At low concentrations, roxithromycin is bacteriostatic, but at higher concentrations it is bactericidal. Roxithromycin possesses intracellular bactericidal properties because of the higher intracellular concentrations that are achieved as it is concentrated in polymorphonuclear leukocytes and macrophages (Drugbank,

2013; MacDougall & Chambers, 2011; Medsafe, 2014). The resistance mechanism of roxithromycin as well as for the other 14-membered macrolides exists when the ribosomal subunit is altered in the resistant bacteria, which in turn prevents binding to the target site. Cross-resistance between different macrolides can occur (Markham & Faulds, 1994:305-306).

### **3.3.2 Dosage of roxithromycin**

The adult oral dosage of roxithromycin is 300 mg once daily or 150 mg twice daily, 15 min before meals, for a period of 5 - 10 days usually. For children weighing 6 - 40 kg, 5 - 8 mg/kg is given daily. Dosages need to be reduced for patients with renal or hepatic impairment (Martindale, 2014). Currently there are no topical roxithromycin dosage forms on the market.

### **3.3.3 Pharmacokinetics of roxithromycin**

Roxithromycin has a good pharmacokinetic profile when compared to its parent compound erythromycin. Little information is known about the topical application of roxithromycin and its effects as there are no current products of its nature, but there is sufficient information available on the oral dosage forms of roxithromycin. It has high plasma and tissue drug concentrations and it also allows for extended dosage intervals because of its long half-life. Roxithromycin has a half-life of approximately 12 h, with a protein binding of 92 - 96% (mainly  $\alpha$ 1-acid glycoprotein) at a concentration of 4.2 mg/l (Medsafe, 2014). Due to roxithromycin's poor aqueous solubility, it only has an oral bioavailability of 50% (Medsafe, 2014). The peak plasma concentrations are reached within 1 - 2 h and range between 6.61 and 7.9 mg/l, following the dosage of a 150 mg oral film-coated tablet (Markham & Faulds, 1994:298, 307). Thereafter, the mean plasma concentration is 9.3 mg/l and the area under curve (AUC) is 7 mg.h/l at steady state following a 150 mg dose twice a day (Medsafe, 2014).

A single oral dose of 150 mg roxithromycin shows that concentrations of the API are detected in the respiratory system, genital tracts, paranasal sinuses, tonsils, synovial fluid, as well as the skin (Medsafe, 2014).

### **3.3.4 Spectrum of activity and target sites of roxithromycin**

The spectrum of roxithromycin is similar to that of erythromycin, but it proves to be more effective against some gram negative bacteria such as *Legionella pneumophila*. Roxithromycin is used in the treatment of respiratory tract, skin and soft tissue and urinary infections, as well as against opportunistic infections associated with acquired immunodeficiency syndrome (AIDS) patients such as *Mycobacterium avium complex* (Drugbank, 2013; Markham & Faulds, 1994:308). It also has an antibacterial spectrum against gram positive cocci such as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Staphylococcus aureus* (except Methicillin-resistant *Staphylococcus aureus* (MRSA)).

Roxithromycin exhibits activity against gram positive bacilli such as *Listeria monocytogenes* and *Corynebacterium spp.*, gram negative cocci such as *Neisseria meningitides* and gram negative bacilli such as *Bordetella pertussis* and *Moraxella catarrhalis*. Roxithromycin also shows activity against *Ureaplasma urealyticum*, *Chlamydia trachomatis*, *Helicobacter pylori*, *Gardnerella vaginalis*, *Haemophilus ducreyi*, *Mycoplasma pneumonia* and *Propionibacterium acnes* (Bryskier, 1998:2-14; Drugbank, 2013).

The bacterial strains are said to be susceptible if the minimal inhibitory concentration (MIC) of roxithromycin is equal to or less than 1 mg/L, but if the MIC value is greater than 8 mg/l then organisms are said to be resistant (Medsafe, 2014).

The topical application and activity of roxithromycin against *P. acnes* is focused on in this study as it is the bacteria responsible for the formation of acne. The target site for roxithromycin is the sebaceous follicles, found in the dermis where the accumulation of *P. acnes* occurs (Gollnick, 2003:1585; Menon, 2002:4). By being applied topically to the skin, it can reach *P. acnes* directly for the bacteristatic effect. The topical application of roxithromycin aims to deliver the API into the skin layers only and to avoid the systemic circulation and its related side effects.

### **3.3.5 Toxicity and interactions of roxithromycin**

Even though the target site for the topical application of roxithromycin is the dermis, it may be possible for a certain amount of roxithromycin to reach systemic circulation. With systemic toxicity, the most common side-effects that present are abdominal pain, diarrhoea, nausea and vomiting, while headaches, rashes, altered smell and taste senses and liver function abnormalities occur less frequently. In comparison to erythromycin, roxithromycin has a much lower affinity for the isoenzymes of cytochrome P450 and is relatively safe with very few interactions. Keeping the aforementioned still in mind, it can still interact with a number of drugs such as cyclosporine, theophylline, disopyramide, midazolam, digoxin, warfarin, terfenadine and ergot alkaloids (Drugbank, 2013; Martindale, 2014; Medsafe, 2014).

### **3.4 Solid state properties of active pharmaceutical ingredients**

Roxithromycin's poor aqueous solubility limits its therapeutic effect and results in an obstacle for formulators. It is more economical to chemically re-design and manipulate a drug molecule than to move through the processes of developing a new drug (Biradar *et al.*, 2006:22). In roxithromycin's case, it is crucial to develop a formulation which overcomes the low solubility problems and this can be done by using solid-state amorphous forms of roxithromycin which, as a result, may lead to improved API delivery (Liebenberg & Aucamp, 2013).

Pharmaceutical compounds are most commonly used in the solid-state (Vippagunta *et al.*, 2001:4). A given solid-state drug may exist in various solid-state forms, each with different

characteristics. A compound considered to be in the solid-state and consisting of the same drug molecules can be classified in either one of the three different groups, namely, crystalline, a pseudopolymorph (solvates, desolvates and hydrates) or amorphous solids (Lee *et al.*, 2008:580). Polymorphism and amorphism gives rise to a variety of modern drug molecules that have shown to be vital in the formulation of appropriate pharmaceuticals (Byrn *et al.*, 1999:14).

### 3.4.1 Polymorphism

Vippagunta *et al.* (2001:4) defined a crystalline solid as "...an ideal crystal in which the structural units, termed unit cells, are repeated regularly and indefinitely in three dimensions in space...". The molecules within the crystal lattice are arranged in different orders and are packed in both long and short-range orders. The regular and periodic molecules which aggregate together are known as the long-range order, while the short-range order is the organisation of molecules which lie adjacent to each other (Brown, 2001:13; Cui, 2007:5).

The term polymorphism can be broken up in to two Greek words, namely "poly" meaning many and "morph" meaning shape. This gives rise to a simple description of polymorphism defined as the existence of more than one crystalline form of a pure compound, having different arrangements and/or conformations of molecules in its solid state (Bernstein, 2002:2; Purohit & Venugopalan, 2009:882; Yu *et al.*, 1998:118). If the difference in crystal forms is due to its difference in conformation, it is termed conformational polymorphism and if it is because of its packing, it is referred to as packing polymorphism (Purohit & Venugopalan, 2009:882).

Grant (1999:1-33) stated that different crystal lattices will result in different physical properties, including the molecular packing, spectroscopic properties, thermodynamic properties such as free energy, melting point, vapour pressure and solubility, mechanical properties such as hardness, tableting, compatibility and tensile strength and kinetic properties, such as stability and dissolution rate (Purohit & Venugopalan, 2009:883). The physical property focused on in this study is the solubility of the API with the goal of improving it by preparing different solid state forms of roxithromycin.

One of the crystalline forms is always more thermodynamically stable (lowest free energy) at a certain temperature, while the other form is metastable. The stable polymorph is the least soluble in solvents, has the slowest dissolution rate and is less reactive than the metastable polymorphs with high free energy. Solubility differences may enable the metastable form to convert to the more thermodynamically stable polymorph once the activation energy barrier is overcome. The transformation of solid-state forms can also be caused by temperature change during manufacturing or by exposing the metastable state to increase pressure (during tableting) (Byrn *et al.*, 1999:266). Considering this, it is advisable to use the stable polymorph for pharmaceutical formulation and to continuously control its crystal form, because it has less

potential of phase transformations during production and storage. Crystalline solids have an advantage over the amorphous forms, since they are more stable but this also results in them being less soluble (Borka, 1991:16-18; Brittain & Grant, 1999:280-281; Kratochvíl, 2011:131; Vippagunta *et al.*, 2001:4).

It is almost certain that any organic compound can exist as different polymorphs and the choice of polymorph is important in determining whether the solid state form will be a good candidate for providing the appropriate therapeutic response. It is therefore necessary to study the different polymorphs and their properties (Haleblian & McCrone, 1969:929).

### **3.4.1.1 Types of polymorphism**

Two types of polymorphs can be distinguished based upon their stability with respect to their pressure and temperature ranges. In the case of only one polymorph being stable at all pressures and temperatures below the melting point, while all other polymorphs are considered unstable, the polymorphs are known as monotropes. Conversely, enantiotropies are present when one of the polymorphs is stable over a particular pressure and temperature, while another polymorph is stable over different ranges (Grant, 1999:18-19; Purohit & Venugopalan, 2009:884).

#### **3.4.1.1.1 Solvates**

The formation of a solvate occurs when a compound is crystallised with the incorporation of an appropriate solvent within the crystal structure (Vippagunta *et al.*, 2001:4). Solvates are classified into two groups, according to the binding position of the solvent. The first of the two groups is the more stable polymorphic solvates, where a crystal is formed with the solvent as an integral part of the crystal lattice. If it is desolvated, the crystal falls apart because the solvent holds the crystal together and as a result the polymorphic solvate will transform into a different crystal form. The second group, pseudopolymorphic solvates, contains voids in the crystal filled with the solvent which does not form part of the crystal structure and when desolvation takes place the same crystal form is retained (Bernstein, 2002:5; Byrn, 1982:6-9; Florence & Attwood, 2009:19).

#### **3.4.1.1.2 Desolvates**

A desolvated solvate is formed when the solvent is removed from the solvate and continues to retain its original crystal structure of the solvate crystal form from which it is originally derived (Byrn *et al.*, 1994:1148). Desolvates are less ordered than crystalline structures and because analytical studies indicate that they are unsolved materials, they are difficult to characterise (Byrn *et al.*, 1995:946).

It is also stated that there are three observations which can be made in order to determine if the polymorph is a desolvate, namely: (1) the form is obtainable from only one solvent, (2) if compared to other forms, it has a lower density and (3) the form converts into an unsolvated structure upon heating it (Byrn *et al.*, 1995:951).

#### **3.4.1.1.3 Hydrates**

As discussed above, a solvate is a crystal structure containing a solvent, therefore, if the solvent incorporated into the solvate is water, a hydrate is formed (Vippagunta *et al.*, 2001:4). Hydrate formation occurs in about one third of all APIs. Due to the small size of the water molecule, it fills the voids in the crystals with ease and links the API molecules to the crystal lattice due to its capability of hydrogen bonding. Even though a compound is water soluble, the activity of the water in the medium determines if a hydrate structure will form (Vippagunta *et al.*, 2001:15).

Three classes of crystalline hydrates are classified. The first class is isolated site hydrates, which are water molecules isolated from direct contact with each other. The second class is the channel hydrates which consist of water molecules that are part of the lattice. They can be situated next to the water molecules of adjacent cells which result in channel formation throughout the lattice. The third class is the ion-associated hydrates classified as the coordination of metal ions with water (Vippagunta *et al.*, 2001:15).

### **3.4.2 Amorphous solids**

Crystalline forms of compounds often exhibit poor physical and chemical properties and this has driven formulators in the direction of the development of amorphous forms to overcome the limitations and improve the pharmaceutical properties of APIs (Threlfall, 1995:2452).

Contrary to polymorphs, amorphous forms of solid states are not crystalline, but rather consist of disordered arrangements of molecules possessing no crystal lattice or unit cell and as a result have zero crystallinity (Grant, 1999:8). Amorphous solids are distinguished from crystalline structures by the absence of long-range, molecular packing. However, amorphous solids show arranged, molecular packing in a short range order, which cannot be classified as crystal and rather termed as non-crystalline structures (Yu, 2001:30). Amorphous solids are referred to as liquid-like due to the fact that they are structurally identical to a liquid, but at the same time they exhibit properties of a solid (Threlfall, 1995:2452).

#### **3.4.2.1 Preparation of amorphous solids**

One pharmaceutical ingredient may have different methods for preparing its amorphous solid forms and this may result in the formation of different amorphous solid-states for each API (Threlfall, 1995:2452). The preparation of amorphous materials is challenging because of their

metastable state which may change and result in an unwanted pharmaceutical product. Since the growing interest in amorphous forms being incorporated into dosage forms, more attention should be focused on the preparation, characterisation and stability of amorphous forms (Bernstein, 2002:253).

Amorphous forms of drugs can be prepared from a number of different procedures such as lyophilisation (freeze drying), spray drying, precipitation by changing the pH, rapid solidification from the melt, removal of solvent from solvate (desolvation), quench cooling of a glass from liquid (the melt), or mechanical techniques such as grinding of the crystalline solid form, milling, compression or granulation (Byrn *et al.*, 1999:22; Cui, 2007:11; Descamps & Willart, 2008:905-908; Guillory, 1999:183-226; Vippagunta *et al.*, 2001:3-26). Taking the above-mentioned methods into consideration, Hancock & Zografi (1997:1) stated that the most frequently used methods for the preparation of amorphous forms is super-cooling of the melt, vapour condensation, precipitation and mechanical stress.

### **3.4.2.2 Specific properties of amorphous solids**

As previously mentioned, amorphous forms of API's, which are more soluble than the crystalline solids, are usually said to be thermodynamically unstable because they possess higher potential energy which causes it to convert back to the stable form. This increased solubility is of great significance in the formulation and delivery of APIs with poor aqueous solubility such as roxithromycin. The increased solubility will, as consequence result, in improved topical delivery if the other properties exhibited by the amorphous form are desirable. The kinetics of the solid controls the rate of the conversion and if the conversion is delayed due to slow kinetics, then the amorphous form can be used in pharmaceutical products (Byrn *et al.*, 1995:952; Byrn *et al.*, 1999:22; Cui, 2007:11; Wu *et al.*, 2010:4). A study conducted by Aucamp *et al.* (2013:23) showed that the solubility of the amorphous form decreased by 43% at 720 min, which indicated that the decreased solubility was due to the solution-mediated phase transformation to the more stable roxithromycin monohydrate.

Amorphous forms also tend to have higher molecular mobility as they are spread in unstructured conformations throughout the solid-state form. This characteristic makes them more chemically reactive which results in a faster degradation rate. This rate depends on the energy level present as well as the mobility of the molecules in the compound (Bernstein, 2002:253; Byrn *et al.*, 1999:22; Cui, 2007:11; Wu *et al.*, 2010:4).

Amorphous forms also have a higher ability to extensively take up water (hygroscopic) (Byrn *et al.*, 1999:952; Yu, 2001:28). In an experiment by Aucamp *et al.* (2012:473), on amorphous forms of roxithromycin, it was shown that increased humidity levels do not alter the amorphous form's solid-state properties and that increased levels of moisture did not induce

recrystallisation of the amorphous form. It should be noted that moisture has a negligible effect on the amorphous forms of roxithromycin.

When considering amorphous forms of the same API, the solid state properties (solubility, melting point, dissolution, etc.) may differ from each other. This is often termed as poly-amorphism, however, this is still a much debated term and a substantial amount of research is necessary to prove this term's accuracy (Byrn *et al.*, 1995:952).

### **3.4.2.3 Amorphous forms of roxithromycin**

Previous successful methods used to prepare a more soluble amorphous form of roxithromycin include freeze drying, spray drying and homogenisation. Two other methods have been investigated for the preparation of amorphous forms of roxithromycin and these involve super-cooling (quench cooling) of the melt as well as desolvation of a solvent (Aucamp *et al.*, 2012:467).

Two patents were registered by researchers from the Centre of Excellence for Pharmaceutical Sciences (Pharmacem) of the North-West University for two amorphous forms of roxithromycin, which proved to increase the poor aqueous solubility of the API. The first amorphous form is the non-crystalline glass form of roxithromycin and the second is the chloroform desolvated amorphous form.

#### **3.4.2.3.1 Amorphous glass form of roxithromycin**

The preparation of the 'glassy' form of roxithromycin was prepared by Liebenberg *et al.* (2013), with the purpose of improving its solubility. Roxithromycin as a glassy form exhibited a vast improvement in its solubility in phosphate buffer (pH 6.8), acetate buffer (pH 4.5) and distilled water. Roxithromycin's solubility in water improved 18.8-fold ( $\pm 1800\%$ ) compared to roxithromycin raw material (Liebenberg *et al.*, 2013). This improved aqueous solubility might lead to an increase in the delivery after topical application of roxithromycin.

The preparation of roxithromycin 'glass' consists of melting the roxithromycin monohydrate raw material on a glass microscope slide or in a glass Petri dish with a hot plate or in an oven at a temperature of approximately 120 °C. This is followed by quench cooling of the melt on an aluminium block to room temperature. This process subsequently forms a stable, 'glassy' amorphous form (Aucamp *et al.*, 2012:468).

#### **3.4.2.3.2 Chloroform desolvated amorphous form of roxithromycin**

Liebenberg & Aucamp (2013) prepared the chloroform desolvated amorphous form of roxithromycin to improve the aqueous solubility of roxithromycin. Roxithromycin showed

improved solubility in acetate buffer (pH 4.5), phosphate buffer (pH 6.8) and distilled water. This amorphous chloroform desolvated form of roxithromycin illustrated that the water solubility of roxithromycin improved by 505.9% (Liebenberg & Aucamp, 2013). This improvement could also lead to increased topical delivery and bioavailability.

The method involves the process of slow evaporation (desolvation) of an organic solvent from a saturated solution made with roxithromycin, followed by desolvation of the resulting form to obtain a stable amorphous form of roxithromycin. During the desolvation process, solvent loss from the crystalline structure takes place at temperatures lower than the melting point of the solid and as a result, the crystal lattice collapses to form an amorphous solid (Aucamp *et al.*, 2012:468).

### **3.4.3 Importance of controlling the crystal forms**

As mentioned earlier, it is important to regulate and control solid-state forms and their behavioural aspects because any phase change due to interconversion of polymorphs, formation of hydrates or solvates, desolvation of solvate or a degree of crystallinity or amorphous change can affect the bioavailability of the drug (Nerurkar *et al.*, 2000:575-610).

In order for a new drug to be approved, the drug substance guideline of the United States Food and Drug Administration (FDA) declares that the detection of polymorphs, hydrates and amorphous forms of a drug substance should be done with the use of “appropriate” analytical procedures and huge emphasis is placed on the need for control over the crystal form during the various stages of drug development (Byrn *et al.*, 1995:945). If a change in crystal form results in a change in thermodynamically properties, the dissolution, permeation and efficacy of the substance will be altered (Nerurkar *et al.*, 2000:575-610).

### **3.5 Carrier systems**

There has been a vast growth in the interest and development of different delivery systems which can improve therapeutic efficacy of new, as well as existing, drugs. Vesicular drug delivery has the ability to improve the delivery and bioavailability of medication especially those of poorly soluble drugs (Bansal *et al.*, 2012:704).

Vesicular systems can be defined as “the formation of highly ordered assemblies of one or several concentric lipid bilayers when the amphiphilic building blocks are met with water...” (Bansal *et al.*, 2012:704). A diverse range of amphiphilic building blocks exist with the main aim of preventing drug degradation and loss, preventing harmful side-effects and maximising the availability of the drug at the site where therapeutic action is needed. Hydrophilic and lipophilic drugs can be incorporated into these carriers (Bansal *et al.*, 2012:704-705).

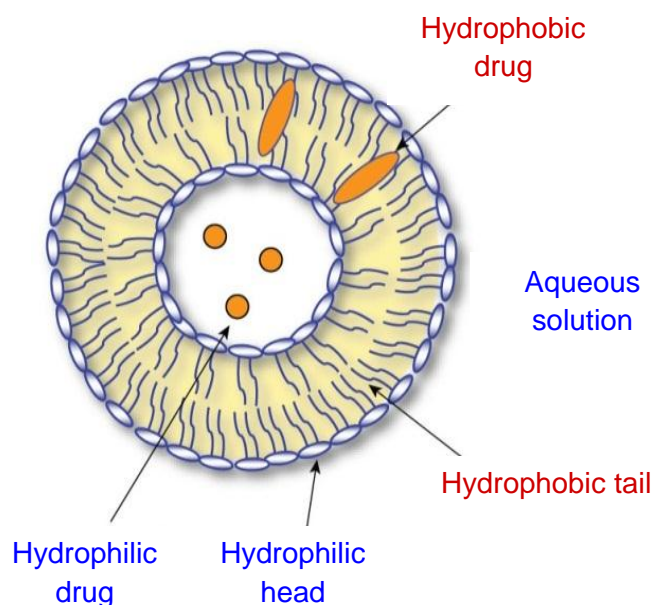
Vesicles can (1) serve as carriers which deliver drug molecules across intact skin, (2) act as penetration enhancers and may alter the intercellular lipids within the stratum corneum layer, (3) act as a depot for sustained release of drugs and (4) serve as a rate-limiting barrier for systemic absorption which provides a controlled delivery system (Alexander *et al.*, 2012:33). With the many possible uses of vesicles systems, the major problem which still exists is their stability, which may in turn limits their use (Bansal *et al.*, 2012:705).

As we already know, the penetration of drugs across the skin is limited because of the organised structure of the stratum corneum (Foldvari, 2000:418). The formulation of vesicular systems may be the solution to the poor solubility and limited skin penetration experienced by roxithromycin. Vesicles are prepared by using a variety of surfactants and lipids. The method of preparation, composition of ingredients and technique affect the physicochemical properties of vesicles, namely charge, size, lamellarity and elasticity (Kumar & Rajeshwarrao, 2011:209). They are most commonly composed of non-ionic surfactants and phospholipids and the resultant vesicles are referred to as niosomes and liposomes (Alexander *et al.*, 2012:33).

### **3.5.1 Liposomes**

Liposomes are microscopic vesicles which consist of one or more concentric bilayers (hydrophobic) surrounded by a phospholipid membrane (either neutral or charged) which encloses an interior aqueous (hydrophilic) volume. They are able to accommodate a wide variety of drugs with different physicochemical properties (polarity, size and charge). Drugs can either localise in the liposomal bilayer with its hydrocarbon chain core (hydrophobic), the surface of the liposome, or in the internal aqueous core (Bansal *et al.*, 2012:705; Escobar-Chávez *et al.*, 2012:212). An illustration of a structure of a liposome can be seen in Figure 3.3.

Electron microscopic investigations showed that liposomes with a diameter of up to 600 nm can penetrate the skin, but they remain in the stratum corneum if they are 1000 nm or larger (El Maghraby *et al.*, 2008:212). Liposomes have an average diameter range of 50 - 150 nm and a membrane thickness of 5 - 6 nm. The shapes and sizes of the liposomal vesicles that may form depend on the lipids used and the preparation technique. Unilamellar vesicles can be small (25 - 100 nm), medium (100 - 500 nm), large (0.1 - 10  $\mu\text{m}$ ) or giant in size. Oligolamellar vesicles, large multilamellar vesicles (100 - 300 nm) and multivesicular vesicles (500 nm to microns) may also be formed (Bansal *et al.*, 2012:705; Escobar-Chávez *et al.*, 2012:212; Varun *et al.*, 2012:635). Previous studies suggest that liposomes show very little drug entry into the deeper skin tissues because the liposomes remain in the upper layers of the stratum corneum and do not cross the granular layers of the epidermis. For this reason, they do not often reach systemic circulation (Elsayed *et al.*, 2007:2; Williams, 2003:127).



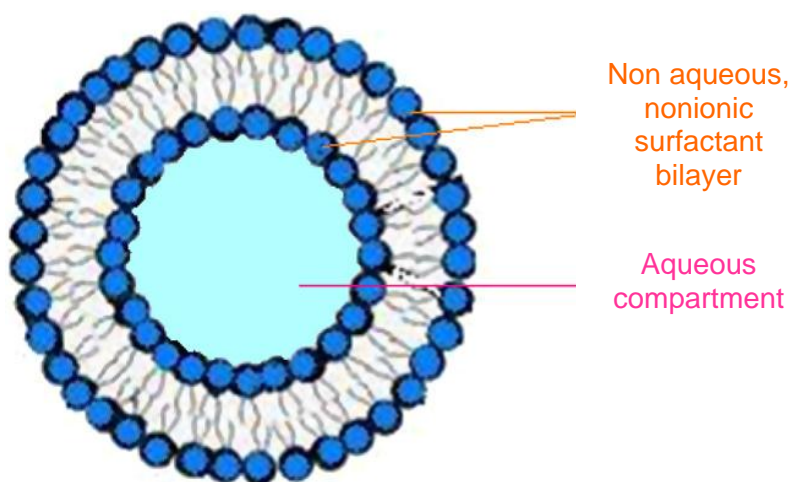
**Figure 3.3:** Structure of a liposome (adapted from Lembo & Cavalli, 2010).

There are many methods which may be used to produce liposomes, but the most common is the film hydration method (Williams, 2003:124). The main component of liposomal membranes of the vesicle consists of dipalmitoylphosphatidylcholine (DPPC), phosphatidylcholine (PC), phosphatidylserine (PS) or phosphatidylethanolamine (PE). Other compounds, dipalmitoylphosphatidylglycerol (DPPG) and cholesterol, are also added to improve the properties. Cholesterol fills up the holes in the membrane making it less permeable, as well as stabilises the membrane and makes it more rigid which aids under stressed conditions (Bansal *et al.*, 2012:705; Elsayed *et al.*, 2007:2; Varun *et al.*, 2012:634). It must be remembered that a decrease in cholesterol concentration in the bilayers, increases the bilayer's fluidity which results in increased transport across the stratum corneum (Elsayed *et al.*, 2007:2).

Many advantages of liposomes as vesicle systems exist. One such advantage is that phospholipids are well tolerated by the body and are thus ideal models for biological membranes. Liposomes have the ability to increase the solubility of poorly soluble drugs 100 - 10 000-fold. An added advantage is that hydrophilic, as well as hydrophobic, drugs can be delivered by liposomes. Many disadvantages exist for liposomes which in turn raise many stability issues. Phospholipids can hydrolyse easily because of the presence of the ester bonds. The formation of ice crystals in liposomes, the leakage of drug, oxidation or hydrolysis of cholesterol and phospholipids can cause stability problems (Bansal *et al.*, 2012:705, 709).

### 3.5.2 Niosomes

Niosomes have recently been reported as agents for topical delivery of drugs in acne treatment. Niosomes are liposomes prepared from non-ionic surfactants (Williams, 2003:128-129). These vesicle systems are prepared by hydrating synthetic non-ionic surfactants, with or without cholesterol. Niosomes are formed by self-assembly of non-ionic surfactants in an aqueous phase and they produce unilamellar, multilamellar and spherical systems as well as polyhedral structures (Bansal *et al.*, 2012:705-706). Non-ionic surfactants are known to improve the solubility of compounds which are poorly soluble (Kumar & Rajeshwarrao, 2011:209). These systems can carry hydrophobic, amphiphilic or lipophilic drugs (Bansal *et al.*, 2012:705-706). They have an average particle size of 10 - 1000 nm (Escobar-Chávez *et al.*, 2012:217). Vesicles are generally classified as three types: (a) small unilamellar vesicles with a size of 25 - 50 nm, (b) multilamellar vesicles with a size larger than that of 50 nm and (c) large unilamellar vesicles being larger than 100 nm (Varun *et al.*, 2012:635). The structure of a niosome is seen in Figure 3.4.



**Figure 3.4:** Structure of a niosome (Adapted from Kumar & Rajeshwarrao, 2011:209).

Niosomes can be made by many methods, but the thin film hydration method is most commonly referred to. The main component of the niosome is the non-ionic surfactant. The non-ionic surfactants which can be used for niosomes are sucrose esters, alkyl glyceryl ethers, polyoxyethylene alkyl ethers, polyoxyethylene acetyl ethers and polyoxyethylene-4-lauryl ethers, spans and tweens. The surfactants hold a penetration enhancing effect which directly disrupts the lipids in the stratum corneum layer (Mahale *et al.*, 2012:47; Williams, 2003:128-129) The maximum amount of surfactant/lipid used in niosome production is usually 1.0 - 2.5% w/w (10-30 mmol/l). When compared to other surfactants, non-ionic surfactants are generally less irritating to tissue, less toxic and stable at physiological pH and function as wetting agents, solubilisers, permeability enhancers and emulsifiers. The hydrophilic-lipophilic balance (HLB) of the surfactant plays an important role in the entrapment efficiency of a vesicle. The highest

entrapment efficiency is produced when the surfactant HBL is 8.6, while those with an HBL of 14 - 17 are not suitable for niosome preparation. The entrapment efficiency is decreased as the HBL value of the surfactant decreases from 8.6 to 1.7. Span 60 has an HBL of 4.7 which yields a high entrapment efficiency. It is necessary to add cholesterol to the surfactant if the HBL is lower than 6 to ensure bilayer vesicle formation. Addition of cholesterol provides added stability, increases rigidity and prevents leakage of vesicles. Sometimes a small amount of dicetyl phosphate (anionic surfactant) is added to increase size, stabilise and provide charge to the vesicles (Bansal *et al.*, 2012:705; Kumar & Rajeshwarrao, 2011:209-210; Mahale *et al.*, 2012:49; Varun *et al.*, 2012:633, 636).

Niosomes are nontoxic, biodegradable and more biologically compatible than free drugs. Other advantages are improved therapeutic efficacy of drugs, enhanced skin penetration and target delivery of drugs and because of their hydrophilic, lipophilic and amphiphilic properties; they can accommodate a wide range of solubilities. Niosomes are the preferred choice when compared to liposomes, because they exhibit higher chemical stability due to the fact that the surfactants in the niosomes are more chemically stable than phospholipids. They are cheaper and have less storage and handling limitations due to the absence of phospholipids which are prone to oxidative degradation. Disadvantages of niosomes are that fusion, aggregation, breaking of vesicles, leakage and sedimentation can also occur, as with liposomes, during their production and storage. These factors decrease the drug efficacy and stability of vesicles (Bansal *et al.*, 2012:706, 709; Rahimpour & Hamishehkar, 2012:145; Varun *et al.*, 2012:635-636). Taking the disadvantages of niosomes into account, they are still being investigated as better carriers for APIs because they lack many of the disadvantages associated with conventional liposomes (Varun *et al.*, 2012:636). For the reasons mentioned above, niosomes were chosen as a suitable vesicle system for the topical delivery of roxithromycin in this study.

### **3.5.3 Ufosomes**

Ufosomes, also known as fatty acid vesicles, are liposomes made of unsaturated fatty acids. Fatty acid vesicles are defined as colloidal suspensions composed of closed lipid bilayers made of fatty acids, as well as their ionised (soap) species. Ufosomes always contain two types of amphiphiles, namely, the ionised form (negatively charged soap) and the unionised form and the ratio of the two forms in the formulation is critical for the stability of the vesicle (Bansal *et al.*, 2012:710). It was reported by Morigaki & Walde (2007:77), that they produce large unilamellar vesicles with oleic acid/oleate with an average particle size of 100 nm.

Ufosomes have a more dynamic nature because they are composed of single-chain amphiphiles instead of double-chain phospholipids, as seen in liposomes, and therefore the concentration of non-associated monomers which are in equilibrium with vesicles is higher in

the ufosomes. The formation of these ufosomes is restricted to a very narrow pH range (7 - 9) because half of the carboxylic acid must be ionised, which varies depending on the chemical structure of the fatty acids used (Morigaki & Walde, 2007:75-76). Fatty acids spontaneously form bilayer membranes when the pH of an aqueous solution it is placed in is close to the pKa of the membrane-incorporated acid. At a lower pH, oil droplets condense and at a higher pH micelles form (Chen & Szostak, 2004:988). It should also be noted, that in dilute systems (> 95 wt.% water), one fatty acid/soap structure type can easily transition into another by changing the pH (Morigaki & Walde, 2007:77).

It has been stated that ufosomes present with limitations due to it being unstable in the presence of divalent cations (Morigaki & Walde, 2007:77). This being said, ufosome membranes are still more stable than liposomes, but fatty acid vesicles are largely unexplored in the pharmaceutical industry and little is known about the permeability of these vesicles (Bansal *et al.*, 2012:710; Morigaki & Walde, 2007:78).

### **3.5.4 Provesicular drug delivery**

Provesicular systems are drug delivery systems formulated with the intention of overcoming the stability problems which ordinary vesicular systems are faced with. Provesicular systems consist of a water soluble, porous powder as the drug carrier which is dissolved in a suitable organic solvent to produce a granular free-flowing material. It can avoid the problems experienced with aqueous vesicle systems such as liposomes, niosomes and ufosomes (Bansal *et al.*, 2012:706, 709).

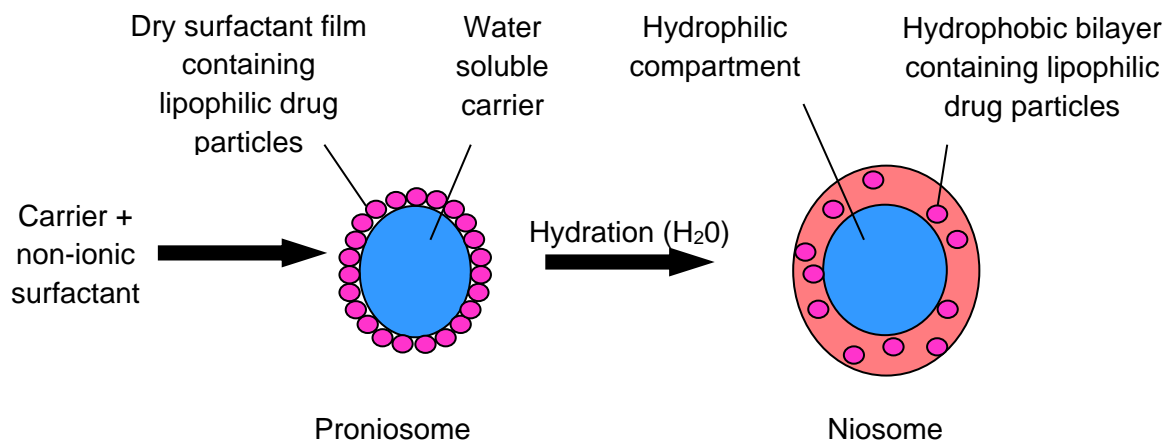
#### **3.5.4.1 Proliposomes**

Proliposomes are an alternative to the conventional liposomes. It can be defined as a mixture of drug and lipids, with the intention of producing multi-lamellar liposomes with the addition of an aqueous media entrapping hydrophobic drugs (Basavaraj & Betageri, 2014:5). The formulation consists of phospholipids, drugs dissolved in organic solvent and a carrier consisting of a water soluble porous powder. The drug and lipids are coated to the soluble carrier and this process produces a free-flowing granular product. The hydrophilic molecules remain in the aqueous core of the micelles and hydrophobic drugs remain in the lipid layer to avoid exposure to the external environment (Basavaraj & Betageri, 2014:6).

Proliposomes have a few of advantages over normal liposomes in that they are more stable and these free flowing powders have the ability to be incorporated into solid dosage forms. This results in suitable large scale industrial manufacturing (Basavaraj & Betageri, 2014:5; Kumar & Rajeshwarrao, 2011:214).

### 3.5.4.2 Proniosomes

Proniosomes are dry water-soluble carrier vesicles coated with a non-ionic surfactant, which are hydrated and agitated with an aqueous phase before use to produce a niosome dispersion, as illustrated in Figure 3.5 (Bansal *et al.*, 2012:710; Kumar & Rajeshwarrao, 2011:214).



**Figure 3.5:** Formation of a niosome from a proniosome (Adapted from Mahale *et al.*, 2012:51).

Preparation of proniosomes can be done by coating a water soluble carrier (e.g. sorbitol) with a thin, dry film of non-ionic surfactant (e.g. Span 60), which results in a dry porous powder. The niosomes are then formed by adding an aqueous phase to the powder at a temperature higher than the mean phase transition temperature for the formation of niosomes (Mahale *et al.*, 2012:50-51).

Proniosomes are more stable during storage, sterilisation, transportation, distribution and dosage, because unlike niosomes, it is a dry, free-flowing product. Being dry, proniosomes also reduce physical stability problems such a fusion, aggregation of vesicles and leakage from vesicles (Bansal *et al.*, 2012:710; Kumar & Rajeshwarrao, 2011:214).

### 3.5.4.3 Pro-ufosomes

Pro-ufosomes are vesicular systems in which no literature could be found, but it is prepared by the same method as the proniosomes. The carrier material is coated with a thin layer of fatty acid and upon use, an aqueous phase is added which dissolves the carrier and the formation of ufosomes (fatty acid vesicles) is expected.

## 3.6 Conclusion

Over the past few decades there has been a great interest in developing techniques and delivery systems which will improve the topical delivery of drugs into the skin. Roxithromycin is

a promising candidate for topical delivery as it falls between most of the optimal physicochemical parameters for effective topical delivery. The log P and melting point is optimal for topical delivery, but the molecular weight of roxithromycin is rather high and its solubility is poor for successful delivery into the skin. It is evident, from previous studies conducted by Liebenberg *et al.* (2013), that roxithromycin showed considerable increase in solubility. Based on literature, it was decided that amorphous forms of roxithromycin would be used to increase the solubility of the API and that these amorphous forms would be encapsulated into nano-sized vesicles to further improve the solubility and enhance delivery into the skin. Topical delivery of APIs, by means of encapsulating into liposomes (vesicle system), has stimulated great interest in the pharmaceutical world. It is also known that vesicle systems increase drug delivery and bioavailability of poorly soluble drugs (Bansal *et al.*, 2012:704). Due to the advantages over liposomes mentioned in this chapter, it was decided that niosomes, proniosomes, ufosomes (fatty acid vesicles) and pro-ufosomes would be used in this study to determine if there was increased delivery of API into the skin. This study focused mainly on the topical delivery of roxithromycin and based on the above information, amorphous forms of roxithromycin encapsulated in vesicles was seen as a hopeful possibility for future drug development.

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# **Article for publishing in Die Pharmazie**

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## *Chapter 4*

Chapter 4 is written as a short review which was accepted for publishing in Die Pharmazie (doi: 10.1691/ph.2016.5165). The master's student contributed to the article by completing all the experimental work, with the expert assistance of Prof. M Aucamp. The author guidelines and restrictions for the publication of this article are found in Annexure F.

## A novel RP-HPLC method for the detection and quantification of roxithromycin in topical delivery studies

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A novel HPLC method with UV detection for the identification and quantification of roxithromycin (ROX) during *in vitro* skin penetration studies has been developed and validated. The method proved to be simple and rapid with isocratic elution (flow rate: 1.0 mL/min) of ROX, using a  $C_{18}$  column and UV detection at 205 nm. The mobile phase consisted of 0.06 M potassium di-hydrogen orthophosphate buffer (pH adjusted to 7.4 with sodium hydroxide) and acetonitrile in a 50:50 (v/v) ratio. This method showed linearity across the concentration range of 5 – 1000  $\mu\text{g/mL}$  with a correlation coefficient of 0.9999. An average recovery of 101.78 % was obtained. Limit of detection (LOD) and lower limit of quantification (LLOQ) values proved that ROX can still be detected at a concentration level of 0.3  $\mu\text{g/mL}$  and accurately quantified at a concentration of 0.5  $\mu\text{g/mL}$ . The specificity testing during method validation proved that this method is suitable for the accurate detection and quantification of ROX even when combined with different compounds typically used during the formulation of topical delivery systems.

### 1. Introduction

Roxithromycin (ROX) is a semi-synthetic 14-membered ring macrolide antimicrobial agent (Bryskier 1998). It shows antimicrobial activity against *Propionibacterium acnes* (Gollnick 2003; Menon 2002). In a recent study ROX was included into delivery systems for the topical treatment of acne. The topical delivery systems included niosomes, pro-niosomes, ufosomes and pro-ufosomes. Proper investigation of the incorporated drug into such delivery systems requires a suitable and robust analytical method. Up to now, no analytical method has been reported for the qualitative and quantitative analysis of ROX incorporated into topical delivery systems. During preliminary studies it became evident that the analytical method for ROX as stipulated in the British Pharmacopoeia (BP, 2015), will not be suitable due to the overlapping of the peaks of the solvent, active ingredient and formulation compounds. Furthermore, *in vitro* skin permeation studies usually require that the analytical method must be sensitive enough to detect very low drug concentrations. It became evident that the pharmacopoeial method will not suffice for the accurate quantification of low concentration levels of ROX. Therefore the need to develop and validate a reversed phase HPLC method that would be sufficiently sensitive, robust and simple for the accurate detection and quantification of ROX during *in vitro* skin penetration studies.

### 2. Investigations, results and discussion

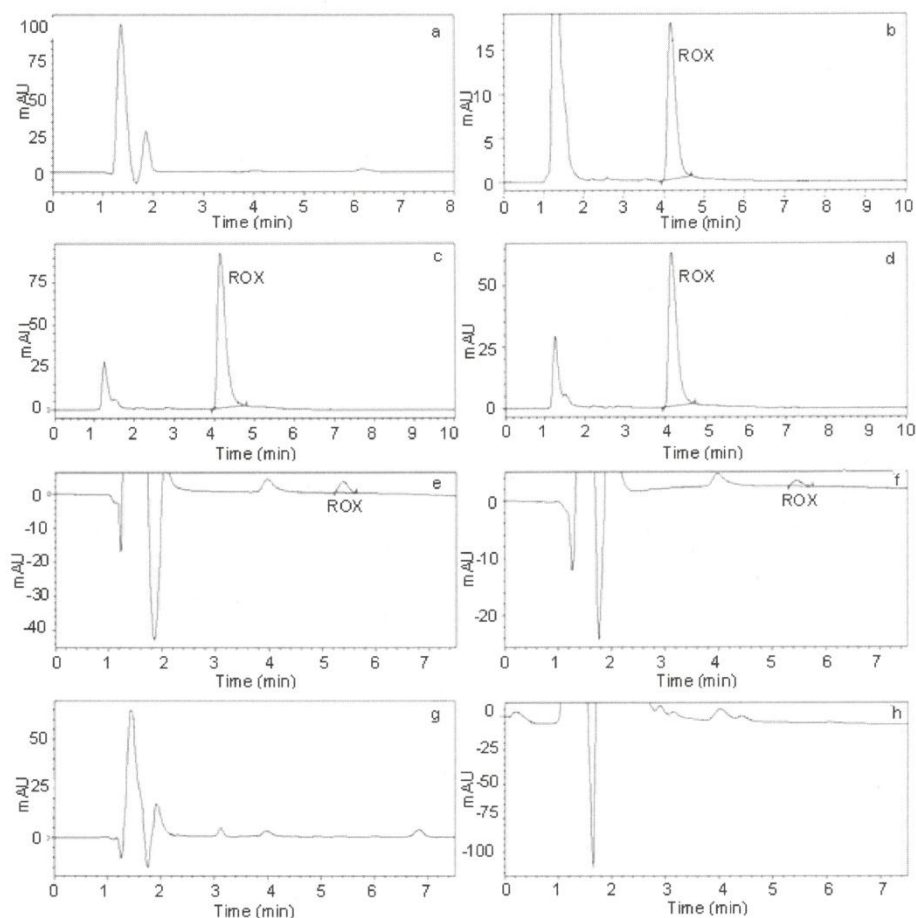
The method was validated in terms of linearity, range, precision, accuracy, recovery, specificity, ruggedness, limit of detection (LOD) and lower limit of quantification (LLOQ). Nine calibration standard solutions, ranging from 5 – 1000  $\mu\text{g/mL}$  were prepared, each solution was analysed in duplicate. A regression plot of peak area response versus ROX concentration provided a regression equation of  $y = 2550.6x - 3584.6$  with a correlation coefficient ( $r^2$ ) of 0.9999. The correlation coefficient demonstrates that this method, within the concentration range tested, provided an excellent correlation between the peak area and ROX concentration. The accuracy of the proposed method was determined by recovery experiments. An average recovery of 101.8 % (%RSD = 1.5) was determined from accuracy testing. Since the mean recovery falls

between the required limits of 98.0 – 102.0 % it can be deduced that this analytical method was accurate. The LLOQ limit is defined as the minimum concentration at which the analyte can still be quantified with acceptable accuracy and precision (RSD < 15.0 %). The LLOQ was determined by the experimental analysis of samples with known concentrations (0.1 – 5.0  $\mu\text{g/mL}$ ). The LLOQ limit was determined to be 0.5  $\mu\text{g/mL}$  (%RSD = 4.8). Any concentration below this determined limit yielded an unreliable peak interrupted by noise. The LOD limit is defined as the lowest concentration of the drug that is still detectable, but cannot be quantitated. The LOD limit for this analytical method was determined to be 0.3  $\mu\text{g/mL}$  (RSD = 10.1 %). The precision of the proposed method was assessed as intra-day and inter-day variation and the repeatability results are provided in the Table.

**Table: Summary of intra-day (repeatability) data obtained for ROX**

| Theoretical concentration ( $\mu\text{g/mL}$ ) | Intra-day measured concentration ( $\mu\text{g/mL}$ ) |      |
|--|---|------|
|  | Average   | %RSD |
| 100  | 100.45  | 1.19 |
| 250  | 258.91  | 1.85 |
| 750  | 761.24  | 1.24 |

During the inter-day precision testing a mean recovery of 101.4 % (1.3 %) over 3 days was obtained. It was furthermore proved that ROX remain stable during HPLC analysis over a 24 h period with a %RSD of 0.4 % between hourly injections. This proved that the method is robust and acceptable to be used by a variety of analysts and in different analytical laboratories. As described, ROX was included into topical delivery systems. It was therefore necessary to determine if any of the compounds used to prepare the vesicle systems will interfere with the ROX peak during membrane release and transdermal diffusion studies. Specificity samples were prepared which contained the compounds of the formulated vesicle systems. For the niosomes and proniosomes sample mixtures were made which included Span® 60, cholesterol, phosphate buffer solution (PBS) (pH 7.4) and sorbitol. While for the ufosomes and



a) PBS with no ROX added, b) 100 µg/mL ROX in PBS, c) specificity test solution for the ingredients incorporated in the niosomes and proniosomes, d) specificity test solution for the ingredients incorporated in the ufosomes and pro-ufosomes, e) 15 µg/mL ROX in ethanol, f) solution from epidermis-dermis sample containing ROX dissolved in ethanol, g) solution from tape stripped skin samples where no ROX was detected and h) solution of the receptor phase (PBS pH 7.4) of topical diffusion studies containing no ROX.

pro-ufosomes sample mixtures were made containing sodium oleate, cholesterol, PBS (pH 7.4) and sorbitol. Furthermore, solutions were made containing tape strips (stratum corneum-epidermis) with added ROX in ethanol to test the specificity of the method during the analysis of ROX during tape stripping. The Fig. depicts chromatograms obtained during specificity testing.

It can be seen from the chromatograms that none of the additives in the vesicle systems or possible compounds from the tape strips and skin interfered with the detected ROX peak. Furthermore, the retention time of ROX in the sample solutions compared very well with the retention time of ROX in the standard solutions. This proves that the specificity of this analytical method is excellent. From the validation results and parameters it is evident that this method is a rapid, simple, precise and reliable procedure for the determination of ROX during membrane release and topical delivery studies.

### 3. Experimental

ROX with purity 99.5 % (anhydrous basis), was purchased from DB Fine Chemicals (Johannesburg, South Africa). A Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system, equipped with a SIL-20AC auto-sampler fitted with a sample

cooler, a UV/VIS PDA detector (SPD-M20A) and a LC-20AD solvent delivery module was used during the method development and validation. A Phenomenex® Luna C18 (5 µm), 150 x 4.6 mm column was used as stationary phase. The mobile phase consisted of 0.06 M potassium di-hydrogen orthophosphate buffer solution with the pH adjusted to 7.4 with NaOH (sodium hydroxide) solution. The buffer was mixed with acetonitrile in the ratio 50:50. Isocratic elution was used with a flow rate of 1.0 mL/min, a detection wavelength of 205 nm and an injection volume of 10 µL.

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# **Article for publishing in Pharmaceutical Research**

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## *Chapter 5*

Chapter 5 is written in article format for the purpose of publishing the article in the Pharmaceutical Research. The master's student contributed by performing all experimental work and writing of the article. US English and Vancouver Style referencing was used for the article. The complete author guidelines for publishing are found in Annexure G.

# **Topical delivery of roxithromycin solid-state forms entrapped in vesicles**

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## **Abstract**

**Purpose:** Recently, considerable interest developed in using newer or improved antibiotics for the treatment of acne vulgaris. The purpose of this study was to encapsulate solid-state forms of roxithromycin, with proven improved aqueous solubility, into vesicle systems for dermis targeted topical delivery. The investigated vesicles included: niosomes, proniosomes, ufosomes and pro-ufosomes.

**Methods:** Preliminary studies were used to identify formulations which would yield optimal vesicles. The different solid-state forms of roxithromycin were incorporated into the vesicle systems. The characterization of the vesicles was done with transmission electron microscopy (TEM), light microscopy, droplet size, droplet size distribution, pH, zeta-potential and entrapment efficiency percentage (EE%). Finally, comparative release and topical diffusion studies were performed in order to evaluate if targeted topical delivery was obtained and if the solid-state amorphous forms of the API resulted in improved topical delivery of roxithromycin.

**Results:** Niosomes, proniosomes, ufosomes and pro-ufosomes containing different solid-state forms of roxithromycin (i.e. crystalline and amorphous) were successfully prepared and characterized. The vesicles showed optimal properties for topical delivery. All carrier systems had topical delivery to the epidermis-dermis, whilst no roxithromycin was found in the receptor compartment or stratum corneum-epidermis. The niosomes were the leading formulation and the two amorphous forms had better topical delivery than the crystalline form.

**Conclusion:** Successful targeted delivery of roxithromycin was obtained in the dermis, where the activity against *Propionibacterium acnes* is needed. The amorphous forms seemed to have held their solid-state form during formulation and in the vesicles, showing improved topical delivery in comparison to crystalline roxithromycin monohydrate.

**Keywords:** Vesicles, Pro-vesicles, Amorphous, Roxithromycin, Topical delivery

## **Abbreviations**

***P. acnes*** *Propionibacterium acnes*

**API** Active pharmaceutical ingredient

**KH<sub>2</sub>PO<sub>4</sub>** Potassium di-hydrogen phosphate

**HPLC** High performance liquid chromatography

**PBS** Phosphate buffer solution

**PVDF** Hydrophilic polyvinylidene fluoride

**TEM** Transmission electron microscope

**Log D** Octanol-buffer distribution coefficient

**EE%** Entrapment efficiency percentage

**ANOVA** Analysis of variances

**LOD** Limit of detection

**LLOQ** Lower limit of quantification

## 1 Introduction

Acne vulgaris is a chronic inflammatory skin disease, which affects most of the population at some point in their life. The disease develops in the pilosebaceous units of the dermis and is characterized by irregular desquamation in hair follicles, increased sebum production in the sebaceous glands and inflammation caused by infiltration of *Propionibacterium acnes* to the area<sup>1,2</sup>. The rationale for the use of antibiotics in the treatment of acne is based on the bacteriostatic effect of the inflammatory agent, *P. acnes*<sup>3</sup>. In recent studies, there has been interest in conducting trials to test the efficiency of newer antibiotics, such as roxithromycin, for acne treatment<sup>3</sup>.

Roxithromycin is a semi-synthetic macrolide antibiotic having bacteriostatic, as well as bactericidal, effects on bacterial organisms by terminating their protein synthesis and growth<sup>4-6</sup>. Roxithromycin has a rather broad spectrum of activity, but in this study, the focus is shifted to its activity against *P. acnes* as this is the micro-organism responsible for causing inflammatory acne<sup>7</sup>. The dermis is the specific area targeted, as it is the region which contains the sebaceous follicles where the accumulation of this *P. acnes* micro-bacterium occurs<sup>7,8</sup>.

One problem which exists with roxithromycin, is its poor aqueous solubility which limits its therapeutic capability. According to Williams<sup>9</sup>, an API (active pharmaceutical ingredient) which is both lipophilic and hydrophilic is ideal for topical delivery as it is able to pass through the lipid layers as well as the aqueous domains of the skin. Roxithromycin has an experimental aqueous solubility of 0.0335 mg/ml at 25 °C, which is poor considering that for an API to optimally penetrate the skin, it requires an aqueous solubility of above 1 mg/ml<sup>10,11</sup>. By using different solid-state amorphous forms of roxithromycin, these insolubility problems may be overcome<sup>12</sup>. Different crystal lattices of the same compound result in altered physico-chemical properties, including that of solubility<sup>13</sup>. Researchers from the Centre of Excellence for Pharmaceutical Sciences (Pharmacem) of the North-West University, South Africa, registered two patents which proved that two amorphous forms of roxithromycin had increased aqueous solubility in comparison to the crystalline form. A 'glassy' amorphous form of roxithromycin exhibited a 1800% increase in aqueous solubility in water, whilst the chloroform desolvated amorphous form showed a 505.9% increase<sup>14,15</sup>.

There has been great interest and growth in developing delivery systems which improve therapeutic effects of drugs. Drug delivery by vesicles has the capability of improving the bioavailability of drugs, particularly those of poor solubility such as roxithromycin<sup>16</sup>. Vesicle systems are formed when amphiphilic components are met with water and arrange themselves into ordered assemblies of one or more lipid bilayers<sup>16</sup>. Vesicles can be made from various lipids including non-ionic surfactants, which result in one of the more popular vesicle systems, niosomes<sup>17</sup>. Niosomes are made from the hydration of non-ionic surfactants and often contain cholesterol<sup>9</sup>. They can act as carriers for lipophilic or hydrophilic drugs with a particle size of 100-1000 nm<sup>16,18</sup>. Roxithromycin is lipophilic with a log P of 2.9, which predicts it will be delivered to the skin in the lipophilic bilayer surrounding the inner aqueous compartment of the vesicles<sup>19</sup>. Ufosomes are another type of liposome prepared from fatty acids. These fatty acid vesicles (ufosomes) are suspensions of fatty acid and ionized (soap) species which form lipid bilayers with an average particle size of 100 nm<sup>16,20</sup>.

Pro-vesicular delivery systems are vesicles developed to overcome the instability problems associated with the vesicles suspended in aqueous solutions, as seen with niosomes and ufosomes. Pro-vesicles are systems consisting of a dry, porous powder which is water soluble. The carrier dissolves when an aqueous solvent is added to it before the use of the system<sup>16</sup>. The dry aqueous soluble carriers of pro-niosomes are coated with non-ionic surfactants and niosomes are formed on aqueous hydration and agitation of these systems.<sup>16,21</sup> Pro-ufosomes are vesicle systems for which no information could be found, but it was prepared using the same method as proniosomes.

Another obstacle formulators continue to face today, is overcoming the skin's great barrier, the stratum corneum, as it is this layer of skin which is problematic for the successful penetration of drugs<sup>22</sup>. The skin is a large and convenient area for the administration of drugs and also boasts a number of advantages over oral and parenteral API delivery such as: avoidance of first-pass metabolism, dose dumping, systemic adverse effects and inconvenience of administration for the patient<sup>9,23,24</sup>. It is therefore beneficial to develop products for topical delivery which successfully penetrate the stratum corneum. There is hope for great improvement in the topical delivery of roxithromycin by using its amorphous forms encapsulated

into vesicle systems which overcome the solubility drawbacks of the API and increase stratum corneum penetration and topical delivery.

The aim of this study was to determine if the amorphous forms, with increased solubility encapsulated in vesicles, would have successful topical delivery. The three solid-state forms of roxithromycin, namely, roxithromycin monohydrate, roxithromycin 'glassy' amorphous form and chloroform desolvated amorphous form of roxithromycin were each encapsulated into four vesicle systems. The four vesicle systems were niosomes, proniosomes, ufosomes and pro-ufosomes. The topical delivery of all preparations were tested using release and diffusion experiments to determine which of the formulations had the optimal topical penetration into the target area, in this case the dermis.

## **2 Materials and methods**

### **2.1 Materials used in this study**

Roxithromycin monohydrate (crystalline form) was purchased from DB Fine Chemicals (South Africa). The ingredients used in the preparations of the vesicle systems were Span 60, sodium oleate, cholesterol and sorbitol, all purchased from Sigma Aldrich (Germany). Methanol and chloroform were of analytical grade (ACE Chemicals, South Africa) and the potassium dihydrogen orthophosphate AR ( $\text{KH}_2\text{PO}_4$ ) was purchased from LabChem (South Africa). Ultrapure water, with a resistivity of  $18.2 \text{ M}\Omega\cdot\text{cm}^{-1}$ , was used in all experiments in this study and was obtained from a Rephile Direct Pure UP water purification system (USA). The acetonitrile used as a component of the mobile phase during high performance liquid chromatography (HPLC) analysis was of chromatography grade (Merck, South Africa).

### **2.2 HPLC analysis method**

The use of the HPLC analysis method was to determine the concentrations of roxithromycin in all samples obtained during the experiments done in this study. The analysis was performed in a controlled laboratory environment. A Shimadzu (Japan) UFLC (LC-20AD) fitted with a UV/VIS Photodiode Array detector (SPD-M20A) and a LC-20AD solvent delivery module was used, with LabSolutions, LCsolution Release 1.21 SP1 (Shimadzu, Japan), used for data analysis. A Luna  $\text{C}_{18}$   $150 \times 4.6 \text{ mm}$  column ( $5 \mu\text{m}$  particle size) was used as a stationary phase, with an injection volume of  $10 \mu\text{l}$ , a flow rate of  $1 \text{ ml/min}$  with a wavelength set at  $205 \text{ nm}$ . The mobile phase consisted of  $0.06 \text{ M}$   $\text{KH}_2\text{PO}_4$  buffer solution (adjusted to pH 7.4 with sodium hydroxide solution) and acetonitrile (50:50 %v/v). Validation of this method resulted in a correlation coefficient of 0.9999.

### **2.3 Preparation of the amorphous forms of roxithromycin**

Two amorphous solid-state forms of roxithromycin were prepared. The glassy amorphous form of roxithromycin was prepared by evenly distributing crystalline roxithromycin monohydrate in a Petri dish. The sample was subsequently melted in a laboratory oven (Binder, Germany) at  $120^\circ\text{C} \pm 2^\circ\text{C}$ . The melt was removed from the oven and quench cooled on a granite surface to form the amorphous solid<sup>25</sup>. The chloroform desolvated amorphous form was prepared by heating chloroform on a hot plate stirrer to  $60^\circ\text{C} \pm 2^\circ\text{C}$  whilst adding roxithromycin

monohydrate until a saturated solution was obtained. The solution was removed from the hot plate and left for slow evaporation of the chloroform to occur <sup>25</sup>. A dense mass was obtained, which was characterized as the roxithromycin chloroform solvate. The sample was desolvated in a laboratory oven (Binder, Germany), set at 60 °C, ± 2 °C, for a period of 24 h.

### **2.3.1 Light microscopy of solid-state forms of roxithromycin**

Evidence that the amorphous forms were pure was proved by viewing each solid-state form under a Nikon Eclipse microscope (E4000) (Japan), through a cross polarizer. Pure amorphous forms would be confirmed if no birefringence existed (birefringence appears when there are crystalline particles in a sample) <sup>26</sup>.

### **2.3.2 Aqueous solubility of solid-state forms of roxithromycin**

The aqueous solubility of roxithromycin monohydrate, glassy roxithromycin and roxithromycin desolvate were determined. All three forms were added to 5 ml PBS (pH 7) in polytops heated to 32 °C and containing magnetic stirrers. Saturated solutions were obtained for 24 h. The solutions were then filtered through 0.45 µm hydrophilic polyvinylidene fluoride (PVDF) filters and analyzed on the HPLC. The experiment was done in triplicate.

### **2.3.3 Log D of roxithromycin**

The log D of roxithromycin was determined. 15 mg of roxithromycin was accurately weighed and added to 2 ml pre-saturated *n*-octanol in a test tube. The test tube was subsequently rotated in a rotating bath at 32 °C for 10 min. Thereafter, 3 ml pre-saturated PBS (pH 7) was added and rotated for 45 min. The test tubes were then centrifuged at 4000 rpm for phase separation of the *n*-octanol and PBS (pH 7) to occur. The separated phases were placed in polytops where they were then filtered through PVDF filters. The API concentration in each phase was determined using HPLC. The experiment was done in triplicate.

The distribution coefficient between the two phases was calculated using Equation 1:

$$\text{Log D} = \text{concentration in } n\text{-octanol} / \text{concentration in PBS (pH 7)} \quad \text{Equation 1}$$

## 2.4 Determining formulas for optimal vesicle systems

**Table I:** Lipid ratios and sonication times of twelve potential vesicle formulas

| Formulation | Lipid ratio                     | Sonication time |         |         |
|-------------|---------------------------------|-----------------|---------|---------|
|             |                                 | 0.0 min         | 2.0 min | 3.5 min |
| Niosomes    | Span 60:cholesterol (2:1)       | (A1)            | (A2)    | (A3)    |
|             | Span 60:cholesterol (1:1)       | (A4)            | (A5)    | (A6)    |
| Ufosomes    | Sodium oleate:cholesterol (2:1) | (B1)            | (B2)    | (B3)    |
|             | Sodium oleate:cholesterol (1:1) | (B4)            | (B5)    | (B6)    |

All vesicle systems were prepared by the hand-shaking method adapted from New <sup>27</sup> with slight modifications to suit this study. To determine the optimal lipid ratio for niosomes and ufosomes, two different lipid ratios for each system were made and each were sonicated at three different sonication lengths. This resulted in six formulations of each vesicle systems as seen in Table I. The parameters used to determine the optimal lipid ratio was Transmission Electron Microscopy (TEM), light microscopy and droplet size and distribution. Only the vesicles with no sonication were viewed with the TEM (FEI Tecnai G2, FEI, Holland) to determine if vesicles had indeed formed. Thereafter, all vesicles were viewed under the Nikon Eclipse E4000 microscope (Nikon, Japan) to determine if vesicles still existed after sonication. Next the droplet size and distribution was tested using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) to show which formulation had better distribution and therefore, better stability <sup>21</sup>. The optimal lipid ratios were then decided by choosing one of the lipid ratios for niosomes and ufosomes from the table above.

The optimal concentration of entrapped roxithromycin monohydrate was determined by loading each of the optimal lipid vesicles (niosomes and ufosomes) with four different concentrations of drug. The entrapment efficiencies of all ratios were tested in duplicate using the mini-column centrifugation method of New <sup>27</sup>. The formulation with the better entrapment efficiency, as well as physical appearance, was chosen as the final optimal formula for each vesicle system.

## 2.5 Preparation of optimal vesicle systems

The preparation of the twelve optimal vesicle systems were based on the results obtained from determining the optimal formulas of the niosomes and ufosomes containing roxithromycin monohydrate. The pro-vesicular systems' (proniosomes and pro-ufosomes) ratios were exactly the same as the optimal ratios of the vesicle systems (niosomes and ufosomes) and all three solid-state forms of roxithromycin were entrapped in the same way. This was decided in order to minimize possible variation amongst vesicles, which made it easier for comparison and explanation of experiments that followed. Table II shows a summary of the twelve vesicle carriers in this study.

**Table II:** Summary of vesicle preparations in this study

| <b>Roxithromycin forms<br/>encapsulated</b> | <b>Vesicle systems</b> |          |             |              |
|---|------------------------|----------|-------------|--------------|
|   | Niosomes               | Ufosomes | Proniosomes | Pro-ufosomes |
| Roxithromycin monohydrate                   | (1)                    | (2)      | (3)         | (4)          |
| Glassy amorphous form                       | (5)                    | (6)      | (7)         | (8)          |
| Chloroform desolvated<br>amorphous form     | (9)                    | (10)     | (11)        | (12)         |

As mentioned above, the niosomes and the ufosomes were prepared according to the hand-shaking method of New <sup>27</sup>, where the lipid components of the vesicles and the API were dissolved in chloroform/methanol solution and left to evaporate. Upon appearance of a thin film at the bottom of the beaker, PBS (pH 7.4) and a magnetic stirrer were added to completely remove the film in the formation of vesicles. Niosomes were then sonicated for 2.0 min and ufosomes for 3.5 min and left to stand for 2 h before use. The slurry method was used for the preparation of the proniosomes and pro-ufosomes <sup>27</sup>. This method consisted of dissolving the lipid layer components and API in chloroform/methanol and adding it drop-wise to sorbitol powder in order for the vesicles to coat the powder. The chloroform/methanol evaporated leaving a dry powder which was constituted with 5 ml PBS (pH 7.4) before it was needed for

further experiments. Proniosomes were sonicated for 2 min and pro-ufosomes for 3.5 min and left to swell for 2 h before use. Table III shows the ingredients of each optimal vesicle system prepared. The niosomes and proniosomes had a Span 60:cholesterol:roxithromycin ratio of 2:1:1; the ufosomes and pro-ufosomes also had a sodium oleate:cholesterol:roxithromycin ratio of 2:1:1. The preparation of each vesicle system was repeated in triplicate using each solid-state form of roxithromycin.

**Table III:** Ingredients used for optimal vesicles systems

| Ingredients   | Formulation |             |          |              |
|---|-------------|-------------|----------|--------------|
|   | Niosomes    | Proniosomes | Ufosomes | Pro-ufosomes |
| Span 60   | 200 mg      | 200 mg      | -        | -            |
| Sodium oleate   | -           | -           | 200 mg   | 200 mg       |
| Cholesterol   | 100 mg      | 100 mg      | 100 mg   | 100 mg       |
| Chloroform/methanol (2:1)                             | 5 ml        | 5 ml        | 5 ml     | 5 ml         |
| Roxithromycin   | 100 mg      | 100 mg      | 100 mg   | 100mg        |
| Sorbitol  | 1000 mg     | 1000 mg     | 1000 mg  | 1000mg       |
| PBS (0.06 M KH <sub>2</sub> PO <sub>4</sub> at pH 7.4 | 5 ml        | 5 ml        | 5 ml     | 5 ml         |

## 2.6 Characterization of vesicles

Although all vesicles were made according to the optimized formulas discussed earlier, it was still necessary to characterize all twelve vesicle systems of different vesicles containing different solid-state forms of roxithromycin.

### 2.6.1 TEM

The morphology of the vesicles was investigated using a FEI Tecnai G2 TEM (FEI, Holland) <sup>28</sup>. Only the four optimal vesicle systems containing no API were viewed using the TEM as there was a risk of crystallization of API with subsequent damage to the TEM. Each vesicle was freshly prepared, diluted 10x with distilled water and one drop was placed on the carbon-coated

copper grid which was left to dry. A drop of osmium was used to stain the lipid components. Once dry, it was loaded into the TEM and viewed at 5000-20000x magnifications.

### **2.6.2 Light microscopy**

Light microscopy, using a Nikon Eclipse E4000 microscope (Nikon, Japan), was performed in order to determine if all systems containing API consisted of vesicles. The shape and lamellarity of larger vesicles could also be seen <sup>29</sup>. Samples were prepared and diluted 20x with distilled water. A drop was placed on a microscope cover slip and viewed at a magnification of 50x (max. for the instrument). The experiment was done in triplicate for each carrier system.

### **2.6.3 Zeta-potential**

The zeta-potential was tested in order to determine the surface charge of each vesicle system, as surface charges can have an influence on the stability of the droplets <sup>21</sup>. Highly charged droplets prevent aggregation and fusion of vesicles <sup>29</sup>. A Malvern Zetasizer Nano ZS (Malvern Instruments, UK) was used to test the zeta-potential. Fresh samples were diluted (20x with distilled water) and a small amount was placed in a cuvette for triplicate readings. The experiment was repeated twice more.

### **2.6.4 Droplet size and distribution**

The droplet size and distribution of each vesicle system was tested using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The particle size and distribution is important as it has a great influence on the stability as well as entrapment efficiency of a system <sup>21</sup>. The twelve vesicular systems were prepared in triplicate and diluted 20x, where 2 ml was injected into a plastic cuvette. The reading of each sample was taken three times for an accurate average.

### **2.6.5 pH**

The pH of the lipid layers of vesicles in solution was determined using a Mettler Toledo pH meter (Mettler Toledo, Switzerland). The twelve 5 ml vesicle solutions, undiluted, were freshly prepared and the pH readings taken three times. The entire experiment was repeated in triplicate with fresh samples each time

### **2.6.6 Entrapment efficiency**

The entrapment efficiency (EE%) determined the difference between the total amount of free drug and the unentrapped drug to determine the amount of drug encapsulated in the vesicles <sup>30</sup>.

The entrapment efficiency of the twelve vesicle systems were tested using the ultra-centrifugation method<sup>31</sup>. This method was discovered later in the study and proved to be faster and more convenient than the mini-column centrifugation method used for optimizing formulas. A Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) with a 50.2Ti fixed rotor was used. Samples were diluted 25x, placed in the centrifugation tubes and centrifuged at 25000 rpm for 30 min. The clear supernatant was then removed and analyzed on the HPLC, where the entrapment was calculated afterwards using the following equation of Kurakula *et al.*<sup>32</sup>:

$$EE\% = C_t - C_f / C_t \times 100$$

**Equation 2**

Where  $C_t$  is the total amount API and  $C_f$  the amount of free unencapsulated drug (x value calculated from HPLC analysis equation  $y = mx + c$ ).

## **2.7 Membrane release studies**

Membrane release studies were done on all twelve vesicle systems to determine if the API was released from the vesicles. The donor phases during the release studies were samples of vesicles containing the different solid-state forms of roxithromycin and the receptor phase consisted of 0.06 M  $\text{KH}_2\text{PO}_4$  dissolved in distilled water with a pH of 7.4. Each experiment had 12 Franz cells, with 10 being test cells and two being controls. The donor phase and the receptor phase were prepared and the donor phase was placed in a 32 °C water bath (to correlate with the skin surface's temperature) and the receptor phase was placed in a 37 °C water bath (temperature of human blood). Vacuum grease was placed on either side of the Franz cells, a magnetic stirrer was placed in the receptor compartment and a PVDF filter was cut circularly and placed between the compartments. The Franz cells were sealed with vacuum grease and a horseshoe clamp. 2 ml PBS (pH 7.4) was placed in the receptor compartment and 1 ml of the vesicles (donor phase) was placed in the donor compartment and sealed with Parafilm®. The cells were placed on a Variomag® magnetic stirrer plate (Variomag, USA) in a Grant® water bath (Grant Instruments, UK), where the temperature was maintained at 37 °C. Extractions and replacements of the PBS (pH 7.4) took place at extraction times of 1, 2, 3, 4, 5 and 6 h. The extractions were analyzed using HPLC analysis to determine the concentration of

API which permeated through the membrane and in turn, the amount of API released from each formula.

## **2.8 Preparation of skin for diffusion studies**

The skin for the diffusion studies was female Caucasian skin, obtained after surgery of the abdominal area, with informed consent having first been obtained from patients before such use. Ethical approval was granted by the Research Ethics Committee of the North-West University (Reference: NWU-00114-11-A4). Skin was received within 24 h after the abdominal surgery and was transported directly to the North-West University in an ice box. Full-thickness skin was cut (thickness of 400  $\mu\text{m}$ ) using a Dermatome™ (Zimmer LTD, United Kingdom). The dermatomed skin was placed on Whatman® filter paper, closed with foil and stored in a freezer at -2 to -5 °C until needed. Skin over six months old was discarded.

## **2.9 Skin diffusion studies**

Skin diffusion studies were conducted to determine if the API penetrated the skin to reach the systemic circulation (receptor phase). The procedure followed for the diffusion studies was exactly the same as the membrane studies discussed earlier, except that circles of dermatomed skin on Whatman® filter paper was cut and placed between the donor and receptor compartments, with the stratum corneum facing upwards instead of the PVDF membranes. The extraction times also differed. The diffusion studies had only one extraction after 12 h based on pilot studies conducted beforehand.

## **2.10 Tape stripping**

Tape stripping was undertaken to determine if there was API in the stratum corneum-epidermis and epidermis-dermis and to see if the roxithromycin amorphous forms had better topical delivery than the roxithromycin monohydrate. The skin samples were removed from the Franz cells and pinned to a board so that the diffusion area of the skin could be seen. It was dabbed with a paper towel and 16 strips of 3M Scotch® Magic™ tape were used to remove the stratum corneum-epidermis from the skin (first strip was discarded). All 15 tape strips were placed into a polytop and 5 ml ethanol was added to dissolve the API. The remaining epidermis-dermis skin was cut into tiny pieces and also placed into a polytop with 5 ml ethanol. The samples were left to stand for 8 h at 2-5 °C for the API to be extracted from both the stratum corneum-

epidermis and epidermis-dermis. The API concentration in each of samples was determined using HPLC analysis. The process was repeated after all twelve diffusion studies.

### **2.11 Statistical analysis**

Data analysis was performed using the epidermis-dermis results obtained from HPLC analysis as this was the only group of samples in which API concentration was found. Statistics was used to compare which vesicle systems, as well as which solid-state form, of roxithromycin had the better topical delivery. Simple non-parametric tests were performed. The Kruskal-Wallis analysis of variance (ANOVA) compared the different formulations and the multiple comparisons test was used to compare among pairs of formulations. Formulations had a significant difference if their p-value was smaller than 0.05.

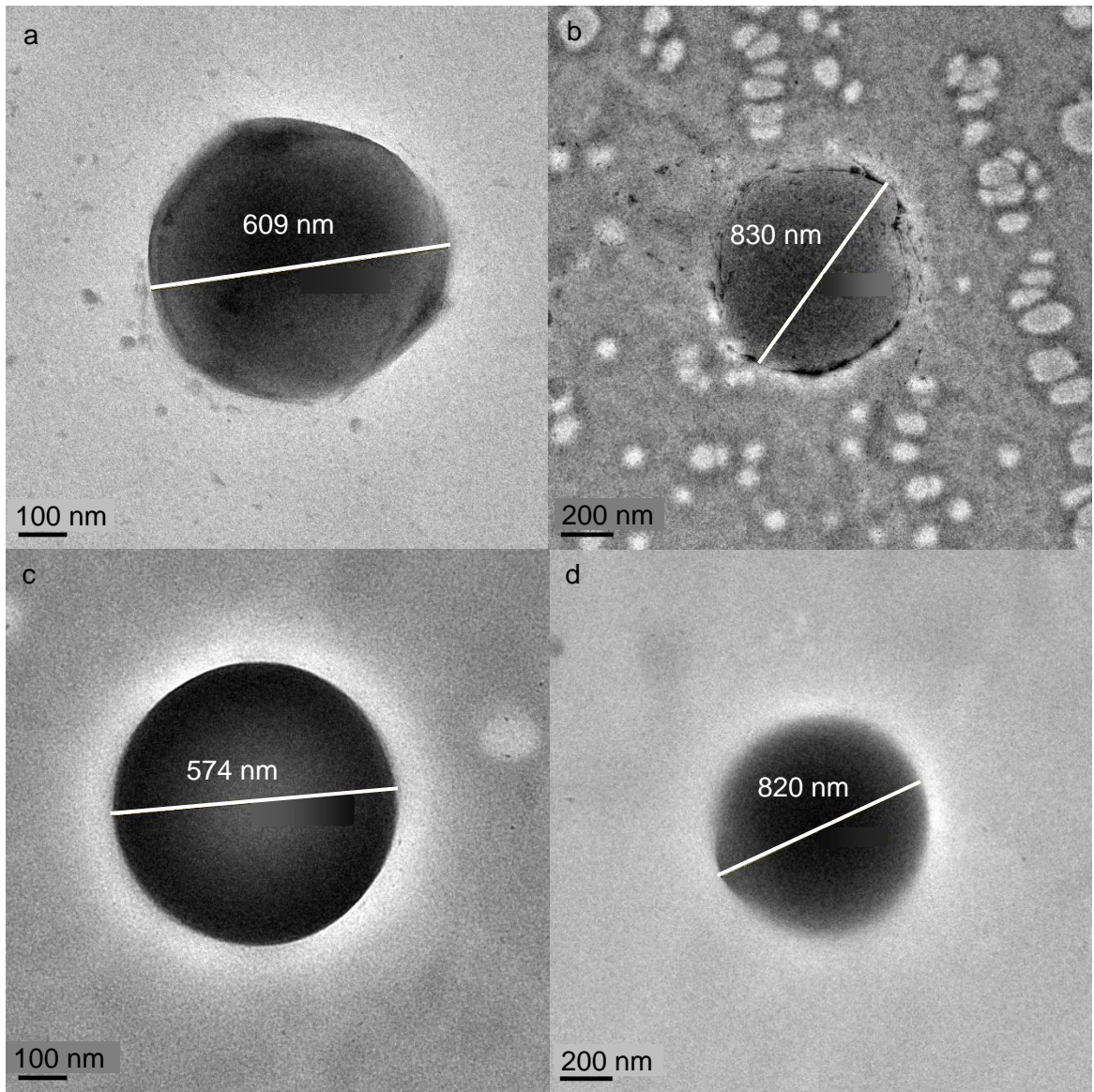
### **3 Results**

#### **3.1 Preparation of amorphous forms**

The preparation of the glassy form of roxithromycin produced a compound which had the appearance of tiny glass flakes. The chloroform desolvate of roxithromycin also resulted in an amorphous form with a rough granular appearance. After viewing the amorphous forms through the polarizer of the light microscope, no birefringence existed. The aqueous solubility of the three solid-state forms in PBS (pH 7) at 32 °C were in very close range of one another with a value of 1.98 mg/ml for roxithromycin monohydrate, 1.88 mg/ml for the glassy roxithromycin amorphous form and 1.92 mg/ml for the chloroform desolvated amorphous form of roxithromycin. The log D for roxithromycin was found to be 1.52 which was optimal for topical delivery<sup>9</sup>.

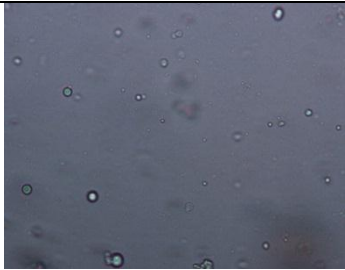
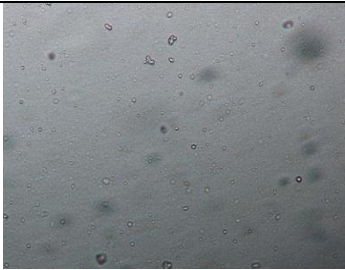

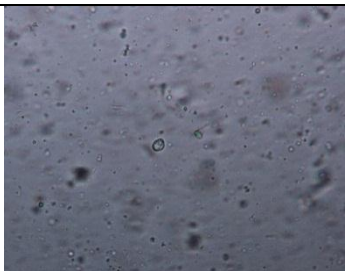

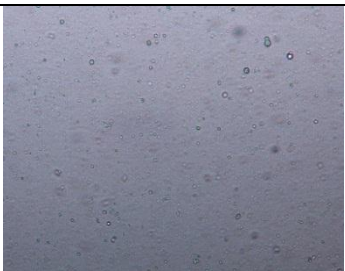





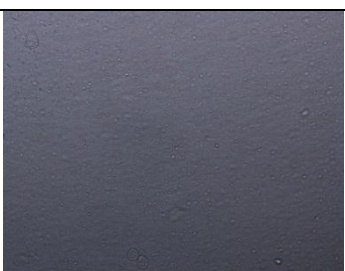
#### **3.2 Decision for optimal vesicle formulations**

The TEM images in Figure 1 reveal that all four samples of non-sonicated vesicles without API did indeed form vesicles.



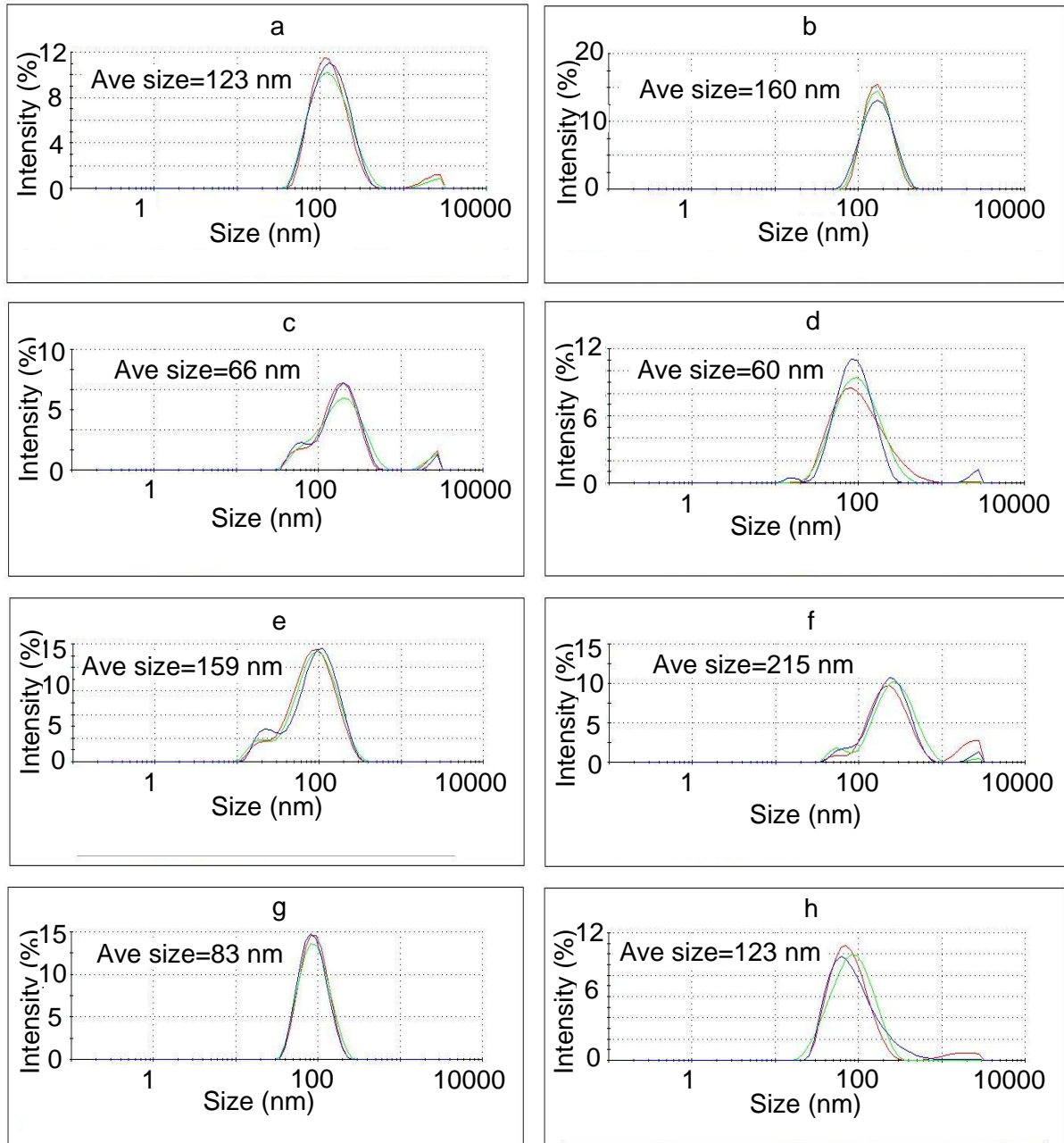
**Figure 1**

**Table IV:** Photomicrographs of vesicle appearances using light microscopy

| Ratio       | No sonication   | 2.0 min sonication   | 3.5 min sonication  |
|-------------|---|--|---|
| Niosome 2:1 |    |    |    |
| Niosome 1:1 |    |    |    |
| Ufosome 2:1 |   |   |   |
| Ufosome 1:1 |  |  |  |

It can be seen from Table IV that all sonicated samples still contained vesicles. The micrographs indicate the non-sonicated samples of all formulations had larger droplets. The niosomes sonicated for 2.0 min and 3.5 min had very similar micrographs while for ufosomes, the samples sonicated for 3.5 min appeared to have smaller and more uniform droplets than those sonicated for only 2.0 min.

Reading 1 — Reading 2 — Reading 3 —



**Figure 2**

The droplet size of the non-sonicated samples could not be measured as the data quality was too poor for accurate readings and this ruled them out as potential formulas. The remaining eight sonicated formulas were tested. From Figure 2 it can be seen that both niosomes with a lipid ratio of 2:1 (a) and 1:1 (b) sonicated at 2.0 min displayed very good droplet size distribution with droplet sizes of 123 nm and 160 nm, respectively. Niosomes sonicated for 3.5 min for ratios 2:1 (c) and 1:1 (d) had poor droplet size distribution. It was clearly evident that ufosomes 2:1 sonicated for 3.5 min (g) had the best droplet size distribution for the ufosomes, with an

average droplet size of 83 nm. Ufosome ratios of 2:1 (e) and 1:1 (f) sonicated for 2.0 min had poor distribution and ufosome ratio 1:1 (h) sonicated for 3.5 min had poor repeatability of results.

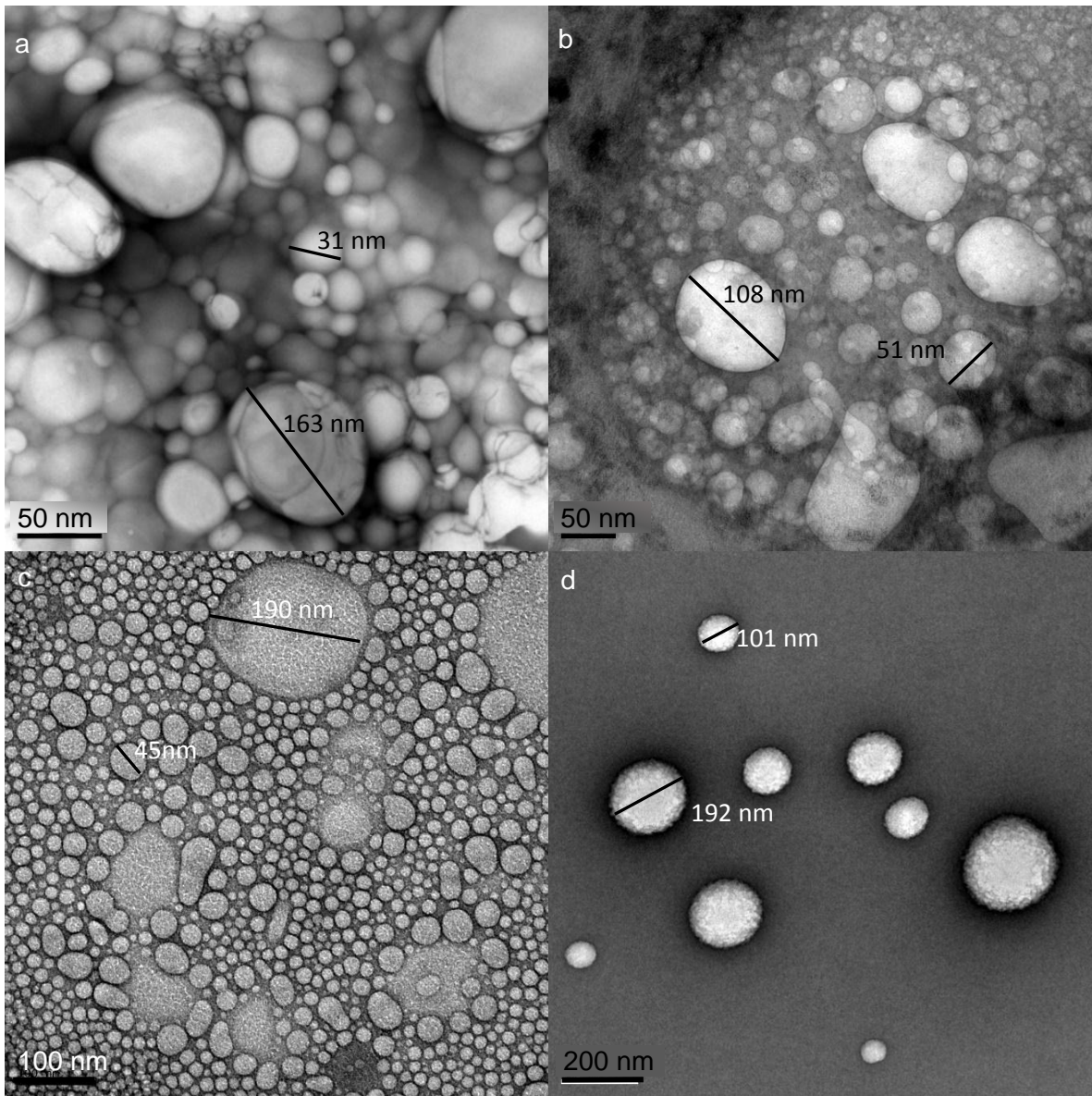
Table V contains the EE% of the potential niosome and ufosome formulations as well as their physical appearance after the drug loading.

**Table V:** EE% and physical appearance of potential niosome and ufosome formulas

| <b>Niosomes</b>                      |                 |                            |
|--------------------------------------|-----------------|----------------------------|
| <b>Span 60:cholesterol:API</b>       | <b>Average%</b> | <b>Physical appearance</b> |
| 2.0:1.0:0.5                          | 51.68           | Good suspension            |
| 2.0:1.0:1.0                          | 59.81           | Good suspension            |
| 2.0:1.0:2.0                          | 52.16           | Good suspension            |
| 2.0:1.0:4.0                          | 63.84           | Good suspension            |
| <b>Ufosomes</b>                      |                 |                            |
| <b>Sodium oleate:cholesterol:API</b> | <b>Average%</b> | <b>Physical appearance</b> |
| 2.0:1.0:0.5                          | 38.69           | Good suspension            |
| 2.0:1.0:1.0                          | 84.36           | Good suspension            |
| 2.0:1.0:2.0                          | 92.99           | Thickish gel               |
| 2.0:1.0:4.0                          | 94.31           | Thick gel                  |

### 3.3 Characterization of vesicles

Figure 3 illustrates the TEM photomicrographs of the morphology of all four optimal vesicle systems containing no API.



**Figure 3**

All the niosome and proniosome images taken using light microscopy looked similar to the niosomes (2:1) sonicated for 2.0 min photomicrographs taken during the decision for optimized formulas and all the ufosome and pro-ufosome formulations appeared the same as ufosomes (2:1) sonicated for 3.5 min images, as seen in Table IV.

**Table VI:** Summary of characterization results of twelve optimal vesicle systems

| Vesicles | API         | Average zeta-potential | Average droplet size | Average pH | Average %EE |
|----------|-------------|------------------------|----------------------|------------|-------------|
| 1        | Monohydrate | -50.06±17.81           | 164.08±51.17         | 6.96±0.06  | 77.78±3.27  |
|          | Glass       | -42.28±13.70           | 160.56±52.52         | 7.16±0.11  | 83.42±4.37  |
|          | Desolvate   | -53.80±17.26           | 127.49±27.14         | 7.21±0.22  | 82.74±3.15  |
| 2        | Monohydrate | -68.42±7.07            | 125.57±49.68         | 7.27±0.04  | 66.64±8.99  |
|          | Glass       | -65.24±2.92            | 77.68±49.96          | 7.26±0.06  | 50.88±7.61  |
|          | Desolvate   | -56.22±5.07            | 121.92±18.14         | 7.23±0.01  | 63.83±5.67  |
| 3        | Monohydrate | -73.16±11.11           | 156.66±14.69         | 8.73±0.11  | 96.27±1.24  |
|          | Glass       | -99.62±24.37           | 140.33±10.34         | 8.52±0.23  | 96.51±0.77  |
|          | Desolvate   | -100.3±21.99           | 112.94±14.42         | 8.78±0.04  | 94.58±2.06  |
| 4        | Monohydrate | -80.27±17.99           | 102.50±10.27         | 8.56±0.05  | 93.41±2.59  |
|          | Glass       | -87.92±13.93           | 70.48±8.07           | 8.46±0.39  | 92.81±1.70  |
|          | Desolvate   | -72.04±4.75            | 100.09±26.77         | 8.51±0.08  | 94.73±1.91  |

1 niosome; 2 proniosomes, 3 ufosomes and 4 pro-ufosomes

The zeta-potential, droplet size, pH and EE% of all twelve formulations are summarized in Table VI. The droplet size distribution of the vesicles all looked very similar to the droplet distribution curves seen earlier in Figure 2, with niosomes' and proniosomes' droplet distribution resembling that of (a) and ufosomes and pro-ufosomes resembling (g). The pH of the lipid surface of niosomes and proniosomes was about 7 for each of the formulas, whilst ufosomes and pro-ufosomes had a pH of about 8.5.

### 3.4 Membrane release studies

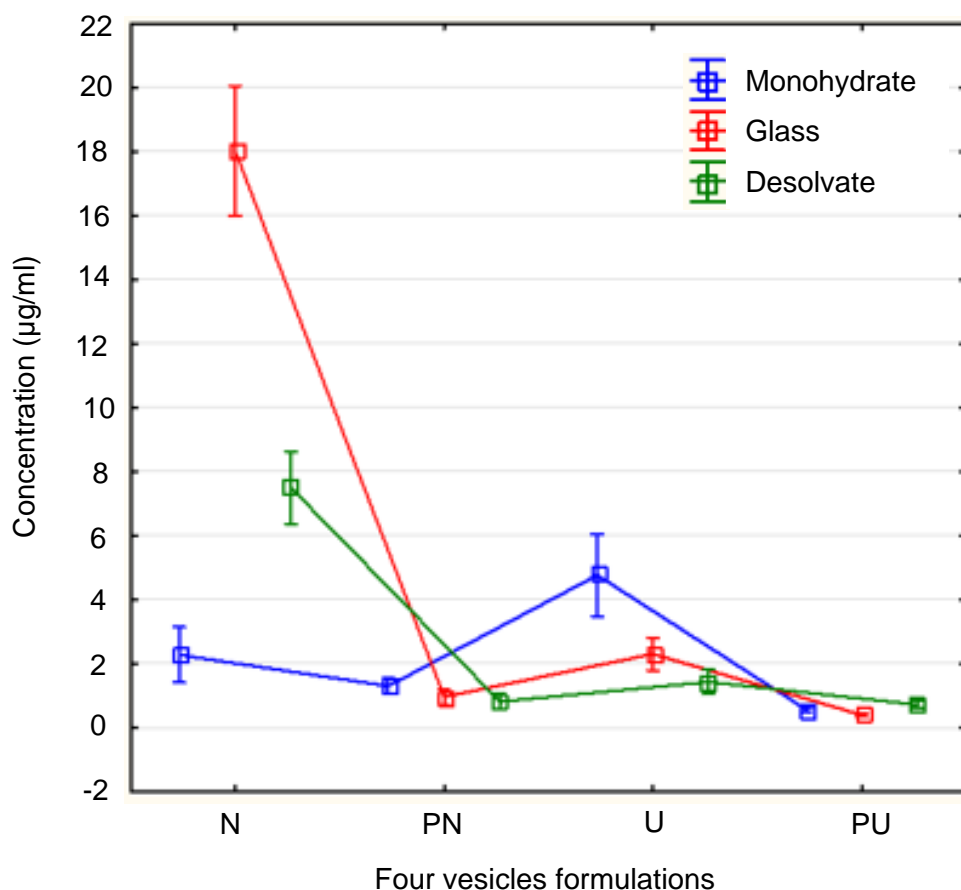
The average flux for each vesicle system, as well as the average percentage, released after a 6 h membrane release study can be seen in Table VII.

**Table VII:** Average flux and average %diffused for all formulations after a 6 h membrane release study

| <b>Formulation</b>      | <b>Average flux<br/>(<math>\mu\text{g}/\text{cm}^2\cdot\text{h}</math>)</b> | <b>Average %API<br/>diffused</b> |
|-------------------------|---|----------------------------------|
| Niosome monohydrate     | 184.54 $\pm$ 19.32  | 3.11 $\pm$ 0.31                  |
| Niosome glass           | 169.86 $\pm$ 18.29  | 2.79 $\pm$ 0.32                  |
| Niosome desolvate       | 183.50 $\pm$ 18.45  | 3.22 $\pm$ 0.30                  |
| Proniosome monohydrate  | 90.14 $\pm$ 8.47  | 1.62 $\pm$ 0.15                  |
| Proniosome glass        | 112.34 $\pm$ 10.12  | 1.80 $\pm$ 0.16                  |
| Proniosome desolvate    | 75.62 $\pm$ 10.42   | 1.26 $\pm$ 0.15                  |
| Ufosome monohydrate     | 20.75 $\pm$ 3.87  | 0.36 $\pm$ 0.07                  |
| Ufosome glass           | 21.29 $\pm$ 2.36  | 0.36 $\pm$ 0.04                  |
| Ufosome desolvate       | 17.58 $\pm$ 2.89  | 0.28 $\pm$ 0.05                  |
| Pro-ufosome monohydrate | 56.29 $\pm$ 10.64   | 0.81 $\pm$ 0.13                  |
| Pro-ufosome glass       | 25.72 $\pm$ 1.39  | 0.45 $\pm$ 0.02                  |
| Pro-ufosome desolvate   | 29.22 $\pm$ 4.12  | 0.55 $\pm$ 0.08                  |

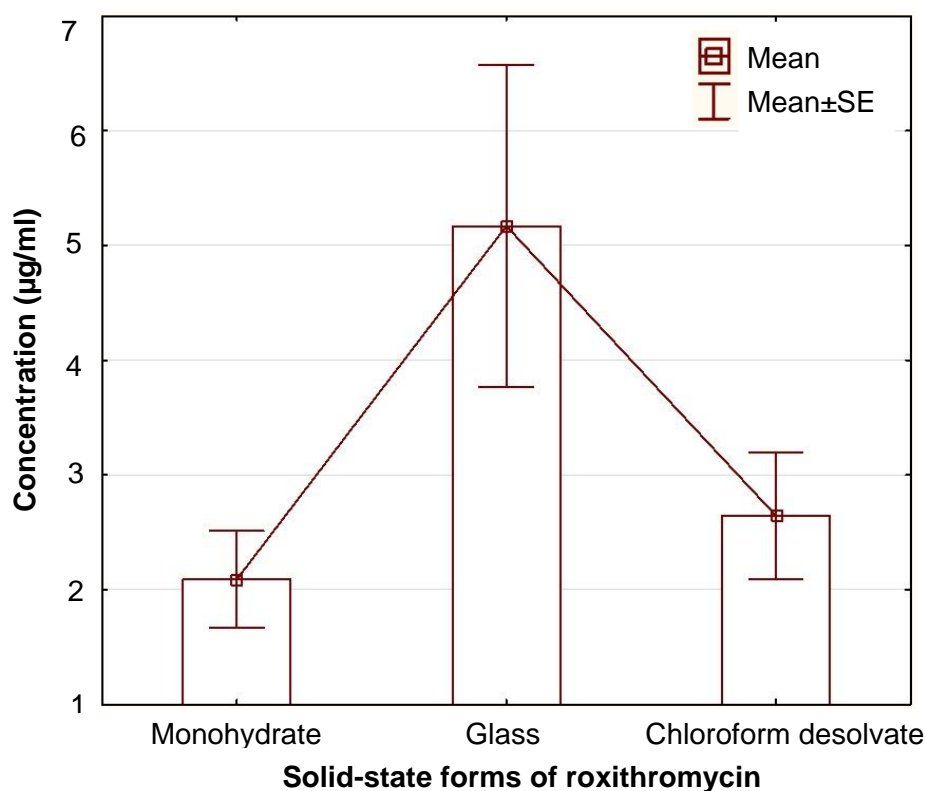
### 3.5 Skin diffusion studies

The %API diffused in the receptor phase was only detected in four of the formulations, namely niosomes with glass (0.0053 $\pm$ 0.0003%; n = 7), niosomes with desolvate (0.0031 $\pm$ 0.0007%; n = 10), proniosomes encapsulating roxithromycin monohydrate (0.0133 $\pm$ 0.0035%; n = 10) and pro-ufosomes containing roxithromycin monohydrate (0.0104 $\pm$ 0.0051%; n = 10). No API was found in the stratum corneum-epidermis layer of the skin.



**Figure 4**

Figure 4 is a graph derived from the ANOVA statistical analysis of the concentrations of API found in the epidermis-dermis skin layer after the 12 h diffusion study. In order from highest to lowest API delivery was niosomes glass ( $18.016 \pm 5.36 \mu\text{g/ml}$ ), niosomes desolvate ( $7.497 \pm 3.57 \mu\text{g/ml}$ ), ufosomes monohydrate ( $5.445 \pm 3.23 \mu\text{g/ml}$ ), ufosomes glass ( $2.303 \pm 1.45 \mu\text{g/ml}$ ), niosomes monohydrate ( $2.273 \pm 2.58 \mu\text{g/ml}$ ), ufosomes desolvate ( $1.432 \pm 1.12 \mu\text{g/ml}$ ), proniosomes monohydrate ( $1.304 \pm 0.73 \mu\text{g/ml}$ ), proniosomes glass ( $0.968 \pm 0.66 \mu\text{g/ml}$ ), proniosomes desolvate ( $0.816 \pm 0.73 \mu\text{g/ml}$ ), pro-ufosomes desolvate ( $0.719 \pm 0.33 \mu\text{g/ml}$ ), pro-ufosomes monohydrate ( $0.569 \pm 0.22 \mu\text{g/ml}$ ) and pro-ufosome glass ( $0.400 \pm 0.16 \mu\text{g/ml}$ ) with the least amount of API. The results of the comparison of the three solid-state forms of roxithromycin, regardless of the vesicle type it was encapsulated in, is illustrated in Figure 5.



**Figure 5**

### 3.6 Statistical analysis

Results obtained from the Kruskal-Wallis ANOVA by ranks analysis of API concentrations found in the epidermis-dermis, illustrated the statistical significance between formulation pairs. There was only statistical significance for the niosome ( $p = 0.0001$ ) and the ufosome ( $p = 0.0128$ ) vesicle systems. The multiple comparisons tests performed on niosomes indicated showed that there was a statistical difference between roxithromycin monohydrate and the glass ( $p = 0.00005$ ) and for ufosomes there was a difference between the roxithromycin monohydrate and the desolvate ( $p = 0.01035$ ). The remaining pairs had no statistical significances ( $p > 0.05$ ).

The Kruskal-Wallis ANOVA by ranks was also tested on all three solid-state forms which proved to have significant differences with roxithromycin monohydrate ( $p = 0.002$ ), roxithromycin glass ( $p = 0.000$ ) and roxithromycin desolvate ( $p = 0.0000$ ). For the multiple comparisons tests on roxithromycin, there was only a significant difference for ufosomes and pro-ufosomes ( $p = 0.000966$ ), while the glass API group had a difference between the niosomes and proniosomes ( $p = 0.003279$ ), niosomes and pro-ufosomes ( $p = 0.000027$ ) and ufosomes and pro-ufosomes

( $p = 0.024711$ ). The niosomes and proniosomes ( $p = 0.000056$ ) and niosomes and profosomes ( $p = 0.000193$ ) had statistical differences in the desolvate amorphous group. The remaining groups displayed no significant differences.

## **4 Discussion**

### **4.1 Preparation of amorphous forms**

The two amorphous forms were successfully prepared and displayed no birefringence when viewed with a microscope under polarized light, which concluded they were pure amorphous forms without the presence of the crystalline form of roxithromycin <sup>26</sup>. The experimental results mentioned in the patents of Liebenberg *et al.* <sup>14</sup> and Liebenberg & Aucamp <sup>15</sup>, indicated that the roxithromycin amorphous forms exhibited increased aqueous solubility, but in this study the values were all similar to one another. It is suggested that during this 24 h solubility experiment, performed in PBS (pH 7) at 32 °C, the amorphous forms converted back to the stable crystalline form which resulted in the close solubility values <sup>13</sup>. The log D value of roxithromycin showed to be within the optimal range (log D of 1-3) for topical delivery <sup>9</sup>. The value obtained in this study correlated well with the results found in literature (log D of 1.7) <sup>19</sup>.

### **4.2 Decision for optimal vesicle formulations**

The TEM images indicated that niosomes with a lipid ratio of 2:1 displayed better membrane formation than the niosomes ratio 1:1. Both formulations formed almost perfect spheres. The ufosomes formed perfect spheres with thick uniform membranes, but ufosomes ratio 2:1 appeared to have a stronger lipid membrane than ufosomes 1:1.

The TEM revealed all four formulations did produce vesicles, but proof that vesicles still existed after different sonication times was obtained by using light microscopy on the same non-sonicated and sonicated samples. With a magnification of 50x, only the larger droplets could be seen and it must be kept in mind that there were many other smaller molecules present in each formulation.

A better indication of droplet size was measuring the droplet size and droplet size distribution. For a stable formulation, a narrow uniform droplet size distribution is optimal <sup>21</sup>, therefore sodium oleate:cholesterol lipid ratio of 2:1, sonicated for 3.5 min, was chosen as the optimal lipid formula for ufosomes. The ufosomes 2:1 also displayed a stronger lipid membrane during TEM analysis than the 1:1 ratio. The niosomes ratio 2:1 also appeared to have a better formed membrane than the 1:1 ratio. Niosomes had two formulas which revealed optimal droplet distribution, but the Span 60:cholesterol 2:1 sonicated for 2.0 min was chosen as the optimal

niosome lipid preparation based on the ufosome decision. By having the same lipid ratios for both niosomes and ufosomes, unnecessary variations were minimized in order to make comparisons between the two vesicles easier.

The last step in the decision for an optimal formula was loading an appropriate concentration of the API into the chosen lipid ratios. The EE% of four possible concentrations of roxithromycin in niosomes and ufosomes was tested. All the niosome ratios had fairly close EE% with good physical appearances. The ratio of 2:1:1 had fairly good entrapment and increasing the amount of drug had little or no increase in EE%. The ufosomes' EE% increased as the concentration of drug increased, but the physical appearance of the ratios 2:1:2 and 2:1:4 were poor with the formation of thickened gels. As a result, the ratio of 2:1:1 for both niosomes (59.81 EE%) and ufosomes (84.36 EE%) were chosen as the final optimal formulas for each vesicle system and the preparation of proniosomes and pro-ufosomes were also based on these ratios.

#### **4.3 Characterization of vesicles**

The TEM revealed that all final formulations prepared had indeed formed vesicles and the images compared well to those obtained by Ammar *et al.*<sup>28</sup>. From the photomicrographs, niosomes displayed a dense arrangement of vesicles with various sizes; the cracks seen in the membranes may be due to the fusion of smaller vesicles. The pro-niosomes also consisted of various spherical vesicles. The ufosomes had a large amount of very densely packed vesicles mostly in the 50 nm size range, with a few larger particles in between. The pro-ufosomes appeared to be more spaced out with a rougher membrane appearance than the rest of the carriers.

The results obtained from the light microscopy of all the vesicles encapsulating the three solid-state forms of roxithromycin was similar to the photomicrographs taken of the optimal formulas during the process of deciding on the optimized formulas, as discussed above. The droplet size of the sonicated samples was very small, so the particles seen using light microscopy with magnification of 50x were not very clear.

The zeta-potential values of all carrier systems were well into the negative axis, which concluded that the chances of aggregation and fusion of any of the vesicles was rather slim and that all the membranes were stable<sup>21,29</sup>. The ufosomes and pro-ufosomes were slightly more

charged than the niosomes and proniosomes and as a result, the particles had a stronger repelling force on one another.

The droplet size distribution of the vesicles revealed narrow distribution curves, which had a positive influence on the stability of the vesicles <sup>21</sup>. Niosomes and proniosomes sonicated for 2.0 min had a fairly larger average particle size than the ufosomes and pro-ufosomes sonicated for 3.5 min, which was attributed to the different sonication times needed for good size distribution curves.

The pH of all the systems illustrated niosomes and proniosomes had a slightly lower pH than ufosomes and pro-ufosomes, probably due to the latter consisting of sodium oleate rather than Span 60. Nevertheless, all systems had a pH very close to that of the buffer (pH 7.4) used in experiments which guaranteed no crystallization or salt formation.

Lastly, the entrapment efficiency of all systems illustrated that ufosomes and pro-ufosomes had the best entrapment efficiency, followed by niosomes and proniosomes. The better entrapment efficiency can be explained by referring to a similar study conducted by Agarwal *et al* <sup>29</sup>. The difference seen was attributed to the API having greater affinity to the sodium oleate of ufosomes and pro-ufosomes than to the Span 60 of niosomes and proniosomes, which resulted in more API being entrapped in the aforementioned. It should also be mentioned that not one solid-state form had the best overall entrapment as it varied for each type of vesicle.

#### **4.4 Membrane release studies**

The membrane release studies revealed API was released from all twelve formulations and this ruled out any problems related to the release from vesicles if no API was found in the skin during topical diffusion studies. The release results illustrated that the API was better released from the niosomes and proniosomes, which again was due to the API having lower affinity to the Span 60 than for sodium oleate (ufosomes and pro-ufosomes) <sup>33</sup>. In general, a trend according to the type of vesicle system can be seen with niosomes having by far the best release, followed by proniosomes, then pro-ufosomes and ufosomes having the least amount of API release of all vesicles. The release trend was grouped according to vesicle types, whose results were very close, rather than the solid-state form of API as no form had a leading release concentration. It was important to remember that the samples with the best release were not

necessarily expected to have the best topical diffusion as there were other factors which also needed to be taken into consideration.

#### **4.5 Skin diffusion studies**

The topical diffusion study was able to distinguish the concentration of roxithromycin in the receptor phase (representing the bloodstream), the stratum corneum-epidermis and the epidermis-dermis. No quantifiable amounts (only traces) of API were found in the stratum corneum-epidermis, which shows that the API successfully penetrated through this tough layer and only four vesicles showed small API concentrations in the receptor phase, which may have been because of differences in the hydration, damage and age of skin used from different donors causing differences in API delivery <sup>9</sup>. The percentages diffused were very small but worth mentioning as they are above the lower limit of quantification (LLOQ) obtained during HPLC validation of this study (0.5 µg/ml). The topical diffusion studies revealed targeted delivery to the epidermis-dermis only, which proved very successful as this is the target area for roxithromycin to have activity against acne causing micro-organisms <sup>7,8</sup>.

Once again, as with the membrane studies, there was a noticeable trend which was grouped together by vesicle type rather than by the type of solid-state form of roxithromycin encapsulated. The niosomes were the vesicle system which had the best overall topical delivery regardless of which solid-state form it contained, the ufosomes had the second best API diffusion, followed by the proniosomes and lastly, the pro-ufosomes. When comparing the delivery of the four vesicle systems, roxithromycin monohydrate had the highest delivery from the ufosomes, while the glass and chloroform desolvate were best delivered from the niosomes. There are many factors to take in to consideration when discussing the reason for this result. Firstly, niosomes may have had better API delivery than the ufosomes because of the better API release from the formulas which resulted in a larger concentration for diffusion from the surface of the stratum corneum to the epidermis-dermis <sup>9</sup>. The same can be said about the proniosomes and their better topical delivery in comparison to pro-ufosomes. As mentioned above, the affinity of the API for the sodium oleate (ufosomes and pro-ufosomes) was much greater than for the Span 60 (niosomes and proniosomes), which illustrated that even though the ufosomes and pro-ufosomes had the best entrapment efficiencies (which usually results in

better delivery), the API was poorly released because of its great affinity to these vesicles <sup>33</sup>. Ufosomes had the second best API topical delivery despite their poor release from vesicles, which shows that the API from these vesicles had exceptionally good diffusion through the skin as many more droplets had to release API for a fair amount to diffuse to the target area compared to niosomes and proniosomes which had excellent release. This good diffusion may be attributed to the many very small particles being densely packed, as illustrated on the TEM images, compared to the other vesicles which made it possible for many droplets to line the stratum corneum therefore increasing the available surface area for API release <sup>33</sup>. The morphology of the vesicles also revealed the “cracks” in the niosomes’ membranes, which may have contributed to the easier release of API from these vesicles. The properties of the ingredients of the vesicles also played a major role in the topical delivery. The Span 60 (niosomes and proniosomes) had a lower melting point and as a result, a better solubility than the sodium oleate (ufosomes and pro-ufosomes) <sup>34,35</sup>. Compounds with lower melting points, along with better solubility, tend to have better skin penetration and topical delivery and this explains why the API from niosomes had better delivery than ufosomes and proniosomes had better delivery than pro-ufosomes <sup>9</sup>. Components of the Span 60 membranes may have also diffused after the release of the API due to its good solubility and its penetration enhancing effect. The Span 60 and sodium oleate are also known as penetration enhancers for lipophilic drugs and each ingredient may have had different degrees of penetration enhancement <sup>33</sup>. Span 60 and sodium oleate are also known as gelling agents which have the ability to form gels when added to other components <sup>36,37</sup>. The reason for the proniosomes and pro-ufosomes having the lowest topical delivery may be due to the thickening of both formulas after the 12 h diffusion studies. The thick gel prevented optimal release and delivery, which may only have occurred with these two formulas because of the extra sorbitol powder which contributed to the total mass of the systems.

When a comparison was done between the better topical deliveries of the three solid-state forms of roxithromycin, regardless of which vesicle it was in, the two amorphous forms of roxithromycin had higher topical delivery than the crystalline monohydrate. The roxithromycin

glass was by far the form which had the highest dermal concentration, followed by roxithromycin chloroform desolvate and in last place, was the crystalline roxithromycin monohydrate.

The results correlated well with the aim of this study for improving the topical delivery of the roxithromycin crystalline form by using its two amorphous forms. The increased topical delivery results suggested that the amorphous forms retained their theoretical improved solubilities, determined by Liebenberg & Aucamp<sup>15</sup> and Liebenberg *et al.*<sup>14</sup>, during the 12 h diffusion studies while being encapsulated in the vesicles. The use of amorphous forms and vesicles proved successful in increasing topical delivery of roxithromycin.

## **5 Conclusions**

This study illustrated that roxithromycin and its two solid-state amorphous forms were capable of being entrapped into selected vesicles (niosomes, proniosomes, ufosomes and pro-ufosomes). These vesicles were all successful in releasing the drug for diffusion into the skin and all systems had targeted delivery specifically into the epidermis-dermis where the therapeutic effect of roxithromycin against *P. acnes* is needed. The niosomes, in total, delivered the highest amount of API followed by ufosomes, proniosomes and lastly, pro-ufosomes. Higher roxithromycin concentrations were found in the dermis with the amorphous forms in comparison to the crystalline form, which suggests that the amorphous forms increase the topical delivery of roxithromycin.

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## **Disclaimer**

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

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## Legends to Figures

**Figure 1:** TEM photomicrographs of formation of vesicles. a) Niosome with Span 60:cholesterol (2:1), b) niosome with Span 60:cholesterol (1:1), c) ufosome with sodium oleate:cholesterol (2:1) and d) ufosome with sodium oleate:cholesterol (1:1).

**Figure 2:** Droplet size distribution of lipid ratios of niosomes and ufosomes. a) Droplet distribution of niosomes (ratio 2:1) sonicated for 2.0 min, b) droplet distribution of niosomes (ratio 1:1) sonicated for 2.0 min, c) size distribution of niosomes (ratio 2:1) sonicated for 3.5 min, d) size distribution of niosomes (ratio 1:1) sonicated for 3.5 min, (e) droplet size distribution of ufosomes (2:1) sonicated for 2.0 min, (f) droplet distribution of ufosomes (1:1) sonicated for 2.0 min, (g) size distribution of ufosomes (2:1) with a sonication time of 3.5 min and (h) size distribution of ufosomes (1:1) with a sonication of 3.5 min.

**Figure 3:** TEM images of optimized vesicles containing no API. (a) Niosomes sonicated for 2.0 min, (b) proniosomes sonicated for 2.0 min, (c) ufosomes with a sonication time of 3.5 min and (d) pro-ufosomes sonicated for 3.5 min.

**Figure 4:** Concentration ( $\mu\text{g/ml}$ ) of the sum of three forms of the API per vehicle system delivered topically in the epidermis-dermis after the 12 h skin diffusion study. N represents the niosomes, PN represents the proniosomes, U represents the ufosomes and PU represents the pro-ufosomes.

**Figure 5:** Total sum of the epidermis-dermis concentrations ( $\mu\text{g/ml}$ ) of each solid-state form of roxithromycin after the 12 h skin diffusion studies regardless of what vesicle system it was encapsulated in.

The stratum corneum, also known as the outermost layer of the skin, is composed of many unique mixtures of lipids, making it a strong permeability barrier which counteracts components that may pass through the skin. Consequently, it serves as an important protective layer of the skin as well as the most difficult obstacle to overcome in topical and transdermal delivery (Baroni *et al.*, 2012:259). Acne is a huge problem faced by many people and the cause is attributed to the accumulation of *P. acnes* in the sebaceous glands found in the dermis (Gollnick, 2003:1585; Menon, 2002:4). Topical antibiotics have a direct effect on the *P. acnes* in the sebaceous glands and reduce the inflammation (Williams *et al.*, 2012:164). One of these newer antibiotics investigated for this purpose is roxithromycin, but its poor solubility makes it unfavourable for topical delivery (Medsafe, 2014). Due to this, its amorphous forms, with increased solubility (Liebenberg & Aucamp, 2013; Liebenberg *et al.*, 2013), were investigated in this study to determine if there was improved topical delivery, as it was found that compounds with an aqueous solubility of above 1 mg/ml had good topical/transdermal diffusion (Williams, 2003:37). Vesicle systems were also implemented to increase the penetration of the poorly soluble roxithromycin (Bansal *et al.*, 2012:704). Experiments were performed to determine optimised vesicles, to characterise preparations and to determine topical and/or transdermal diffusion through the skin.

The objectives of the study included the following:

- Development and validation of an HPLC method in order to quantitatively determine the concentration of roxithromycin in each vesicle system.
- Preparation of the glassy form of roxithromycin and the chloroform desolvated amorphous form of roxithromycin.
- Determination of the aqueous solubility and the log D of roxithromycin and its two amorphous forms.
- Optimisation of the vesicle formulations and encapsulation of the three solid-state forms into the four optimised formulas, namely, niosomes, proniosomes, ufosomes and pro-ufosomes to prepare twelve systems.
- Characterisation of the vesicle systems in terms of droplet size and distribution, zeta-potential, pH, EE%, TEM and light microscopy.
- Performing membrane studies to determine if roxithromycin and its two amorphous forms were released from the different vesicle systems.

- Performing transdermal diffusion studies followed by tape stripping to determine whether roxithromycin and its two amorphous forms were delivered systemically and/or topically, respectively.

A new HPLC method was developed and validated with the expertise of Dr Marique Aucamp. This method proved to be accurate, repeatable, precise and reliable for its use throughout this study and could successfully analyse the concentrations of API in all experimental samples, including the samples from the Franz cells during diffusion studies.

The glassy amorphous form of roxithromycin and the chloroform desolvated form of roxithromycin were both prepared and images of the solid-state forms, viewed through a polariser of a light microscope, proved they were indeed pure amorphous forms as they exhibited no birefringence as seen with the crystalline form (Stieger *et al.*, 2012).

The aqueous solubility of all three solid state forms were tested in phosphate buffer solution (PBS) (pH 7 at 32 °C). The expected result of the amorphous forms having increased solubility as found with Liebenberg & Aucamp (2013) and Liebenberg *et al.* (2013) did not correlate, as all the values in this experiment were more or less the same. The similar values suggest the amorphous forms converted back to their stable crystalline form while in PBS solution for 24 h (Purohit & Venugopalan, 2009:891). The log D was calculated as 1.52, which is in the optimal range of 1 - 3 for topical delivery (Williams, 2003:36).

The optimised lipid formulas for niosomes and ufosomes were tested using the TEM, light microscopy and droplet size distribution on four potential formulas of each. Once an optimal lipid ratio with a chosen sonication time was determined, four different concentrations of roxithromycin monohydrate were encapsulated in niosomes and ufosomes and the EE% of each was determined. The formula with a high EE% along with a good physical appearance was chosen as the optimal formula. The formulas for the proniosomes and pro-ufosomes were based on the results of optimised formulas for the niosomes and ufosomes. The decided ratio for niosomes and pro-niosomes was Span 60:cholesterol:roxithromycin (2:1:1) and the optimal formula for the ufosomes was sodium oleate:cholesterol:roxithromycin (2:1:1). The three solid-state forms of roxithromycin were each encapsulated into one of the four vesicle systems to yield twelve preparations for characterisation and for the release and diffusion studies.

The characterisation of all twelve vesicles showed very similar results to those seen with the optimised formulas. The droplet size and distribution for each sample was small and narrow which ensured optimal system stability (Kumar & Rajeshwarrao, 2011:213). The droplet size of the ufosomes and pro-ufosomes was smaller than that of the niosomes and proniosomes, as a longer sonication time was needed to achieve good distribution. The zeta-potential of all systems were highly negative, indicating a very slim chance of aggregation and fusion of

vesicles (Agarwal *et al.*, 2001:44). The pH of all the systems was relatively close to 7.4 (pH of PBS used), which suggested no salt formation or precipitation of vesicles occurred during diffusion studies. The EE% revealed that ufosomes and pro-ufosomes had the highest entrapment, followed by the niosomes and proniosomes. The reason for this was that the API had a higher affinity for the ufosome and pro-ufosome carriers that were prepared using sodium oleate and therefore they entrapped more API, which was also seen in a similar study conducted by Agarwal *et al.* (2001:49). The TEM and light microscopy images showed well-formed vesicles with ufosomes having the smallest, most densely packed arrangement of vesicles and niosomes having small cracks in the membranes. In general, the characterised vesicles showed optimal properties.

The membrane release studies confirmed the API was successfully released from all the vesicle systems and that release was ruled out as a problem if no topical delivery was achieved. Niosomes had the best release of roxithromycin and its two amorphous forms, proniosomes the second most release, followed far behind by the ufosomes and pro-ufosomes. The lower release of the latter two can again be attributed to the fact that the API had higher affinity for the sodium oleate than for the Span 60, as seen earlier with the EE% results (Dayan, 2005:74).

The skin diffusion studies showed no API was found in the stratum corneum-epidermis and no API was found in the majority of receptor phases which concluded that the API penetrated the stratum corneum-epidermis but did not have systemic delivery. All formulations showed API concentrations in the epidermis-dermis, which was the intended target site for this study. Niosomes was the leading formulation, followed by ufosomes and then proniosomes and pro-ufosomes. The different solid-state forms encapsulated in a single type of vesicle illustrated very similar results, which proved to be a challenge in determining the better solid-state form, but when combining all the results obtained for all vesicles, the two amorphous forms showed better topical delivery than the crystalline monohydrate form of roxithromycin. This suggests that the glass and chloroform desolvate, along with their theoretically improved solubilities retained their amorphous habit during the diffusion studies and consequently, resulted in better topical delivery.

Future recommendations include:

- Comparison of results obtained in this study to roxithromycin solid-state forms not encapsulated in vesicles.
- Test if the amorphous forms remained amorphous after encapsulation into vesicles.
- Formulation of vesicles encapsulating roxithromycin into optimised topical formulations such as creams, gels, ointments for testing in diffusion studies.
- Clinical testing of vesicles encapsulating the API to determine the effect of physiological factors on diffusion.

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# Method validation for HPLC analysis of roxithromycin

*Annexure  
A*

## A.1 Introduction

The validation of an analytical method must be done in order to give evidence that it effectively meets the particular requirements for its intended purpose and ensures reliable results (Araujo, 2009:2224). A validation was conducted to ensure that the analyte could be identified in samples tested throughout the study and this was done by testing samples potentially containing an API against a known concentration of a reference standard (ICH, 2005:1). The validation ensured that the analytical method used for the detection of the API was sensitive and reliable for its intended use (FDA, 2001:2). HPLC was used to analyse and detect roxithromycin encapsulated in vesicle systems for topical delivery.

## A.2. Chromatographic conditions

The analytical method was developed and performed with the assistance of Dr Marique Aucamp from the North-West University (NWU), Potchefstroom. The analysis was performed in a controlled laboratory environment, at a room temperature of 25 °C (ambient temperature). The parameters used in this method are as follows:

**Analytical instrument:** A Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system was 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 done using LabSolutions, LCsolution, Release 1.21 SP1 (Shimadzu, Kyoto, Japan).

**Column:** A Luna C<sub>18</sub> 150 × 4.6 mm column with a 5 µm particle size was used.

**Mobile phase:** The mobile phase consisted of 0.06 M potassium di-hydrogen orthophosphate (KH<sub>2</sub>PO<sub>4</sub>) buffer solution with the pH adjusted to 7.4 with sodium hydroxide (NaOH) solution. The buffer was mixed with acetonitrile in the ratio 50:50.

**Flow rate:** 1 ml/min

**Injection volume:** 10 µl

**Detection wavelength:** 205 nm

**Retention time:** 4.1 min  
**Stop time:** 10.0 min  
**Solvent:** The solvent used in this study consisted of 0.06 M KH<sub>2</sub>PO<sub>4</sub> buffer solution adjusted to pH 7.4 with NaOH.

### A.3 Standard preparation

Standard solutions were prepared by accurately weighing an amount of roxithromycin reference standard and dissolving it in the solvent. In total, nine standard solutions were prepared with concentrations of 5, 10, 25, 50, 100, 250, 500, 750 and 1000 µg/ml. The standard solutions were filtered through a 0.22 µm hydrophilic polyvinylidene fluoride (PVDF) filter into HPLC vials for analysis.

### A.4 Validation parameters

The following parameters were used to validate the analytical method: linearity; limit of detection (LOD) and lower limit of quantification (LLOQ); accuracy; precision; ruggedness and specificity.

#### A.4.1 Linearity

Linearity of an analytical method can be defined as the ability to quantitatively determine results directly proportional to the amount of an API present in a certain range of samples (ICH, 2005:5). In order to prove the method is linear, a specific concentration range should be set (Howard, 2003:8). The range for roxithromycin was 5 - 1000 µg/ml. Nine different concentrations of standards, as mentioned in Section A.3, were prepared and each standard was injected in duplicate. The linearity of the method was determined by plotting the API concentration (µg/ml) versus the peak area ratios (mean value) of all nine standards and a linear regression analysis was performed on the plot. A regression coefficient value ( $R^2$ ) close to one ( $R^2 = 1$ ) indicates that the method has high linearity and is precise and reliable for the analysis of samples (Araujo, 2009:2229). The following linear equation adapted from Araujo, (2009:2229) can be used to describe the data produced by the regression:

$$y = mx + c$$

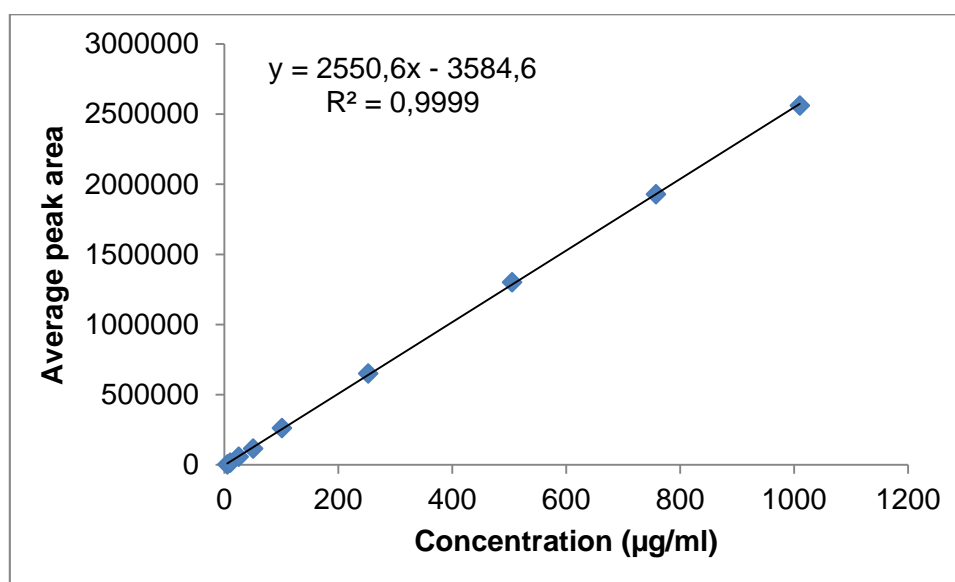
**Equation A.1**

Where:

y: peak area ratio  
m: slope of the line  
x: concentration of analyte (µg/ml)  
c: y-intercept

**Table A.1:** Linearity of roxithromycin

| Standard ( $\mu\text{g/ml}$ ) | Peak area (mean) |
|-------------------------------|------------------|
| 5.05                          | 3336.5           |
| 10.10                         | 15533.0          |
| 25.25                         | 57573.0          |
| 50.50                         | 116316.0         |
| 101.00                        | 262766.0         |
| 252.50                        | 650979.5         |
| 505.00                        | 1301035.0        |
| 757.50                        | 1928854.0        |
| 1010.00                       | 2561112.0        |
| <b>Slope (m)</b>              | 2550.6           |
| <b>y-intercept (c)</b>        | -3584.6          |
| <b>R<sup>2</sup></b>          | 0.9999           |



**Figure A.1:** Linear regression curve of roxithromycin

Table A.1 and Figure A.1 shows that a correlation coefficient ( $R^2$ ) of 0.9999 was obtained for the linearity of roxithromycin for this analytical method. This demonstrated that the method was reliable, accurate and stable within the concentration range of 5 - 1000  $\mu\text{g/ml}$ .

#### **A.4.2 Limit of detection and lower limit of quantification**

The LOD of an analytical method is the lowest amount of an API that can be detected in the sample, but cannot be quantitated as a numerical value by the analytical method. The LLOQ of an analytical procedure can be defined as the lowest possible quantity of an analyte (API) present in a sample which can be quantitatively measured with acceptable accuracy (FDA, 2001:10; ICH, 2005:5).

The LOD and LLOQ were determined by preparing different standards of low concentrations and injecting each of them six consecutive times. It is accepted that the percentage relative standard deviation (%RSD) for LOD and LLOQ should not exceed 20% and 15%, respectively. This being said, the LOD for roxithromycin was 0.3 µg/ml with a %RSD of 10.11%. The peak was detected but was not quantifiable. The LLOQ was observed as 0.5 µg/ml, with a %RSD of 4.82% which ensures minimal variation between samples while maintaining sufficient accuracy and precision. Any concentration below this value yielded an unreliable peak interrupted by noise. These results were viewed as unreliable and rejected if a concentration below the LLOQ was detected during analysis. Table A.2 shows the LOD and LLOQ results for roxithromycin. Standard is abbreviated by STD and standard deviation by SD.

**Table A.2:** Results for the LOD and LLOQ of roxithromycin

| STD<br>µg/ml | Peak<br>area<br>(1) | Peak<br>area<br>(2) | Peak<br>area<br>(3) | Peak<br>area<br>(4) | Peak<br>area<br>(5) | Peak<br>area<br>(6) | Mean     | SD     | %<br>RSD |
|--------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|----------|--------|----------|
| 0.1          | 2023                | 2591                | 0                   | 1898                | 2539                | 2340                | 1898.50  | 885.49 | 46.64    |
| 0.3          | 1872                | 1919                | 1854                | 2271                | 2292                | 2339                | 2091.17  | 211.35 | 10.11    |
| 0.5          | 3888                | 4121                | 4394                | 4338                | 4352                | 3948                | 4173.50  | 201.11 | 4.82     |
| 1.0          | 5110                | 5217                | 5403                | 5146                | 5146                | 5340                | 5227.00  | 108.52 | 2.08     |
| 1.5          | 6248                | 5934                | 6105                | 6155                | 5999                | 6346                | 6131.17  | 139.88 | 2.28     |
| 2.0          | 7956                | 7817                | 7752                | 7732                | 8151                | 7677                | 7847.50  | 161.42 | 2.06     |
| 2.5          | 13470               | 13523               | 13538               | 13655               | 13634               | 13918               | 13623.00 | 146.52 | 1.08     |
| 5.0          | 19619               | 19640               | 19537               | 19863               | 19568               | 19912               | 19689.83 | 144.36 | 0.73     |

#### A.4.3 Accuracy

Accuracy is a measure of the closeness between experimental results obtained after replicate injections and the true reference concentration of the analyte (Araujo, 2009:2226; ICH, 2005:4). Nine standards consisting of three different concentration ranges were prepared as in Section A.3. Three different solutions of each concentration range were prepared and placed into HPLC vials where they were injected in duplicate. For accuracy, expressed as percentage recovery, the mean recovery should fall between 98 - 102%. From Table A.3, it is seen that the average recovery for roxithromycin was 101.78% which concludes that this analytical method was accurate.

**Table A.3:** Results for accuracy of roxithromycin

| Concentration    | Peak area | Peak area | Mean      | Recovery         |        |
|------------------|-----------|-----------|-----------|------------------|--------|
| $\mu\text{g/ml}$ | (1)       | (2)       |           | $\mu\text{g/ml}$ | %      |
| 101.0 (1)        | 265272    | 260260    | 262766.0  | 103.4849         | 102.46 |
| 101.0 (1)        | 256748    | 257383    | 257065.5  | 101.2398         | 100.24 |
| 99.5 (1)         | 252983    | 253072    | 253027.5  | 99.6496          | 100.15 |
| 505.0 (2)        | 1295910   | 1306160   | 1301035.0 | 512.3853         | 101.46 |
| 502.5 (2)        | 1309399   | 1309183   | 1309291.0 | 515.6367         | 102.61 |
| 497.5 (2)        | 1311444   | 1314462   | 1312953.0 | 517.0789         | 103.94 |
| 1010.0 (3)       | 2554511   | 2554193   | 2554352.0 | 1005.9780        | 99.60  |
| 1005.0 (3)       | 2644926   | 2651040   | 2647983.0 | 1042.8520        | 103.77 |
| 995.0 (3)        | 2553448   | 2563550   | 2558499.0 | 997.6347         | 100.26 |
|                  |           |           |           | <b>Mean</b>      | 101.78 |
|                  |           |           |           | <b>SD</b>        | 1.57   |
|                  |           |           |           | <b>%RSD</b>      | 1.54   |

#### A.4.4 Precision

According to the ICH (2005:4), precision of an analytical method is the proximity of a series of values obtained after repeatedly applying the method to the same sample under prescribed conditions. Precision can be divided into intra-day variation and inter-day variation.

##### A.4.4.1 Intra-day variation

Intra-day variation in precision testing assesses the repeatability of the analytical method and expresses the precision within the same day under the same set conditions (Araujo, 2009:2227). Three standards of three different concentrations ranges of roxithromycin were prepared, as discussed in Section A.3. In total, nine standards were prepared in the range of 100 - 750  $\mu\text{g/ml}$  and injected in duplicate; a standard of 1000  $\mu\text{g/ml}$  was prepared for comparison. The acceptance criterion for intra-day precision is a %RSD of  $\leq 2\%$  (McPolin, 2009:84). Table A.4 shows that roxithromycin had a %RSD of 1.91%, indicating the method was precise.

**Table A.4:** Results for intra-day precision of roxithromycin

| Concentration<br>µg/ml | Peak area | Peak area | Mean      | Recovery    |        |
|------------------------|-----------|-----------|-----------|-------------|--------|
|                        | (1)       | (2)       |           | µg/ml       | %      |
| 101.00 (1)             | 265272    | 260260    | 262766.0  | 102.46      | 101.45 |
| 100.50 (1)             | 256748    | 257383    | 257065.5  | 100.24      | 99.74  |
| 99.50 (1)              | 252983    | 253072    | 253027.5  | 98.66       | 99.16  |
| 252.50 (2)             | 651298    | 650661    | 650979.5  | 253.84      | 100.53 |
| 251.25 (2)             | 671293    | 674167    | 672730.0  | 262.32      | 104.40 |
| 248.75 (2)             | 667300    | 669213    | 668256.5  | 260.57      | 104.75 |
| 746.25 (3)             | 1923041   | 1934667   | 1928854.0 | 752.12      | 100.79 |
| 753.75 (3)             | 1981367   | 1986943   | 1984155.0 | 773.68      | 102.64 |
| 761.00 (3)             | 1944662   | 1942837   | 1943750.0 | 757.93      | 99.60  |
|                        |           |           |           | <b>Mean</b> | 101.68 |
|                        |           |           |           | <b>SD</b>   | 1.94   |
|                        |           |           |           | <b>%RSD</b> | 1.91   |

**A.4.4.2 Inter-day variation**

Inter-day precision tests the variations that may occur within the exact laboratory when repeating the experiment on two or more different days (ICH, 2005:5). The variation over three days was tested in this validation. Three different samples of 500 µg/ml, as well as a 1000 µg/ml standard for comparison, were freshly prepared on each of the three consecutive days and injected in duplicate. Table A.5 shows the inter-day precision results for the three individual 500 µg/ml samples. The acceptable %RSD for inter-day precision should be < 5% (Du Preez, 2010a:7). A %RSD of 1.29% was obtained for roxithromycin which was acceptable.

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

|                 | Day 1 (%) | Day 2 (%) | Day 3 (%) | Mean between days |
|-----------------|-----------|-----------|-----------|-------------------|
|                 | (1)100.46 | (1)99.10  | (1)100.97 |                   |
|                 | (2)101.60 | (2)103.30 | (2)102.85 |                   |
|                 | (3)102.91 | (3)100.78 | (3)100.68 |                   |
| <b>Mean (%)</b> | 101.66    | 101.06    | 101.50    | 101.41            |
| <b>SD</b>       | 1.00      | 1.73      | 0.96      | 1.30              |
| <b>%RSD</b>     | 0.98      | 1.71      | 0.95      | 1.29              |

#### A.4.5 Ruggedness

Ruggedness can be divided into two tests performed on the API, namely system repeatability and stability.

##### A.4.5.1 System repeatability

System repeatability determines the precision of the method over a short time interval (Du Preez, 2010b:6). Repeatability was measured by preparing a standard with a concentration of 1000 µg/ml, as mentioned in Section A.3. The standard was then injected six consecutive times on the HPLC, all being on the same day. In order for the system to be repeatable, the peak area and retention times of the analytes should have a %RSD of ≤ 2% (Du Preez, 2010a:8). Table A.6 shows the %RSD of roxithromycin was acceptable with 0.17% for peak area and 0.10% for the retention time, respectively.

**Table A.6:** Results for system repeatability of roxithromycin

| Injection number | Peak area | Retention time (min) |
|------------------|-----------|----------------------|
| 1                | 2554193   | 4.074                |
| 2                | 2553448   | 4.062                |
| 3                | 2563550   | 4.068                |
| 4                | 2561160   | 4.068                |
| 5                | 2564565   | 4.068                |
| 6                | 2560367   | 4.073                |
| <b>Mean (%)</b>  | 2559547   | 4.07                 |
| <b>SD</b>        | 4288.39   | 0.00                 |
| <b>%RSD</b>      | 0.17      | 0.10                 |

##### A.4.5.2 Stability

The stability test is a measure of the chemical stability of an API in a given environment with specific conditions and for a certain time period (FDA, 2001:21). It is necessary to test the stability of the analyte over a period of time. A standard with a concentration of 1000 µg/ml was prepared, as discussed in Section A.3, then placed in a vial for analysis. The standard was injected every hour for a period of 24 h and the peak area of every hour was compared to the peak area at time 0 h. It is accepted that samples should not be used after the time it takes for the degradation of the analyte to fall by 2% (Du Preez, 2010a:7). Roxithromycin proved to be stable over 24 h with a %RSD of 0.40%, as seen in Table A.7. This proved the API would remain stable throughout the diffusion studies performed over 12 h.

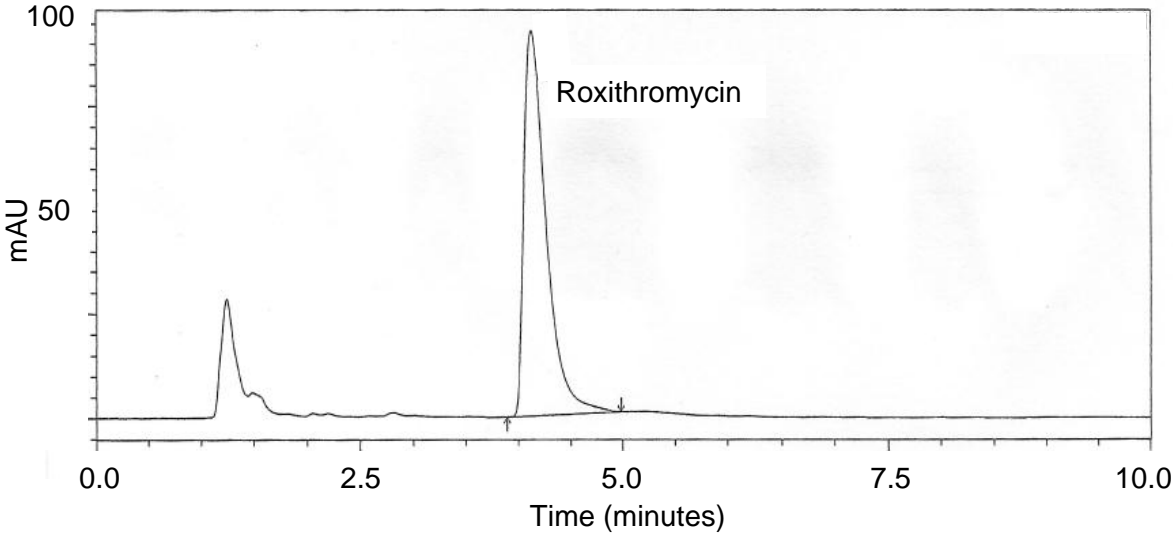
**Table A.7:** Results for stability of roxithromycin

| Time (h)        | Peak area | Recovering (%) |
|-----------------|-----------|----------------|
| 1               | 2569061   | 100.00         |
| 2               | 2567198   | 99.93          |
| 3               | 2562002   | 99.73          |
| 4               | 2566233   | 99.89          |
| 5               | 2554624   | 99.44          |
| 6               | 2575176   | 100.24         |
| 7               | 2571679   | 100.10         |
| 8               | 2560905   | 99.68          |
| 9               | 2574669   | 100.22         |
| 10              | 2553292   | 99.39          |
| 11              | 2582660   | 100.53         |
| 12              | 2550159   | 99.26          |
| 13              | 2573774   | 100.18         |
| 14              | 2568480   | 99.98          |
| 15              | 2555695   | 99.48          |
| 16              | 2570226   | 100.05         |
| 17              | 2554390   | 99.43          |
| 18              | 2557325   | 99.54          |
| 19              | 2566547   | 99.90          |
| 20              | 2561323   | 99.70          |
| 21              | 2535717   | 98.70          |
| 22              | 2550949   | 99.29          |
| 23              | 2570175   | 100.04         |
| 24              | 2576714   | 100.30         |
| <b>Mean (%)</b> |           | 99.79          |
| <b>SD</b>       |           | 0.40           |
| <b>%RSD</b>     |           | 0.40           |

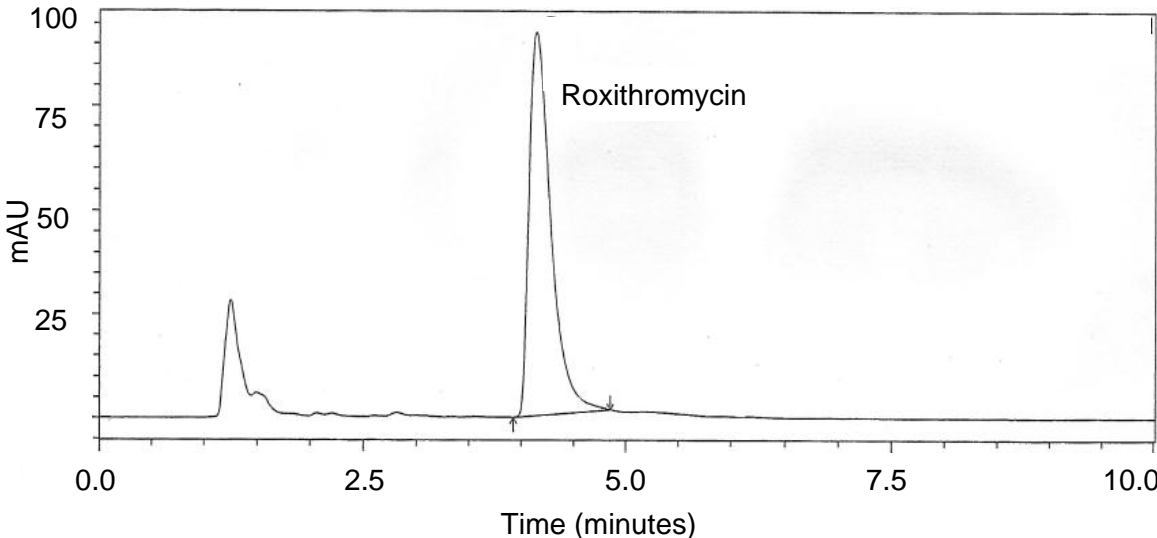
#### A.4.6 Specificity

Specificity can be defined as the assessment of an API in the presence of other expected components without any interference from those components (ICH, 2005:4). To test the specificity, a 1000 µg/ml standard was prepared, as well as two other samples. The first sample consisted of a preparation of niosome reagents such as Span 60, cholesterol, roxithromycin monohydrate, PBS (pH 7.4) and added sorbitol (ingredient present in proniosomes). The second sample contained a preparation of ufosome ingredients such as sodium oleate, cholesterol, PBS (pH 7.4), roxithromycin monohydrate and sorbitol (an extra ingredient used in the preparation of pro-ufosomes). These samples were filtered through a 0.22 µm PVDF filter

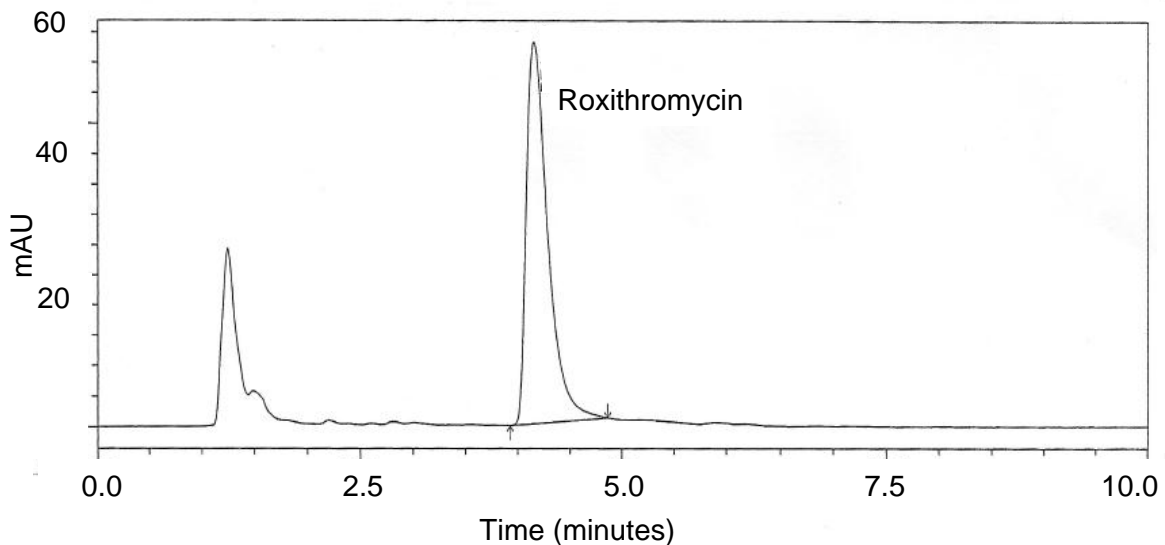
into HPLC vials and injected in duplicate. For acceptable specificity, there should be no peaks interfering with the peak of the analyte in question (McPolin, 2009:84). Figure A.2 shows the roxithromycin standard for comparison and Figures A.3 and A.4 show the results of the specificity tests of niosome, proniosome, ufosome and pro-ufosome ingredients used in preparing vesicles. It can be seen from the chromatograms that none of the additives in the vesicle systems interfered with the peak detected for roxithromycin and the peaks of the two samples appeared to be the same as the roxithromycin standard. This therefore proves specificity of the analytical method.



**Figure A.2:** Roxithromycin standard for specificity test



**Figure A.3:** Specificity results for niosome and proniosome ingredients



**Figure A.4:** Specificity results for ufosome and pro-ufosome ingredients

#### **A.5 Conclusion**

The HPLC method validated was found to have satisfactory results for linearity, accuracy, precision, repeatability, stability and specificity of roxithromycin. The HPLC analytical method was found to be reliable and suitable for determining the concentration of API present in vesicle system samples and the samples tested during membrane studies and topical diffusion studies. This method proved to be an accurate method, not only for measuring roxithromycin monohydrate, but also the glassy form of roxithromycin and the chloroform desolvated form of roxithromycin when encapsulated in vesicle systems and tested topically on skin.

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FDA **see** FOOD AND DRUG ADMINISTRATION

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ICH **see** International Conference on Harmonisation

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# Preparation of the amorphous forms of roxithromycin and vesicular systems used to encapsulate different forms of roxithromycin

*Annexure*  
**B**

## B.1 Introduction

The composition and method of preparation of vesicles affect their physicochemical properties such as size, surface charge (zeta-potential), elasticity and lamellarity. The structure of the vesicle can be modified to yield optimised properties which will provide controlled targeted drug delivery (Kumar & Rajeshwarrao, 2011:208). The topical products prepared in this study consisted of different amorphous forms of roxithromycin encapsulated in various vesicle systems. This annexure discusses the methods of preparing the three forms of roxithromycin and the encapsulation of the forms into four different vesicle systems. The carrier systems made were niosomes, ufosomes (fatty acid vesicles), proniosomes and pro-ufosomes. Tests were first performed on samples of niosomes and ufosomes to determine the optimal method of preparation and ratio of ingredients. The optimised method of preparation was then used for all vesicle and provesicular systems. Table B.1 is a summary of the final twelve vesicle preparations tested in this study, which were prepared based on the results obtained from testing certain parameters of niosome and ufosome optimised formulas.

**Table B.1:** Summary of final twelve vesicles systems

| Roxithromycin forms encapsulated     | Vesicle systems |          |             |              |
|--------------------------------------|-----------------|----------|-------------|--------------|
|                                      | Niosomes        | Ufosomes | Proniosomes | Pro-ufosomes |
| Roxithromycin monohydrate            | (1)             | (2)      | (3)         | (4)          |
| Glassy amorphous form                | (5)             | (6)      | (7)         | (8)          |
| Chloroform desolvated amorphous form | (9)             | (10)     | (11)        | (12)         |

The ingredients, ratio of ingredients and method of preparation used to determine the optimal formulas remained the same in all further experiments of this study.

## **B.2 Preparation of amorphous forms of roxithromycin**

Three solid-state forms of the API were used during this study. The first form was the crystalline monohydrate form of roxithromycin. The two amorphous solid-state forms were also prepared for comparison with the roxithromycin monohydrate; the first amorphous form was the 'glassy' form of roxithromycin and the second was the chloroform desolvated form of roxithromycin.

### **B.2.1 Ingredients used in the preparation of amorphous forms**

Both amorphous forms were prepared using the roxithromycin monohydrate. The ingredients used to prepare the amorphous forms of roxithromycin only included roxithromycin monohydrate powder (purchased from DB Fine Chemicals, South Africa) for the glassy amorphous form and roxithromycin monohydrate powder and chloroform for the preparation of the chloroform desolvated amorphous form.

### **B.2.2 Preparation of amorphous glassy form of roxithromycin**

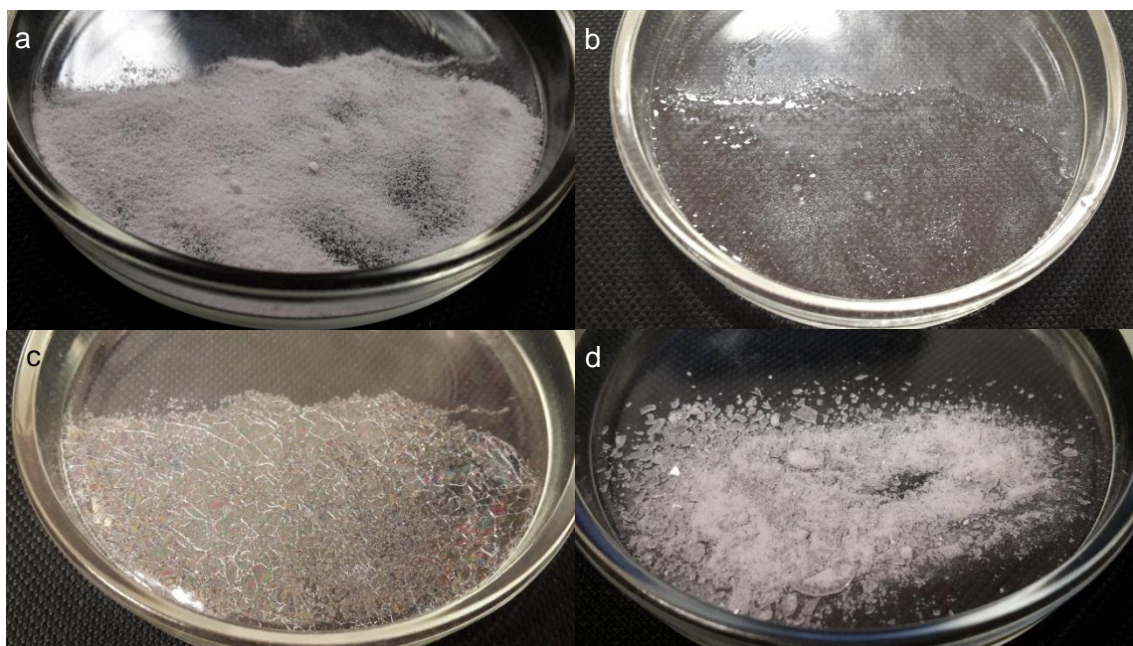
#### **B.2.2.1 Method of preparation**

The method for the preparation of roxithromycin 'glass' consisted of melting the roxithromycin monohydrate raw material in a glass Petri dish, in an oven, at a temperature of approximately 120 °C (Aucamp *et al.*, 2012:468). Once the powder was completely melted, it was quench cooled on a cool granite surface at room temperature. After complete cooling the hardened glass was broken and scraped off the surface of the Petri dish into a suitable storage container.

#### **B.2.2.2 Results**

This process formed a stable, glass amorphous form of roxithromycin as seen in Figure B.1.

The raw material (a) was completely melted (b) on removal of the compound from the oven. On cooling, the melt hardened and cracked. The appearance of the glassy amorphous form was like a piece of glass containing millions of tiny cracks, as seen in (c). After scraping the broken glass into a container, it appeared as thin, little glass flakes (d).



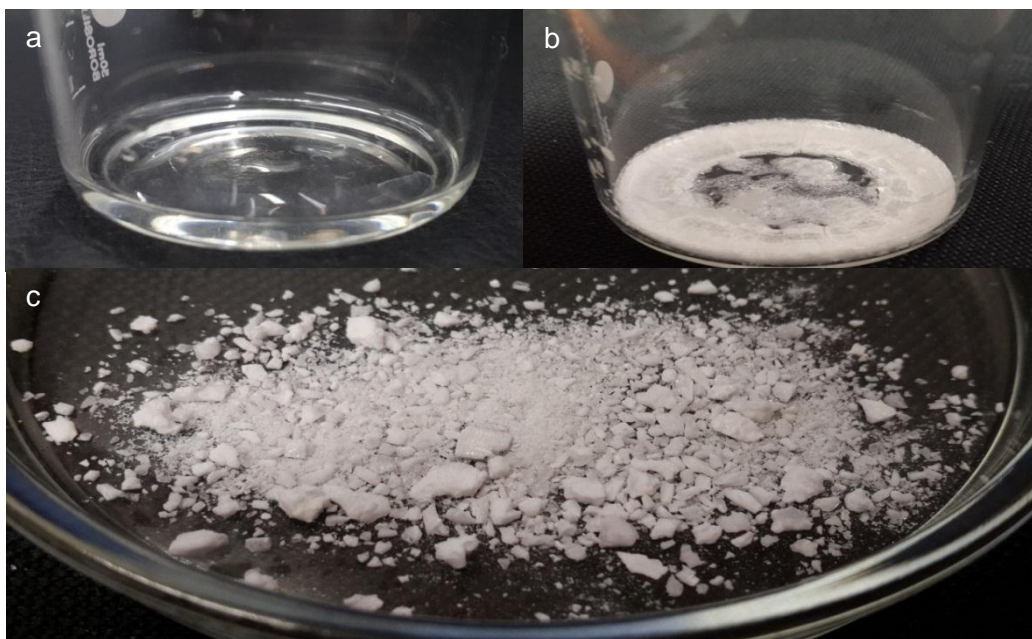
**Figure B.1:** Preparation of glassy amorphous form of roxithromycin. a) Raw material of roxithromycin, b) melted roxithromycin, c) cracked glassy amorphous form of roxithromycin (after quench cooling) and d) glass flakes of glassy amorphous form of roxithromycin.

### **B.2.3 Preparation of chloroform desolvated amorphous form of roxithromycin**

#### **B.2.3.1 Method of preparation**

The method of preparing chloroform desolvated amorphous form of roxithromycin involved the recrystallisation of roxithromycin monohydrate from chloroform. Roxithromycin monohydrate was added to chloroform while stirring with a magnetic stirrer and heating the solution to approximately 60 °C. Roxithromycin monohydrate was continuously added until a saturated solution was obtained (a milky solution) (Aucamp *et al.*, 2012:468). Thereafter, a small amount of chloroform was added until the solution appeared clear once again. The solution was then covered with pierced Parafilm® for evaporation to occur. The solution was left in a fume hood at room temperature (25 °C) for complete evaporation of the chloroform to occur. On removal from the fume hood, the dried mass was broken into granules using a steel rod and then placed in an oven for 12 h at 40 °C to ensure complete desolvation of the amorphous powder before transferral to a final container.

### B.2.3.2 Results



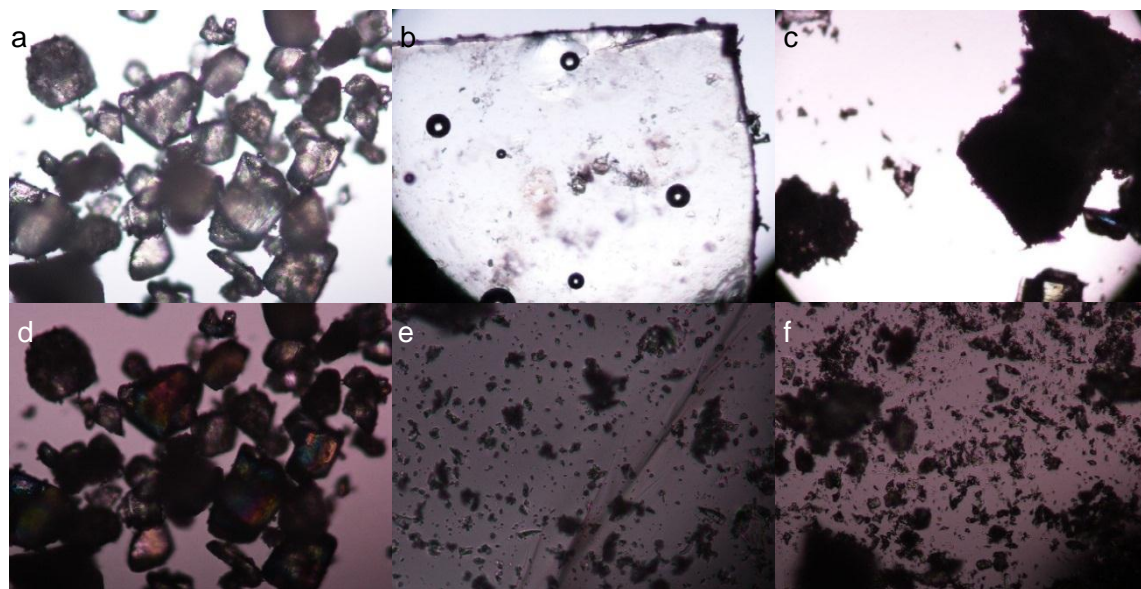
**Figure B.2:** Preparation of chloroform desolvated amorphous form of roxithromycin. a) Roxithromycin monohydrate dissolved in chloroform, b) dense mass of chloroform solvate and chloroform desolvated amorphous roxithromycin powder and c) granules of chloroform desolvated amorphous form after being completely desolvated using an oven.

The roxithromycin monohydrate made a clear chloroform solution, as seen in Figure B.2 (a). A dense white mass was obtained after slow evaporation of the chloroform in a fume hood, which consisted of chloroform solvate and chloroform desolvated amorphous roxithromycin powder (b). After the mass was broken up and dried in the oven, the process formed a stable chloroform desolvated powder with a rough granular appearance (c).

### B.2.4 Appearance of amorphous forms under the light microscope

Light microscopy was used to view the microscopic appearance of the raw and amorphous forms of roxithromycin. A Nikon Eclipse microscope (E4000), containing a Nikon DS-Fi1 camera (Linkam THMS600, Japan), was used to view amorphous samples. A small quantity of each compound was placed on a glass slide and viewed under the microscope at magnification of 20x. The cross polariser was used to determine if the solid-state form was amorphous or if it still contained crystalline particles (Stieger *et al.*, 2012).

### B.2.4.1 Results



**Figure B.3:** Light micrographs of roxithromycin and amorphous forms. a) Roxithromycin monohydrate powder (crystalline), b) flake of glassy amorphous form of roxithromycin, c) granules of chloroform desolvated amorphous form, d) polarised micrograph of roxithromycin monohydrate, e) polarised micrograph of amorphous glass powder and f) polarised micrograph of chloroform desolvate powder.

The light microscopy revealed morphology and solid-state differences of the roxithromycin forms which could not be seen with the naked eye. Figure B.3 shows the crystalline habit of the roxithromycin monohydrate (a), a flake of the glassy form of roxithromycin (b) and the granules of the roxithromycin desolvate (c), as seen under the microscope after preparation without any size reduction of particles. Figure B.3 also shows the polarised images of roxithromycin raw material (d), crushed roxithromycin glass (e) and the crushed chloroform desolvated amorphous form of roxithromycin (f).

From polarised micrographs, the difference between crystalline material and amorphous material can be seen. If the compound is crystalline, birefringence exists (reflection of colours from the crystals) as seen in image (d) with the crystalline roxithromycin raw material. The images of amorphous particles showed no interference colours (appear black) as seen in images (e) and (f) with the two amorphous forms of roxithromycin. If birefringence exists on polarisation of amorphous forms, there is still crystal matter present in the sample, but based on the above information, both amorphous forms of roxithromycin were characterised as purely amorphous (Stieger *et al.*, 2012).

### **B.3 Preparation of vesicular systems**

Four carrier systems were prepared for experimental use in this study, i.e. two vesicular systems (niosomes and ufosomes) and two provesicular carriers (proniosomes and pro-ufosomes). The preparation of the first two vesicle systems were done according to the film hydration method which yields multilamellar vesicles (New, 1990:36-38). The two provesicular systems were prepared using the slurry method (Hazel *et al.*, 2012:176; New, 1990:40-42). A few adaptations were made to the existing methods, based on the literature gathered in order to minimise the variation of ingredients and processes carried out amongst the four vesicle carriers prepared in this study. These changes were tested to determine if the adaptations made for this study were acceptable and if vesicles successfully formed.

#### **B.3.1 The general method used for the preparation of vesicular systems**

The method used for the preparation of the niosomes and ufosomes was adapted from the hand shaking method used by New (1990:36-38), a method also known as the film hydration method (Agarwal *et al.*, 2001:44). The method used consisted of dissolving a lipid mixture in a 2:1 volume ratio of chloroform and methanol. The beaker containing the mixture was then placed on a hot plate at 60 °C (above the lipid's transition temperature) in a fume hood and kept there until all the liquid had evaporated leaving a thin film at the bottom of the beaker. To hydrate the film, PBS (pH 7.4) and a magnet were added to the beaker and stirred with a magnetic stirrer for about 20 min until all of the film had come off the bottom of the beaker to give a milky suspension with no large droplets visible to the eye. The vesicular system was then sonicated, using a sonicator probe, for a specified time in order to achieve a good droplet size distribution (New: 1990:44). The vesicular suspension was left for 2 h at room temperature to allow for optimal swelling of the vesicles (New: 1990:36-38).

#### **B.3.2 The general method used for the preparation of provesicular systems**

The preparation of the provesicular systems was based on the guidelines used by New (1990:41-42), while obtaining the same ingredients and quantities used to prepare the vesicular systems in this study. This method was used to prepare the proniosomes and pro-ufosomes and consisted of dissolving the lipids in a 2:1 chloroform:methanol solution. Sorbitol powder was placed in a separate beaker with a magnet and placed on a magnetic stirring hot plate at 60 °C (higher than the transition temperature of the lipids in solution). A drop of the lipid solution was introduced to the sorbitol. When the drop had evaporated, the rest of the solution was placed drop-wise onto the sorbitol, ensuring that over-wetting and clumping did not occur at any time during the process. When all the solution had been used, the beaker was left to stir the coated sorbitol (without heating) for a further 24 h to ensure complete dryness. Before use,

the provesicles were hydrated with PBS (pH 7.4), sonicated with a probe sonicator for a specified time and left to stand for 2 h.

### **B.3.3 Ingredients used in the preparation of vesicular systems**

#### **B.3.3.1 Span 60 (sorbitan monostearate)**

Span 60 (sorbitan monostearate) is a non-ionic surfactant used as a solubiliser, wetting agent and permeability enhancer (Kumar & Rajeshwarrao, 2011:209). It forms part of the lipid layer of vesicles. Non-ionic surfactants are popular because they have the ability to enhance solubility and in turn, increase bioavailability of poorly soluble drugs (Mahale *et al.*, 2012:47). It exhibits the highest entrapment efficiency compared to similar non-ionic surfactants such as other Spans and Tweens (Kumar & Rajeshwarrao, 2011:210; Mali *et al.*, 2013:587) and has a transition temperature of 56 - 58 °C. During the thin lipid layer formation, the chloroform mixture was heated above this transition temperature (60 °C) (Kumar & Rajeshwarrao, 2011:210). Other properties which make non-ionic surfactants (Span 60) the preferred choice, is their stability, compatibility and absence of toxicity (Mahale *et al.*, 2012:47).

#### **B.3.3.2 Sodium oleate**

Sodium oleate is a fatty acid able to form fatty acid vesicles (ufosomes) at a specific pH (pH = pK<sub>a</sub> of acid bilayer) (Walde *et al.*, 1994:11649). Sodium oleate is a long chain fatty acid that acts as a penetration enhancer. It increases skin penetration and skin permeability by disrupting the stratum corneum lipid layers (Alexander *et al.*, 2012:31; Dayan, 2005:74).

#### **B.3.3.3 Cholesterol**

Cholesterol is known to have an effect on the structure and physical properties of vesicles by enhancing the stability of the lipid bilayer of the vesicle systems (Kumar & Rajeshwarrao, 2011:210). Cholesterol was added to modulate mechanical strength, cohesion and water permeability of the membrane (Mahale *et al.*, 2012:49). Cholesterol also improves the fluidity of vesicles and rigidity of the bilayer which in turn ensures less leakage of the vesicles (Kumar & Rajeshwarrao, 2011:211; Varun *et al.*, 2012:633).

#### **B.3.3.4 Sorbitol**

Sorbitol acted as the carrier for the lipid layer of the provesicular systems by coating the dry sorbitol powder with the lipid soluble components dissolved in the organic solvent. It is acceptable for clinical use and vesicles formed from it have less leakage and lower osmolarity than lower molecular weight compounds (New, 1990:40). On hydration, sorbitol dissolves in the aqueous phase for the formation of vesicles to occur.

### **B.3.3.5 Organic solvents**

Chloroform is an organic solvent which evaporates readily, is convenient to handle and is a popular solvent used in the preparation of vesicle systems (New, 1990:33). Methanol is also an organic solvent which is mostly used as a mixture with chloroform for compounds that are sparingly soluble in chloroform or methanol alone. The volume ratio of chloroform:methanol used as an organic solvent is usually 2:1 (New: 1990:33). This mixture was used as the solvent in this study as it was predicted that most compounds dissolved readily in the combination.

### **B.3.3.6 Phosphate buffer solution**

The most common hydration medium for vesicles and provesicular systems is various phosphate buffer solutions at different pH values (Mahale *et al.*, 2012:48). The PBS used as the hydration medium for vesicles throughout this study was 0.06 M  $\text{KH}_2\text{PO}_4$ , at pH 7.4 (British Pharmacopoeia, 2014). The pH of the buffer depends on the solubility of the encapsulated drug (Kumar & Rajeshwarrao, 2011:210). Roxithromycin is soluble in PBS (Aucamp *et al.*, 2012:468). A pH of 7.4 was chosen due to the fact that the pH in the receptor phase during transdermal studies is also 7.4, which in turn resembles the pH of human blood (Rogers & McCutcheon, 2015:46). Having the same buffers at the same pH in formulas and the receptor phase prevented the formation of salts which might have altered experimental analysis. The hydration medium plays a very important role in the size and formation of vesicles and should always be above the phase transition (gel to liquid) temperature of the system. In this case it was above 60 °C (transition temperature of Span 60) on hydration of vesicles (Kumar & Rajeshwarrao, 2011:210, 212).

### **B.3.3.7 Roxithromycin**

The API that was entrapped in the lipid layer of the vesicular systems was roxithromycin monohydrate (raw material), followed by the entrapment of the two amorphous forms, each in different formulations. The two amorphous forms are the glassy amorphous form of roxithromycin and the desolvated amorphous form of roxithromycin. The percentage of API was kept constant at 2% (w/v) in all final vesicular systems.

## **B.4 Formulating and testing for optimised vesicle preparations**

The preparation of final vesicle carrier systems containing API were first optimised on the basis of results obtained from testing light microscopy, transmission electron microscopy (TEM), vesicle size, size distribution and EE% of various potential preparations of niosomes and ufosomes (Jadon *et al.*, 2009:1188). Vesicles (niosomes and ufosomes) were first prepared without any API encapsulated to obtain optimal lipid ratios, thereafter, the vesicle system formulas containing API (niosomes and ufosomes containing roxithromycin monohydrate) were

optimised. Following this, the two provesicular systems (proniosomes and pro-ufosomes containing roxithromycin monohydrate) were prepared in identical ratios. The remaining amorphous forms of roxithromycin were then encapsulated according to the optimised formulas determined when encapsulating roxithromycin monohydrate.

#### **B.4.1 Preparation of vesicles without API**

The first step entailed determining the preferred lipid ratio by preparing vesicle systems containing only the lipids and no API. To determine the optimal ratios of the lipids used for the preparation of the niosomes and ufosomes, two different combination of ratios were used to prepare each of the two vesicle systems. The two lipid variations of each niosome and ufosome samples were tested using TEM, light microscopy and droplet size and size distribution as a guideline in order to determine which lipid ratio yielded better vesicles. For the lipid layer of niosomes, the ratios of Span 60:cholesterol 2:1 and 1:1 were tested. Each niosome sample was divided into three separate beakers and sonicated for (a) 2.0 min, (b) 3.5 min and (c) no sonication to determine which length of sonication yielded better droplet size distribution for each ratio. For the lipid layer of the ufosomes, the ratios of sodium oleate:cholesterol 2:1 and 1:1 were tested. Each ufosome sample was also divided into three and sonicated at (a) 2.0 min, (b) 3.5 min and (c) not sonicated, to determine the optimal ratio of lipids used as well as the optimal sonication time needed for a good droplet size. Each non-sonicated niosome and ufosome vesicle system containing different ratios of lipids (2:1 and 1:1) were viewed under the TEM (FEI Tecnai G2 transmission electron microscope, FEI, Holland), at much higher magnification, to determine if the droplets seen were indeed vesicles. Thereafter, light microscopy was used on all sonicated samples to see if the formation of vesicles took place in all the preparations and to see which ratios and length of sonication formed better vesicles. A Malvern Zetasizer Nano ZS (Malvern Instruments, UK) was used to determine droplet size and distribution of the sonicated vesicle samples. Using these tests, an optimal lipid formula (and sonication time) without API for each vesicle system was found.

##### **B.4.1.1 Method of preparation of niosomes without API**

The general film hydration method by New (1990:36-38), mentioned in Section B.3.1, was used for the preparation of the niosome vesicles without API. The first niosome sample was prepared by dissolving Span 60 and cholesterol (2:1) in a 5 ml mixture of chloroform and methanol (2:1). Once all the liquid had evaporated, leaving a thin film on the bottom of the beaker, 5 ml PBS (pH 7.4) was added and stirred to form a milky suspension. The sample was then divided into three different polytopes, where the first was not sonicated, the second sonicated for 2.0 min and the third was sonicated for 3.5 min. The samples were left to stand for 2 h before tests were performed. The same process was followed with the second niosome

ratio to be tested, except the ratio of Span 60:cholesterol was 1:1. The process resulted in six niosome samples for testing of an optimal formula.

#### **B.4.1.2 Method of preparation of ufosomes without API**

The guidelines of New (1990:36-38) were used in the preparation of the ufosomes containing no roxithromycin. The first ufosome sample ratio was prepared by dissolving sodium oleate:cholesterol (2:1) in 5 ml chloroform/methanol solution (2:1). The lipid mixture was allowed to evaporate completely, thereafter, PBS (5 ml at pH 7.4) was added to form the vesicle suspension. The suspension was divided, as with the niosomes, and each sample sonicated at 2.0 min, 3.5 min and the last not sonicated at all. They were left to stand for 2 h and then tested. The process was repeated with a 1:1 ratio of sodium oleate and cholesterol. A total of six ufosome samples were prepared for testing optimal ratios.

#### **B.4.2 Tests performed on samples to determine optimal lipid ratio**

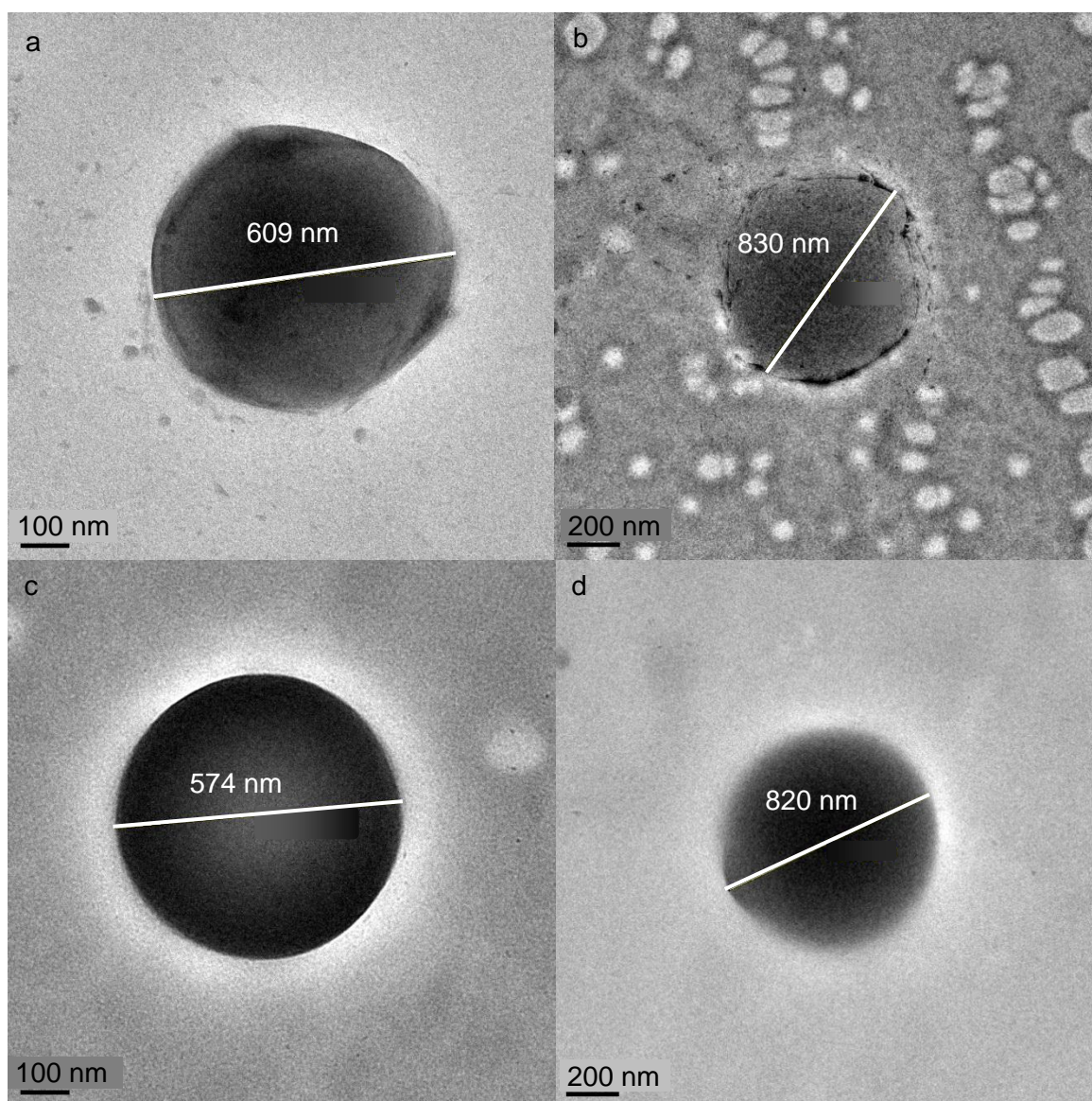
##### **B.4.2.1 Transmission electron microscopy (TEM)**

TEM was performed on the two non-sonicated niosome samples with a Span 60:cholesterol ratio of 2:1 and 1:1 and the non-sonicated ufosome samples with a sodium oleate:cholesterol ratio of 2:1 and 1:1. TEM was used to see if all four systems did in fact produce vesicles at all, before performing other tests on them. A FEI Tecnai G2 TEM (FEI, Holland) fitted with a Gatan bottom mount digital camera was used. It was operated at 120 kV by Dr A Jordaan (Laboratory for Electron Microscopy, Room G14, Building G10, North-West University: Potchefstroom Campus). Each sample was diluted approximately 10x with distilled water and a drop was placed on a copper carbon-coated 300 mesh grid which was left to dry for 15 min. Osmium was used to stain the lipid films of each vesicle and this was left to dry for 30 min before it was inserted into the TEM. Magnifications of vesicles were viewed between 5000 - 15000x.

##### **B.4.2.1.1 TEM results**

The results obtained from the TEM are shown in Figure B.4. It can be seen that vesicles formed for all four of the formulations. The dark regions illustrated are the vesicle membranes, as osmium fixes to the lipid layer of the droplets. The appearance of well-formed vesicles, as well as vesicles with weaker lipid layers, can be seen in the micrographs in Figure B.4. Niosomes with a ratio of Span 60:cholesterol 2:1 (a) and 1:1 (b) formed almost perfect spheres, whilst ufosomes with sodium oleate:cholesterol 2:1 (c) and 1:1 (d) formed perfect spheres. The vesicle membranes of the ratio 2:1, (a) and (c), for niosomes and ufosomes appeared to have formed stronger, thicker lipid membranes, as the osmium fixed more to these membranes to give a darker appearance than the ratio of 1:1 as seen in (b) and (d). The ufosome ratio 2:1 seemed to form the strongest, most uniform lipid layer. The vesicles were relatively large due to

the fact that the non-sonicated samples of each potential formula were used. These samples proved the formation of vesicles and allowed for further testing of the characteristics of formulations.



**Figure B.4:** Vesicle formation viewed using TEM. a) Niosome with Span 60:cholesterol 2:1, b) niosome with Span 60:cholesterol 1:1, c) ufosome with sodium oleate:cholesterol 2:1 and d) ufosome with sodium oleate:cholesterol 1:1.



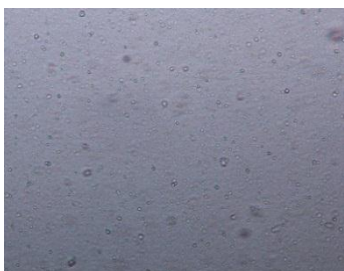
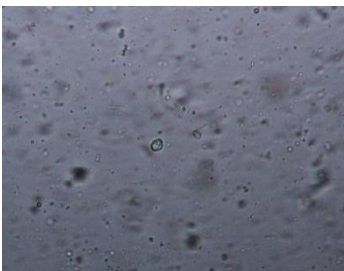


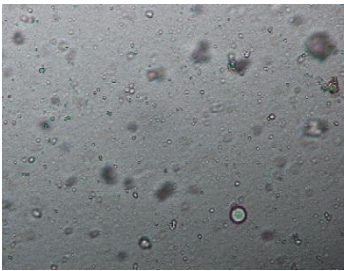


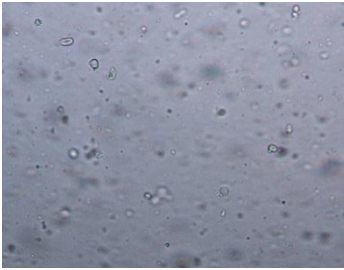


#### B.4.2.2 Light microscopy

Light microscopy was used to determine if vesicles had formed after niosomes and ufosomes were formulated and some sonicated. It was also an indication of the shape of as well as the homogeneity of the vesicles. Light microscopy was used because it was faster, cheaper, more convenient and more readily available than the TEM to confirm vesicle formation. The light microscopy was performed on a Nikon Eclipse E4000 microscope (Nikon, Japan

Linkam THMS600), equipped with a Nikon DS-Fi1 camera. Each vesicle system was diluted 10x with distilled water and a small amount was placed onto a 20 mm microscope slide. The vesicle system was then viewed at a magnification of 50x (maximum for the instrument). Niosomes with a Span 60:cholesterol ratio of 2:1 with no sonication and sonication times of 2.0 min and 3.5 min were each viewed separately on the microscope. Thereafter, the three niosomes with a ratio of 1:1 at different sonication times were viewed. The same was done for the six ufosome preparations containing sodium oleate:cholesterol ratios of 2:1 and 1:1.

#### B.4.2.2.1 Light microscopy results

**Table B.2:** Appearance of vesicles using light microscopy

| Ratio       | No sonication   | 2.0 min sonication   | 3.5 min sonication  |
|-------------|---|--|---|
| Niosome 2:1 |   |   |   |
| Niosome 1:1 |  |  |  |
| Ufosome 2:1 |  |  |  |
| Ufosome 1:1 |  |  |  |

A few micrographs of each sample were taken under the light microscope, but only one was used to represent a sample for the formation of vesicles. Table B.2 displays micrographs of the twelve potentially optimal vesicle systems of niosomes and ufosomes, at various sonication times, at a magnification of 50x. Due to this very low magnification, only the larger vesicles can be seen and it must be kept in mind that there were many more nanovesicles in each sample that were not visible. All twelve formulations contained tiny droplets resembling large vesicles. According to the TEM results in Section B.4.2.1.1, large vesicles were evident in each system, and these were the same non-sonicated samples viewed later with light microscopy which also showed large round particles. This confirmed that all the tiny droplets viewed with light microscopy were indeed systems containing vesicles.

It can be seen that the vesicles are very large in the non-sonicated samples and produce smaller, more uniform vesicles when sonicated. The niosome vesicles sonicated for 2.0 min and 3.5 min look similar in the pictures and therefore the difference between the droplets were determined by the next step of measuring the better droplet size and droplet size distribution between formulations in Section B.4.2.3. The ufosome vesicles were smaller, more uniform and almost impossible to see when sonicate for 3.5 min as compared to the slightly larger vesicles seen after 2.0 min sonication. From the images above, it is evident that all formulas produced vesicles of different particle sizes, but smaller, more uniform droplets are preferred for optimal stability of the preparation. Therefore droplet size and size distribution was an excellent next step in determining the optimal lipid formula for a stable system.

### **B.4.2.3 Droplet size distribution**

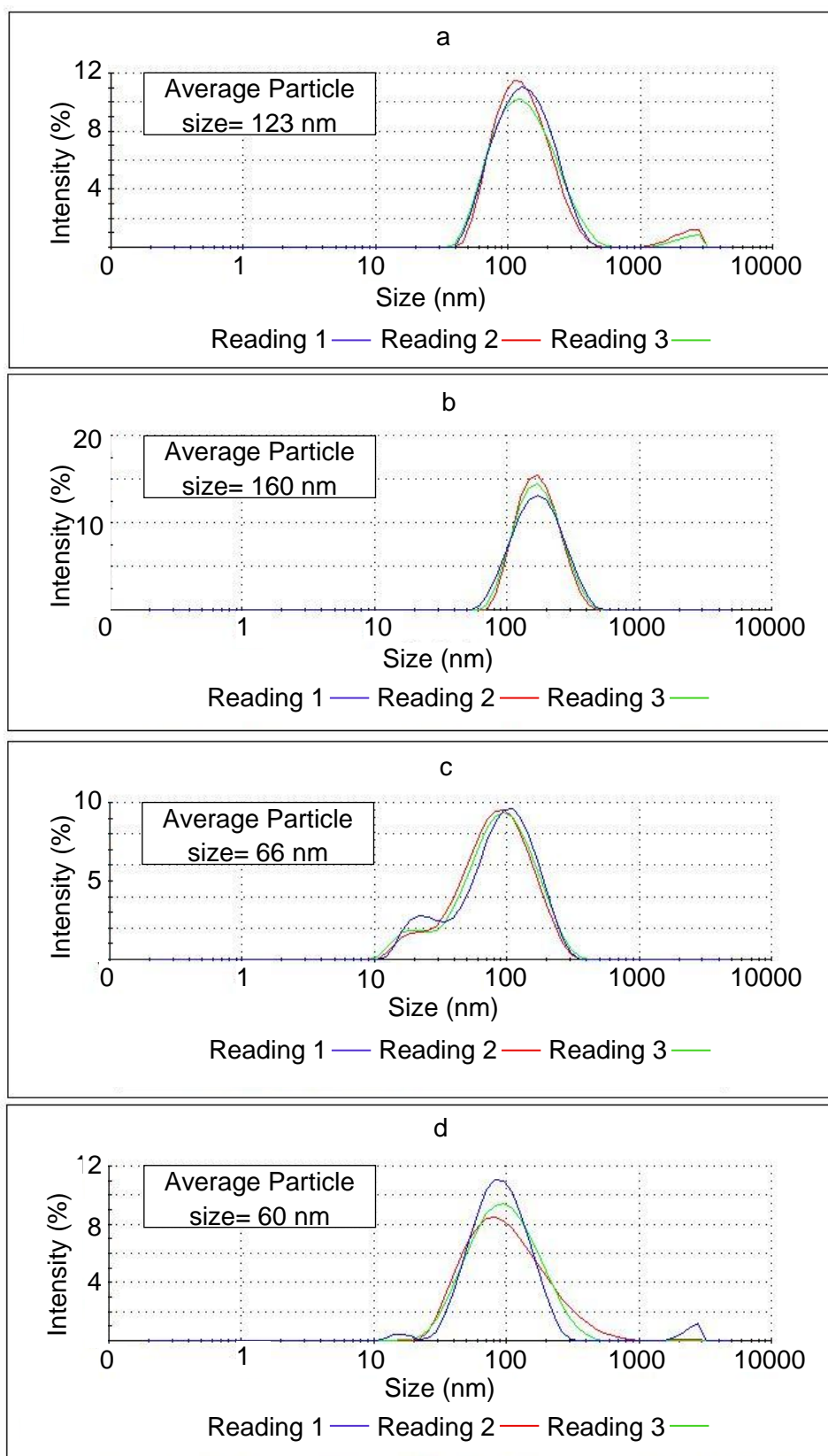
The average droplet size and size distribution was measured to determine which lipid ratio and which sonication duration would yield a stable, uniform vesicle system with droplet sizes in close range of one another for niosomes and ufosomes. Size of droplets influences the stability of vesicle systems and droplet size distribution should be within a narrow range for optimal formulas (Kumar & Rajeshwarrao, 2011:213). The droplet size of the vesicles was determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The six niosome samples were diluted 20x with distilled water and then placed in a plastic cuvette, where the reading of each sample was taken in triplicate. The same process was done with the six ufosome samples.

#### **B.4.2.3.1 Droplet size distribution results**

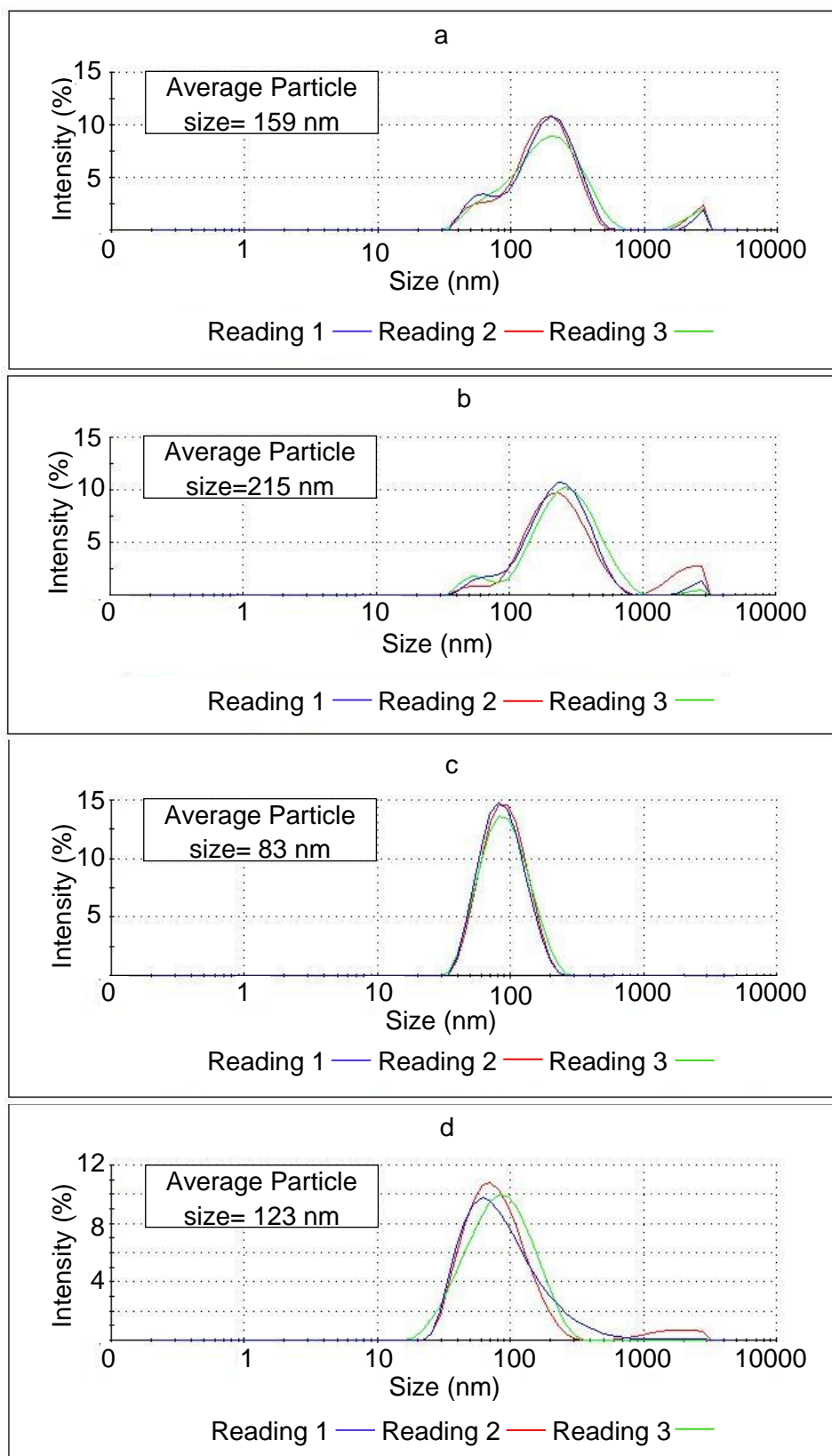
The droplet size and droplet size distribution could not be measured on the samples that were not sonicated as the results showed “data quality poor” for each sample, which could be due to the very large vesicles and particles seen in the samples during light microscopy.

The droplet size and droplet size distribution of the niosome formulations can be viewed in Figure B.5. The droplet size distribution for both niosome formulations sonicated for 2.0 min were good and the curves were repeatable with an average droplet size of 123 nm and 160 nm for the 2:1 (a) and 1:1 (b) ratio, respectively. Both formulas of 2:1 at 3.5 min (c) and 1:1 at 3.5 min (d) showed a poorer droplet size distribution curve. Although the average droplet size of both formulas at 3.5 min were about half of that sonicated for 2.0 min, some droplets were still large while others were tiny and this did not produce the preferred distribution curves. Overall, the size of the niosomes after sonication in this study correlated well with literature. Niosomes formed multi-lamellar vesicles (MLV) with a size of 100 - 300 nm ( $\geq 50$  nm). These types of vesicles have more than one bilayer with good encapsulation of lipophilic drugs. They are stable in long term use and good for drug targeting into specific tissue (Varun *et al.*, 2012:635). The decision for an optimal lipid formula for the niosomes was between both formulas (ratio 2:1 (a) and 1:1 (b)) sonicated for 2.0 min.

The results of the droplet size and distribution of the ufosomes' sonicated samples can be seen in Figure B.6. It is clear from the size distribution graphs that ufosome ratios 2:1 (a) and 1:1 (b) sonicated for 2.0 min had poor droplet size distribution with average droplet sizes of 159 nm and 215 nm, which were fairly large. When sonicated for 3.5 min, both droplet sizes were reduced considerably, but ufosomes 2:1 (c), with an average droplet size of 83 nm, showed better size distribution according to repeatable results obtained by triplicate readings than ufosomes 1:1 (d), with an average droplet size of 123 nm, whose distribution curves are not so precisely repeated. All ufosome formulas had droplet sizes corresponding well to literature, which states ufosomes form MLV (100 - 300 nm) which were reduced in size by a probe sonicator to form a variety of MLV ( $> 50$  nm) and many smaller unilamellar vesicles (25 - 50 nm) in order for them to have a good size distribution curve (New 1990:44; Varun *et al.* 2012:635).



**Figure B.5:** Droplet size distribution of lipid ratios of niosomes. a) Size distribution of niosomes (ratio 2:1) sonicated for 2.0 min, b) size distribution of niosomes (ratio 1:1) sonicated for 2.0 min, c) size distribution of niosomes (ratio 2:1) sonicated for 3.5 min and d) size distribution of niosomes (ratio 1:1) sonicated for 3.5 min.



**Figure B.6:** Droplet size distribution of lipid ratios of ufosomes. a) Size distribution of ufosomes (ratio 2:1) sonicated for 2.0 min, b) size distribution of ufosomes (ratio 1:1) sonicated for 2.0 min, c) size distribution of ufosomes (ratio 2:1) sonicated for 3.5 min and d) size distribution of ufosomes (ratio 1:1) sonicated for 3.5 min.

### **B.4.3 Summary of decision made for optimal lipid ratios**

Once all the results of the twelve samples were analysed, the decision for an optimal lipid ratio for the vesicles had to be decided. The criteria and testing used to make this decision was TEM, light microscopy and droplet size and size distribution.

With its vast magnification, the TEM successfully proved that the two different non-sonicated lipid ratios of niosomes and the two non-sonicated ratios of ufosomes did in fact form vesicles. This provided confidence in the successful method used to produce the different ratios of vesicles. From the TEM micrographs, the ratio 2:1 for niosomes and ufosomes appeared to have thicker, stronger lipid layers than their 1:1 ratio counterparts. The light microscopy showed the larger vesicles present in the twelve formulations at different sonication times and proved that vesicles existed in all the formulations. It was seen that the samples sonicated at 2.0 min and 3.5 min produced smaller and more uniform vesicles than those not sonicated, but the difference in size and size distribution of samples sonicated at 2.0 min and 3.5 min was not clear and size distribution tests had to be performed.

During droplet size distribution the non-sonicated vesicles were ruled out of the study as the samples had large sediments which made droplet size analysis impossible. This left four sonicated samples of niosomes (ratio 2:1 at 2.0 min and 3.5 min and 1:1 ratio at 2.0 min and 3.5 min) and four sonicated samples of ufosomes (ratio 2:1 at 2.0 min and 3.5 min and ratio 1:1 at 2.0 min and 3.5 min) for further testing of droplet size and size distribution. The niosomes 2:1 and 1:1 ratio sonicated at 2.0 min both showed the better size distribution curves. The best droplet size distribution for the ufosomes, without doubt, was the sodium oleate:cholesterol 2:1 ratio sonicated for 3.5 min and it was decided as the optimal lipid ratio for ufosomes used to encapsulate the API. Based on the results from ufosomes, the niosome Span 60:cholesterol 2:1 ratio at 2.0 min sonication was chosen as the optimal lipid formula for niosomes which would encapsulate the API. Although both niosome samples had good size distribution, this formula had the same optimal lipid ratio chosen for the ufosomes (ratio 2:1) above. This made comparison of formulas easier due to minimal variations between niosome and ufosome samples. The only variations which existed between the niosomes and ufosomes was the sonication time and the sodium oleate which replaced the Span 60 when preparing ufosomes.

### **B.4.4 Preparation of vesicles entrapping roxithromycin**

Once the optimal lipid ratios of the vesicle systems were decided on, by the results obtained from microscopy and droplet size distribution, the optimal lipid ratio and sonication time for the niosome and ufosome preparations were kept constant. The fixed lipid ratios were then used to determine the highest amount of roxithromycin that could successfully be entrapped in the vesicle systems. Four different amounts of roxithromycin were added to each of the two lipid

ratios and the entrapment efficiency was tested to determine the optimal drug concentration for each preparation.

#### **B.4.4.1 Method of preparing vesicles entrapping roxithromycin**

The method used to make the niosomes and ufosomes containing API was the same as mentioned in Section B.3.1, except that roxithromycin monohydrate (lipophilic API) was added to the fixed lipid ratio mixture dissolved in chloroform/methanol in four different ratios. For niosomes, the Span 60:cholesterol:roxithromycin monohydrate ratios tested were 2.0:1.0:0.5, 2.0:1.0:1.0, 2.0:1.0:2.0 and 2.0:1.0:4.0 and each preparation was sonicated for 2.0 min. For the ufosomes, the ratio of sodium oleate:cholesterol:roxithromycin monohydrate was also 2.0:1.0:0.5, 2.0:1.0:1.0, 2.0:1.0:2.0 and 2.0:1.0:4.0, each sonicated for 3.5 min. Eight formulations were left to swell for 2 h, then the entrapment efficiency was tested to determine the optimal API concentrations entrapped in the niosomes and ufosomes.

#### **B.4.4.2 Entrapment efficiency test using minicolumn centrifugation**

The EE% of eight preparations (see Section B.4.4.1) was tested to determine what the optimal API load was in niosomes and ufosomes. The method used to determine the entrapment efficiency was adapted from the minicolumn centrifugation method of New (1990:126-127). This method is also known as the Sephadex gel filtration method (Mahale *et al.*, 2012:52). Sephadex (10 g) was added to 120 ml of PBS (pH 7.4) and left overnight to swell at 4 °C. The plungers of eight 3 ml syringes were removed and the syringes were plugged with glass wool. The swollen Sephadex gel was added to fill each barrel, which was then placed into 15 ml centrifugation tubes. The eight barrels were centrifuged at 2000 rpm for 4 min and when removed, the Sephadex had dried and moved away from the sides of the syringe barrel. All eight samples were freshly prepared the morning of the entrapment efficiency test and 0.5 ml of each sample containing roxithromycin monohydrate was placed drop-wise onto the Sephadex minicolumn. The samples were centrifuged at 2000 rpm for another 3 min. Thereafter 0.1 ml PBS (pH 7.4) was dripped on each sample and centrifuged at the above mentioned speed for the same time. The milky elute was removed and discarded, as this was the vesicles which had moved through the column (New, 1990:126); 1 ml PBS (pH 7.4) was then dripped on each barrel and centrifuged for a third time followed by the addition of another 1 ml to each barrel which was centrifuged for the last time. The last 2 ml was collected and filtered through 0.45 µm PVDF filters for HPLC analysis. This experiment was repeated in duplicate. The linear standard curve of roxithromycin ( $y = mx + c$ ) was used to determine the drug concentrations (x) in each of the vesicular systems. The following equation adapted from Kurakula *et al.* (2012:37), was then used to calculate the entrapment efficiency of each vesicular system:

$$EE\% = C_t - C_f/C_t \times 100$$

**Equation B.1**

Where  $C_t$  is the total API concentration and  $C_f$  is the concentration of free unencapsulated drug (x value).

The preparations with the best EE% along with acceptable physical appearance of the vesicle suspension were the optimal preparations chosen for all further vesicular systems in this study.

#### B.4.4.2.1 Entrapment efficiency results

**Table B.3:** Comparative entrapment efficiencies of niosome preparations

| Span60:cholesterol:API | EE%   | Average % | Physical appearance |
|------------------------|-------|-----------|---------------------|
| (a) 2.0:1.0:0.5        | 44.78 | 51.68     | Good suspension     |
|                        | 51.68 |           |                     |
| (b) 2.0:1.0:1.0        | 57.53 | 59.81     | Good suspension     |
|                        | 62.66 |           |                     |
| (c) 2.0:1.0:2.0        | 48.71 | 52.16     | Good suspension     |
|                        | 55.60 |           |                     |
| (d) 2.0:1.0:4.0        | 61.45 | 63.84     | Good suspension     |
|                        | 66.22 |           |                     |

Table B.3 shows the results of the EE% of the niosomes with concentrations in four different ratios. All four ratios formed good liquid suspensions with good physical appearances. The lowest EE% of 51.68% was in (a), while the highest entrapment shown was that of (d) with an entrapment efficiency of 63.84%. This being said, the entrapment efficiency values of all four preparations in the table above proved to be very close. It can be seen in Table B.3 that a ratio of 2.0:1.0:1.0 had a fairly good entrapment efficiency and increasing the drug content, as in (c) and (d), would provide little or no additional entrapment.

**Table B.4:** Comparative entrapment efficiencies of ufosome preparations

| Sodium oleate:cholesterol:API | EE%   | Average% | Physical appearance |
|-------------------------------|-------|----------|---------------------|
| (a) 2.0:1.0:0.5               | 47.04 | 38.69    | Good suspension     |
|                               | 30.33 |          |                     |
| (b) 2.0:1.0:1.0               | 86.18 | 84.36    | Good suspension     |
|                               | 82.53 |          |                     |
| (c) 2.0:1.0:2.0               | 92.76 | 92.99    | Thickish gel        |
|                               | 93.22 |          |                     |
| (d) 2.0:1.0:4.0               | 94.80 | 94.31    | Thick gel           |
|                               | 93.81 |          |                     |

In Table B.4, the results for the ufosomes showed that the physical appearance of (a) and (b) were good liquid suspensions, whereas (c) and (d) were so thick that they appeared as gels due to the fact that there was too much powder for the amount of PBS (pH 7.4) added. The lowest entrapment efficiency was found with (a), which had a very poor EE% of 38.69%. The entrapment efficiency of (b), (c) and (d) were very good and in close range of one another with (d) having the highest entrapment efficiency of 94.31%.

The entrapment efficiency results were the final step in determining which formula for each type of vesicle would be used in this study. In general, the ufosomes had higher entrapment efficiencies than the niosomes. The decision for an optimal formula for niosomes and ufosomes was based on the EE% and the physical appearance of each. It was quite evident for the ufosomes in Table B.4 that (b) showed good physical appearance as well as high entrapment (84.36%) and was chosen as the optimal formula. With 92.99% and 94.31% respectively, (c) and (d) showed higher entrapment than (b), but the physical appearance of these formulas were thick and they had a gel-like appearance which was unfavourable. From Table B.3 the optimal niosome formula chosen was also (b), with an entrapment of 59.81% and a good appearance for a liquid suspension. Although all the niosome ratios formed good suspensions and had entrapments in close range of one another, (b) was chosen because niosomes and ufosomes had to have the same quantity of API in their formulas for comparative diffusion experiments that took place in this study. Therefore, the optimised formula for niosomes was Span 60:cholesterol:API 2.0:1.0:1.0 and for ufosomes it was sodium oleate:cholesterol:API 2.0:1.0:1.0. Both formulas contained 2% (w/v) API.

#### **B.4.5 Final preparation of vesicle systems used in the topical delivery of roxithromycin**

The niosome and ufosome preparations, with an acceptably high entrapment efficiency coupled with a good appearance and droplet size distribution, were chosen as the optimal vesicle preparations for topical delivery of roxithromycin. The ratios used in the preparation of the provesicular systems (proniosomes and pro-ufosomes) were based on the results obtained from the vesicle preparations and the same concentrations were used in order to eliminate any additional factors that would influence the amount of API transported into the skin. Niosomes, ufosomes, proniosomes and pro-ufosomes were prepared based on the optimised formulas, with each vesicle system containing one of the three forms of roxithromycin discussed above. This gave rise to a total of twelve preparations (referred to in Table B.1) to be tested in the topical delivery of roxithromycin.

### B.4.5.1 Preparation of niosomes encapsulating roxithromycin

The final formulation for the niosomes, based on test results above, was a ratio of Span 60:cholesterol:roxithromycin of 2:1:1 with a sonication time of 2 min after the formation of vesicles.

#### B.4.5.1.1 Method of preparation of niosomes

The general method for preparation of niosomes was as described in Section B.3.1. Span 60, cholesterol and roxithromycin were dissolved in chloroform and methanol as stated in Table B.5 and evaporation took place. PBS (pH 7.4) was added to the flask and stirred until the lipid film was removed from the base of the beaker. The niosomes were then sonicated for 2 min using a sonicator probe. The suspension was left to stand for 2 h before use (New, 1990:33-39). The same methods and ingredients were used to prepare the three niosome preparations, except in the two other formulations, roxithromycin monohydrate was replaced with glassy roxithromycin and chloroform desolvated roxithromycin as the API. This resulted in three different niosome preparations each carrying a different solid-state form of roxithromycin.

**Table B.5:** Niosome formula

| Component of vesicle                                   | Amount      |
|--|-------------|
| Span 60  | 200 mg      |
| Cholesterol  | 100 mg      |
| Chloroform:methanol (2:1)                              | 5 ml        |
| Roxithromycin  | 100 mg (2%) |
| PBS (0.06 M KH <sub>2</sub> PO <sub>4</sub> at pH 7.4) | 5 ml        |

#### B.4.5.1.2 Outcome

All three niosome vesicle systems formed good milky suspensions with no sediment or larger droplets present in the samples.

### B.4.5.2 Preparation of proniosomes encapsulating roxithromycin

Another way to prepare niosomes is to coat a water-soluble carrier (sorbitol) with the lipid phase to form a dry formulation which is later hydrated with PBS before use (Mahale *et al.*, 2012:50). The final proniosome formulation was based on the results obtained from the tests performed for the optimal formulation of niosomes. The optimal ratio used for the preparation of proniosomes was Span 60:cholesterol:roxithromycin (2:1:1) with a sonication time of 2 min on hydration with PBS (pH 7.4).

#### B.4.5.2.1 Method of preparation of proniosomes

The method mentioned in Section B.3.2 was used for preparing proniosomes. Span 60, cholesterol and roxithromycin were dissolved in chloroform/methanol as stated in Table B.6. Sorbitol was then added to a separate beaker and placed on a stirring plate which was set at 60 °C. The mixture was added to sorbitol drop-wise and left to dry overnight at room temperature. When reconstitution of proniosomes was needed, 5 ml PBS (pH 7.4) was added to each vial containing the powder and mixed for 30 sec and sonicated for 2 min before use (Hazel *et al.*, 2012:176; New, 1990:40-42). The proniosomes containing glassy amorphous form and chloroform desolvated form were prepared in the same way with roxithromycin being replaced with glassy form of roxithromycin and desolvated form of roxithromycin in the second and third formulation.

**Table B.6:** Proniosome formula

| <b>Component of vesicle</b> | <b>Amount</b> |
|-----------------------------|---------------|
| Span 60                     | 200 mg        |
| Cholesterol                 | 100 mg        |
| Chloroform:methanol (2:1)   | 5 ml          |
| Roxithromycin               | 100 mg (2%)   |
| Sorbitol                    | 1 g           |

#### B.4.5.2.2 Outcome

The preparation of proniosomes formed a white powder very similar in appearance to the sorbitol powder. The grain size of the coated sorbitol powder was slightly larger than the original grain size of pure sorbitol. On hydration of the proniosomes, a good suspension formed with a milky appearance which was whiter and more opaque than the original niosomes.

#### B.4.5.3 Preparation of ufosomes encapsulating roxithromycin

The optimal ratio for the preparation of ufosomes in this study was sodium oleate:cholesterol:roxithromycin in a 2:1:1 ratio. This system was sonicated for 3.5 min after each preparation. It was very important that the pH of the PBS (pH 7.4) used was almost the same as the pKa of the oleic acid (pKa 7-9), as this property was vital for the spontaneous formation of ufosome vesicles (Chen & Szostak, 2004:988).

##### B.4.5.3.1 Method of preparation of ufosomes

Sodium oleate and cholesterol were dissolved in chloroform as referred to in Table B.7. Roxithromycin was added to the mixture and subsequently left to evaporate. The film was

hydrated with PBS (pH 7.4) (New, 1990:40-42; Walde *et al.*, 1994:11650). The ufosomes were sonicated for 3.5 min and left to stand for 2 h. The glassy amorphous ufosomes and the chloroform desolvated amorphous ufosomes were prepared in an identical manner in the same ratios. This resulted in three ufosome formulations.

**Table B.7:** Ufosome formula

| <b>Component of vesicle</b>                            | <b>Amount</b> |
|--|---------------|
| Sodium oleate  | 200 mg        |
| Cholesterol  | 100 mg        |
| Chloroform   | 5 ml          |
| Roxithromycin  | 100 mg (2%)   |
| PBS (0.06 M KH <sub>2</sub> PO <sub>4</sub> at pH 7.4) | 5 ml          |

#### **B.4.5.3.2 Outcome**

The ufosome formulation formed a good suspension with an off-white milky colour and no visibly large droplets present.

#### **B.4.5.4 Preparation of pro-ufosomes encapsulating roxithromycin**

Based on the tests done to determine optimal formulation of ufosomes, the ratio of pro-ufosomes was sodium oleate:cholesterol:roxithromycin (2:1:1) with a sonication of 3.5 min. It was identical to the ufosomes so as to prevent any unnecessary variation when comparing the vesicle systems to the provesicle systems.

##### **B.4.5.4.1 Method of preparation of pro-ufosomes**

Sodium oleate, cholesterol and roxithromycin monohydrate were dissolved in chloroform/methanol as referred to in Table B.8. The solvent was added to the sorbitol in drops and left to evaporate overnight at room temperature (25 °C). When reconstitution of pro-ufosomes took place, 5 ml PBS (pH 7.4) was added to the powder, mixed for 30 sec and sonicated for 3.5 min (New, 1990:40-42; Walde *et al.*, 1994:11650). The same procedure was followed for pro-ufosomes containing glass forms and chloroform desolvated forms of roxithromycin to yield three different pro-ufosome preparations.

**Table B.8:** Pro-ufosome formula

| <b>Component of vesicle</b> | <b>Amount</b> |
|-----------------------------|---------------|
| Sodium oleate               | 200 mg        |
| Cholesterol                 | 100 mg        |
| Chloroform:methanol (2:1)   | 5 ml          |
| Roxithromycin               | 100 mg (2%)   |
| Sorbitol                    | 1 g           |

#### **B.4.5.4.2 Outcome**

The pro-ufosomes formed a dry powder similar, but with larger grains than that of the sorbitol powder. The powder had an off-white/slightly yellow colour. When hydrated, they formed a good suspension with a milky off-white/beige appearance. The sample's droplets appeared more densely concentrated than the ufosomes and had a more opaque appearance.

#### **B.5 Conclusion**

There were a few steps in the process of determining the optimised formulas of the vesicle systems. The optimised formula was chosen based on the testing of important properties using TEM, light microscopy, droplet size distribution and entrapment efficiency of the possible formulas. The optimised formula for niosomes was Span 60:cholesterol:roxithromycin (2:1:1) as this formula showed better properties than the other contenders. The optimised ufosome formulation was sodium oleate:cholesterol:roxithromycin (2:1:1) as the properties of this formula were favourable. The formulas for the provesicular systems (proniosomes and pro-ufosomes) were identical to those of the vesicle systems. The reason for this being to minimise any factors which would affect further experimental results when comparing these formulas to each other in this study.

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## C.1 Introduction

Even though all vesicles were made according to the optimised formulations, it was still necessary to characterise each of the twelve final formulations containing the three different roxithromycin forms. The parameters used to characterise each formula were TEM, light microscopy, droplet size and distribution, zeta-potential, pH and EE%. Characterising the properties helped draw conclusions about the formulas and their results obtained after topical delivery of them through the skin. Before any characterisation testing took place, the stability of roxithromycin monohydrate was tested to determine if any drug degradation took place while it was in the vesicle solutions.

## C.2 Stability of roxithromycin and its two amorphous forms

A stability test was performed on roxithromycin monohydrate. There was no need to test the amorphous forms because in solution, HPLC analysis only detects the presence of an API and not its solid-state form. Roxithromycin monohydrate was prepared at a concentration of 1000 µg/ml, which was then injected in duplicate every hour for 24 h on the HPLC. This was done to determine if the API in the samples was stable or if any degradation took place. The full method used for the stability testing and the results were seen in Annexure A, Section A.4.5.2.

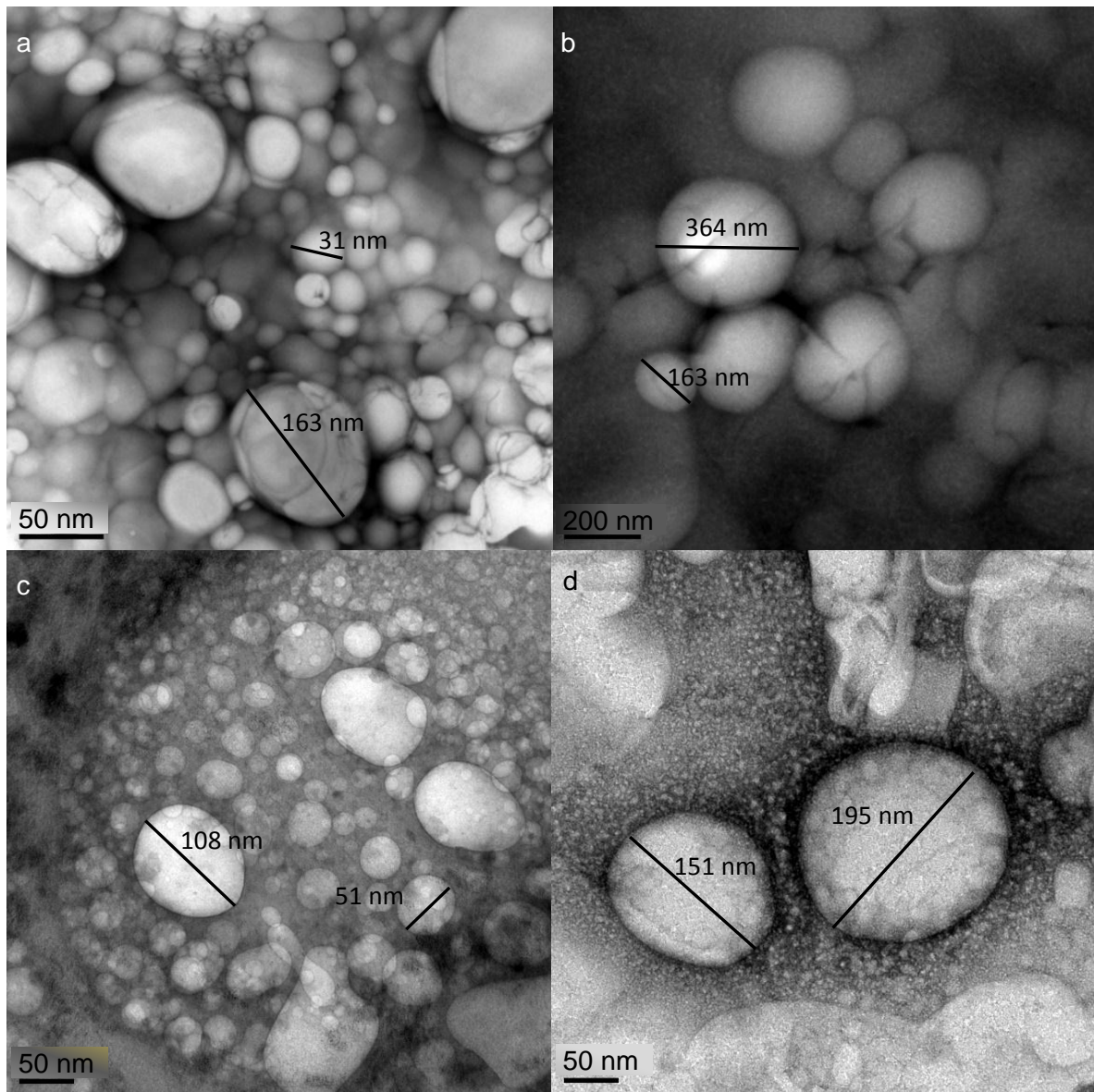
## C.3 Characterisation of vesicular systems encapsulating different forms of roxithromycin

### C.3.1 Transmission electron microscope (TEM)

The formation and morphology of the vesicle systems was viewed by the use of TEM (Ammar *et al.*, 2011:142). It was also used to verify the results of vesicle size measured on the Zetasizer (Mahale *et al.*, 2012:52; Varun, *et al.*, 2012:635). After many trial and error preparations, the final vesicle formulas were viewed using TEM. Only the preparation of the four vesicle types containing no drug could be viewed, as precipitation of API could cause damage to the microscope. A drop of hydrated vesicle (diluted 10x with distilled water) was placed on a carbon-coated 300 mesh copper grid and allowed to dry for 10 min. A drop of osmium was applied to the grid in order to stain the lipid films and thereafter left to air dry for 30 min. Samples were viewed using a FEI Tecnai G2 TEM (FEI, Holland) which was operated with the

expertise of Dr A Jordaan (Electron Microscopy Laboratory, NWU Potchefstroom Campus, G10 Building, Room G14) at 120 kV. Samples were viewed at magnifications of 5000 - 20000x.

### C.3.1.1 TEM results

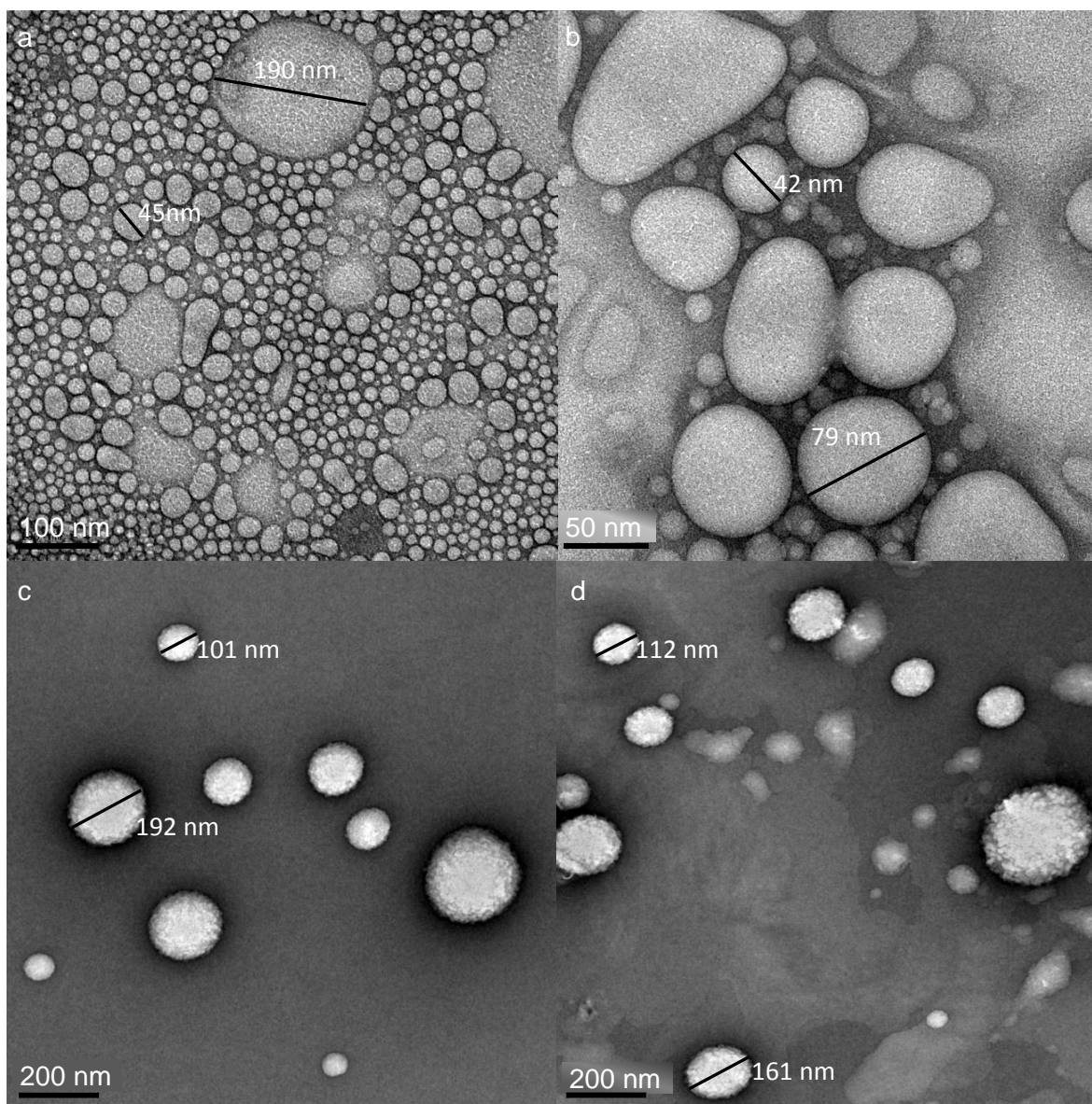


**Figure C.1:** TEM images of niosomes and proniosomes. Niosomes, (a) and (b), with no API sonicated for 2 min and proniosomes, (c) and (d), with no API sonicated for 2 min.

From Figure C.1, the photomicrographic results of the final formulations of niosomes and proniosomes, with no drug, can be seen. Many micrographs of each sample were taken, but only two of each were chosen as representative images. The sonicated niosomes, (a) and (b), showed a dense arrangement of various sized particles. The larger spherical particles appeared to have tiny cracks in them which may have been due to the fusion of many smaller niosomes. Images (c) and (d) show proniosomes with thick lipid membranes and particles of

various sizes in the nanometre range. The particle size of the proniosomes, in general, appeared slightly smaller than those of niosomes.

Figure C.2 shows ufosomes, (a) and (b), with the majority of particles smaller than 50 nm, although a few larger particles also existed. A large amount of particles were present and very densely packed. Images (c) and (d) were the pro-ufosomes with particles fairly far spread out from one another and fewer in number than the other vesicles. The surface of the particles were not smooth like the rest of the vesicles, but had a rather rough, bumpy appearance.



**Figure C.2:** TEM images of ufosomes and pro-ufosomes. Ufosomes, (a) and (b), containing no API sonicated for 3.5 min and pro-ufosomes, (c) and (d), sonicated for 3.5 min.

The shape of all vesicles is assumed to be spherical (Varun, *et al.*, 2012:636). The TEM showed spherical nano-vesicles with edges stained dark with osmium. This compared well to

the images of the shape of vesicles observed by Ammar *et al.* (2011:145) in their formulation of vesicles. The TEM pictures taken by Walde *et al.* (1994:11650) correlated with the results obtained from this study's TEM images having many diameters in the small nanometre range and a few in the larger nanometre range.

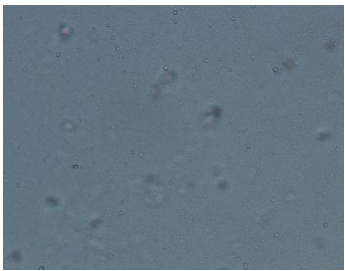
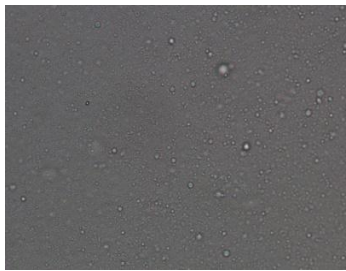
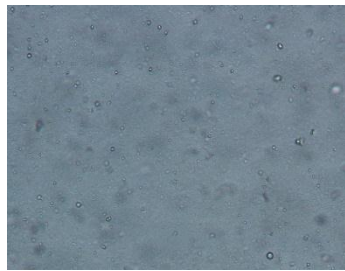
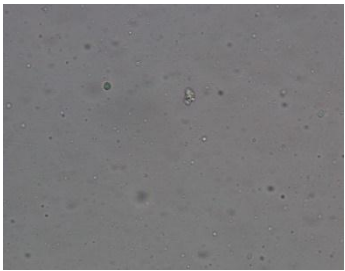
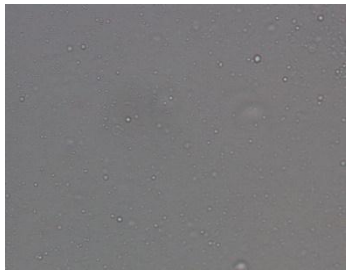
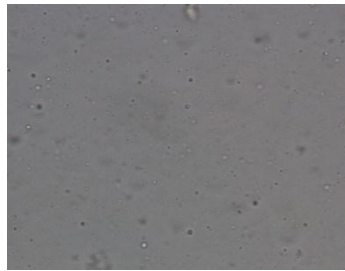
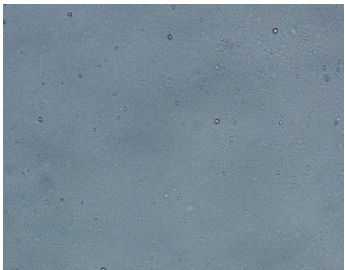

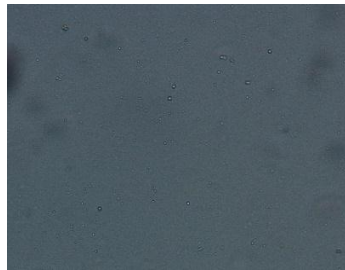

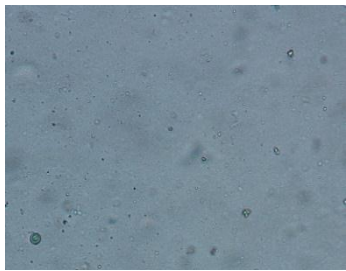
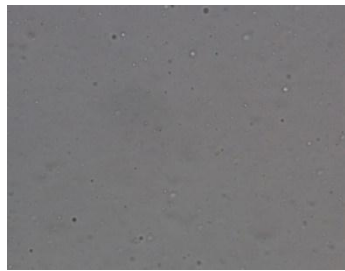
### **C.3.2 Light microscopy**

Light microscopy was done to prove that the formation of vesicles took place in all samples containing the different forms of API by observing the shape and lamellarity of the larger vesicles in a sample (Agarwal *et al.*, 2001:45; Walde *et al.*, 1994:11650). Light microscopy was performed with a Nikon Eclipse E4000 microscope (Nikon, Japan). A thin spread of the vesicle preparation (diluted 20x with distilled water) was placed on a 20 mm glass for microscopic viewing of the vesicles and micrographs were taken at 50x magnification. The method was repeated for many samples of all the vesicles.

#### **C.3.2.1 Microscopy results**

Many micrographs were taken, but only one is shown to represent each vesicle system. The light microscope showed the larger vesicles entrapping roxithromycin and its amorphous forms at a magnification of 50x. Table C.1 shows the twelve vesicles systems which formed tiny spherical particles floating in the solution. Although the particles could clearly be seen under the microscope, it may be more difficult to view them from these micrographs. Some particles are so small they could hardly be seen at this magnification strength. Niosome and proniosome particles were more visible with light microscopy than the ufosome and pro-ufosome vesicle systems, as the particles were generally larger.

**Table C.1:** Micrographs of vesicle systems viewed using light microscopy

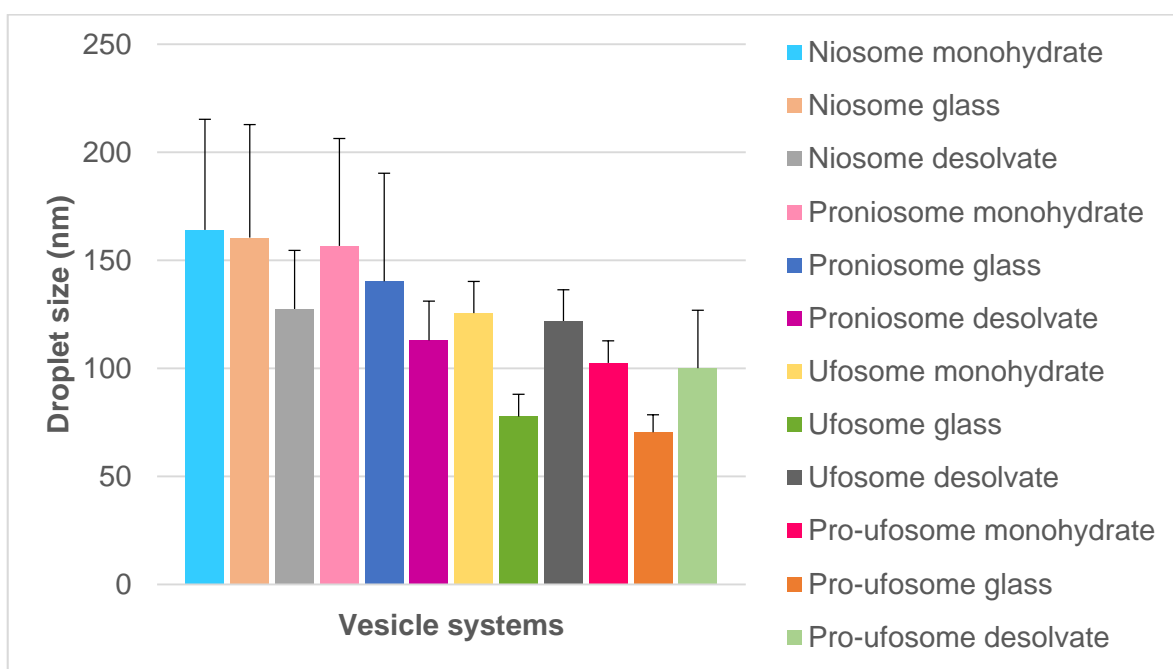
|             | Roxithromycin monohydrate   | Glass  | Desolvate   |
|-------------|---|--|---|
| Niosome     |    |    |    |
| Proniosome  |    |    |    |
| Ufosome     |   |   |   |
| Pro-ufosome |  |  |  |

### C.3.3 Droplet size and distribution

One of the most important parameters of vesicle systems is droplet size and size distribution (Shaji & Bhatia, 2013:154). Size has a large effect on the stability and encapsulation efficiency of the vesicle system (Kumar & Rajeshwarrao, 2011:213). The smaller the vesicles and the narrower their particle size distribution, the more stable the formula is. The size of the vesicles was determined by using the Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The freshly prepared vesicle samples were diluted approximately 20x with distilled water and 2 ml was injected into a plastic cuvette. Three readings were taken from a sample of each vesicle system and the total experiment was repeated in triplicate with fresh samples prepared each time.

### C.3.3.1 Droplet size and distribution results

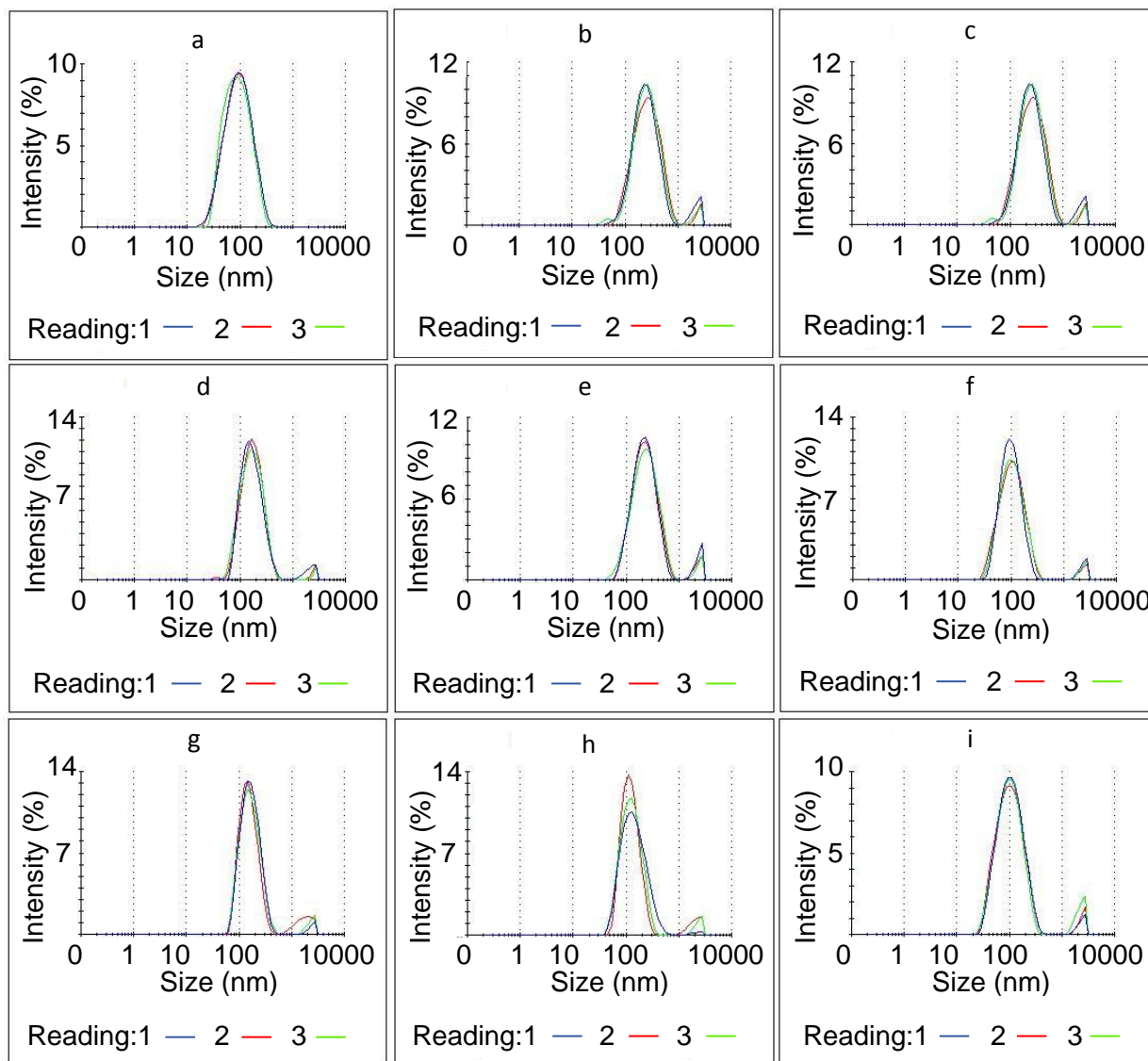
The droplet sizes of each of the twelve vesicle systems were measured in triplicate with new samples prepared for each experiment. During each test, three readings of each sample were taken to ensure a good average droplet size was obtained. Figure C.3 shows the average droplet size of each vesicle system. The droplet sizes of niosomes and ufosomes containing API were fairly similar to the droplet size during the testing of optimal vesicle formulas without API in Annexure B, Section B.4.2.3.1. During preformulation, optimal niosomes without API had an average droplet size of 123 nm, whereas during the characterisation of niosomes containing API (Figure C.3), the average droplet size ranged from 128 nm (niosomes desolvate) to 164 nm (niosomes roxithromycin monohydrate). The ufosomes without API had an average droplet size of 83 nm during optimal formulation, whilst ufosomes with API had an average droplet size ranging between 78 nm (ufosome glass) and 126 nm (ufosome roxithromycin monohydrate). The size results for ufosomes correlated well with the results reported by Morigaki & Walde (2007:77), who stated that fatty acid vesicles prepared from oleic acid produced unilamellar vesicles with an average particle size of 100 nm.



**Figure C.3:** Droplet size results of vesicle systems

From Figure C.3, it is also seen that the niosomes and proniosomes generally have a larger particle size than the ufosomes and the pro-ufosomes. This is attributed to the fact that niosomes and proniosomes were sonicated for 2 min to achieve a good droplet size distribution curve, whereas ufosomes and pro-ufosomes were sonicated for 3.5 min which resulted in smaller particles. With niosomes and proniosomes, the desolvate vesicles had the smallest particle size, whilst those with roxithromycin monohydrate had the largest particle size and with

ufosomes and pro-ufosomes, the glassy form had the smallest particle size and the roxithromycin monohydrate had the largest again. This being said, all particles were in the nanometre range (10 - 1000 nm) which was needed to enhance delivery of the API through the stratum corneum (Kumar & Rajeshwarrao, 2011:209).

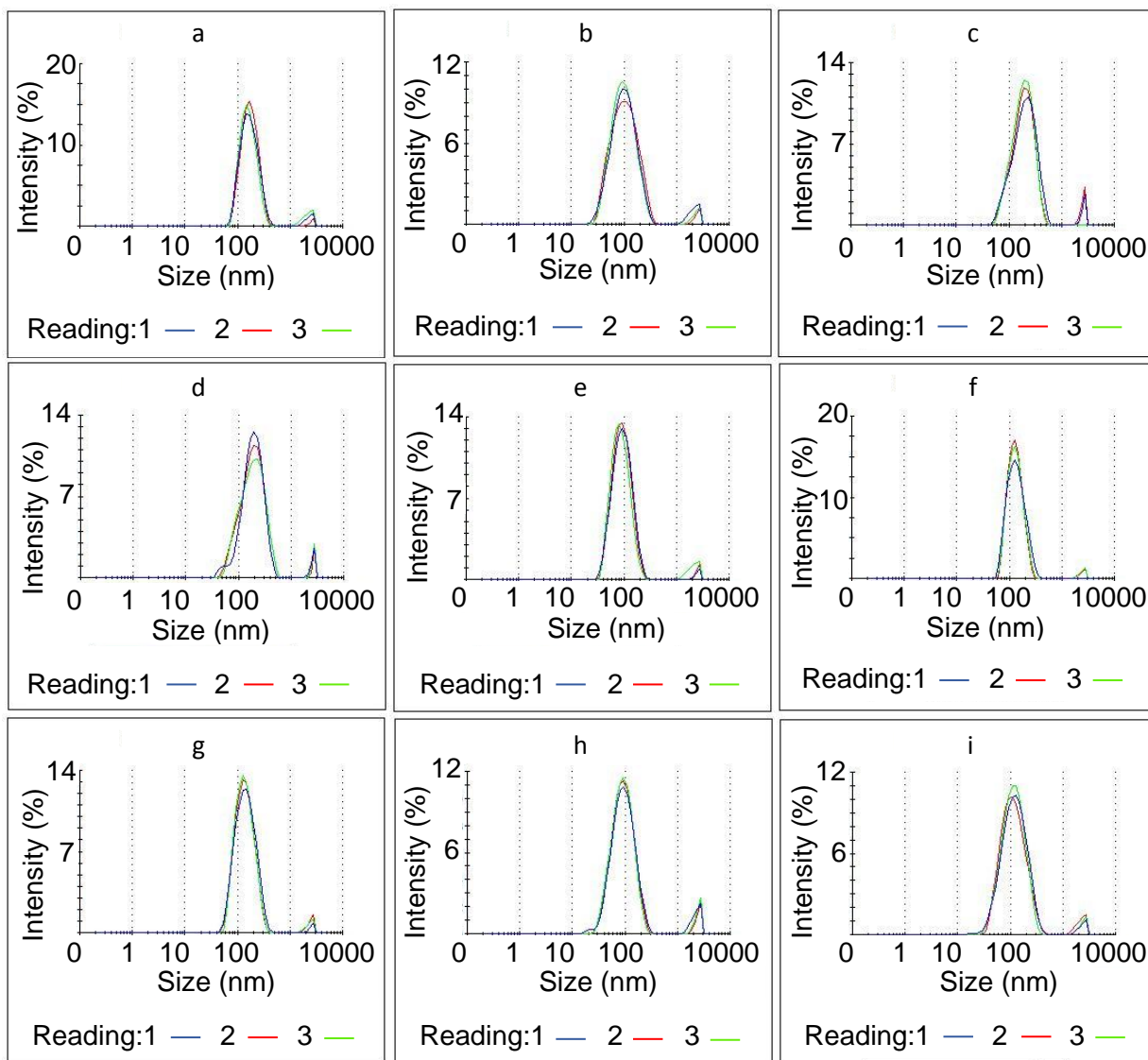


**Figure C.4:** Droplet size distribution curves of niosomes. (a), (b) and (c) are individually prepared niosome samples containing roxithromycin monohydrate, (d), (e) and (f) are individually prepared niosome vesicles encapsulating roxithromycin glass and (g), (h) and (i) are individually prepared niosome carrier systems with roxithromycin desolvate.

When the droplet size was determined, the droplet size distribution for all formulas were also portrayed in graphs which appeared as acceptable smooth curves representing each reading. Three samples of each vesicle preparation were made and three readings of each sample were done in order to achieve a good repeatability. The curves resembled those of niosomes and

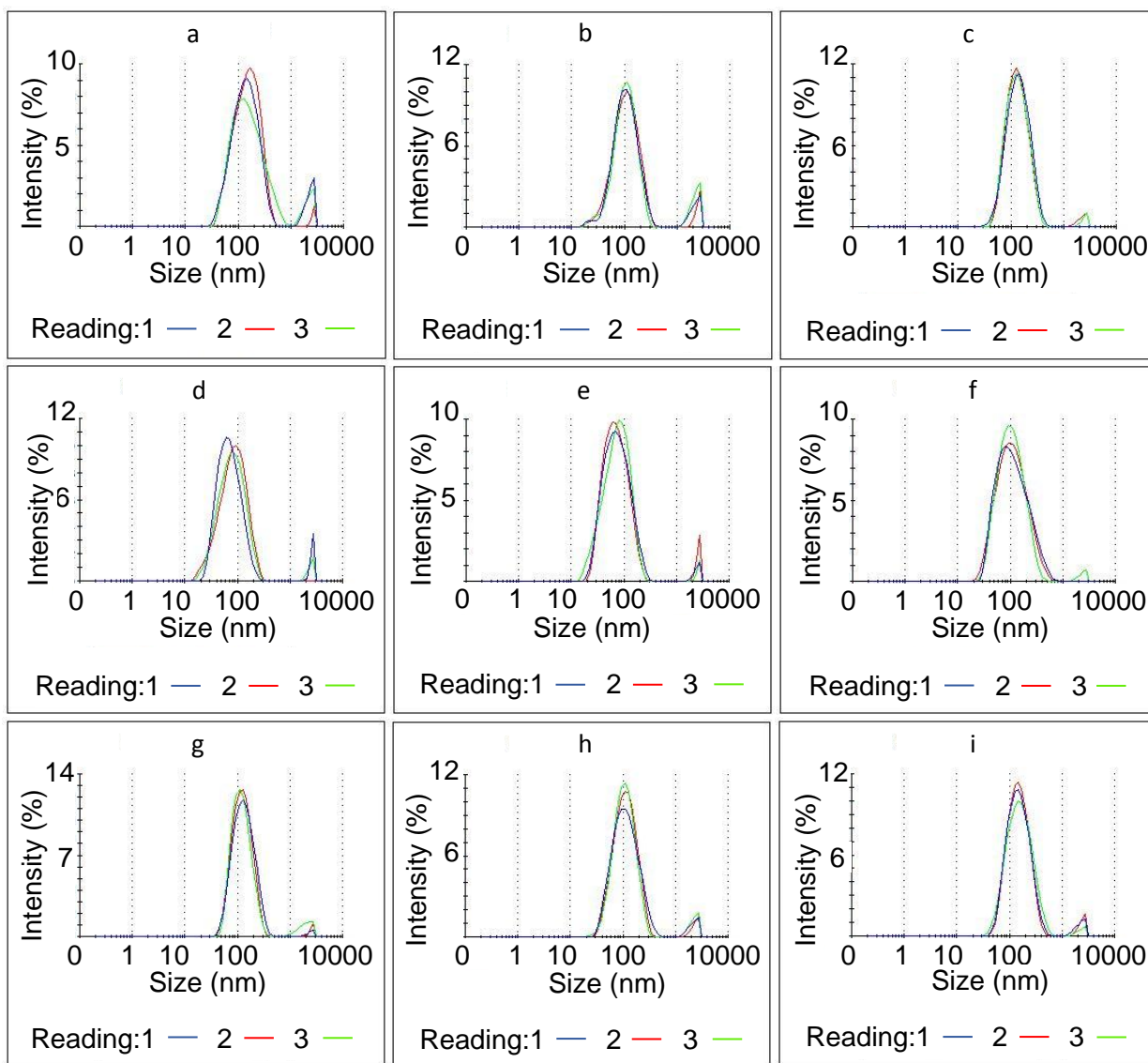
ufosomes without API in Annexure B, Section B.4.2.3.1, which were fairly narrow indicating that most of the particles were in close range of one another. Figure C.4 shows the size distribution of niosomes containing the three forms of roxithromycin.

The droplet size distribution of proniosomes containing roxithromycin solid-state forms are seen in Figure C.5.



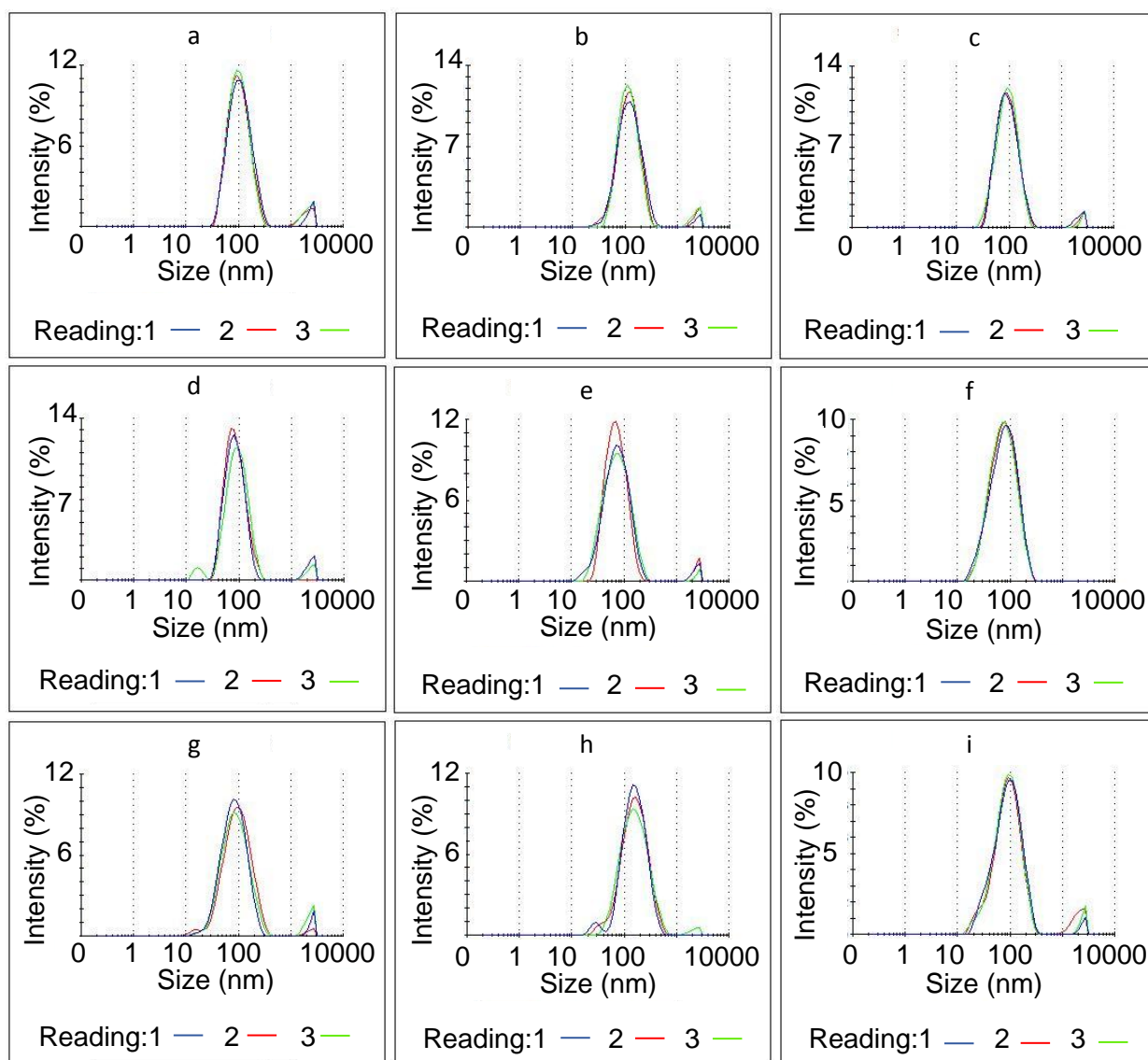
**Figure C.5:** Droplet size distribution curves of proniosomes. (a), (b) and (c) are individually prepared proniosome samples encapsulating roxithromycin monohydrate, (d), (e) and (f) are individually prepared proniosome vesicles containing roxithromycin glass and (g), (h) and (i) are individually prepared proniosome carrier systems encapsulating roxithromycin desolvate.

The results of the droplet size distribution of ufosomes are shown below in Figure C.6. Three samples of each ufosome containing a different solid form of roxithromycin is seen.



**Figure C.6:** Droplet size distribution curves of ufosomes. (a), (b) and (c) are individually prepared ufosome carriers encapsulating roxithromycin monohydrate, (d), (e) and (f) are individually prepared ufosomes containing amorphous roxithromycin glass and (g), (h) and (i) are individually prepared ufosome carrier systems encapsulating roxithromycin desolvate.

In Figure C.7, the droplet distribution of pro-ufosomes encapsulating the three solid forms of roxithromycin is seen.



**Figure C.7:** Droplet size distribution curves of pro-ufosomes. (a), (b) and (c) are individually prepared pro-ufosomes encapsulating roxithromycin monohydrate, (d), (e) and (f) are individually prepared pro-ufosome samples containing roxithromycin glass and (g), (h) and (i) are individually prepared pro-ufosome systems encapsulating amorphous roxithromycin desolvate.

Generally, all the graphs above had fairly narrow distribution curves. The TEM micrographs in Section C.3.1.1 confirmed the vesicle systems had a large variety of many different sized droplets, but the droplet size distribution showed that even though many different particle sizes existed in each system, the majority of particles were around the 100 nm size range which resulted in a good size distribution curve. According to Ammar *et al.* (2011:148), the size distribution of their niosomes was between 50 nm and 980 nm illustrating that even though the majority of droplets resulted in a good size distribution curve, there were still many particles of different sizes in the formulation. A smaller tail at the end of the curve was seen, which showed that each system had a number of large particles which could be the result of droplet

aggregation or a few large sediments present in the sample. Ammar *et al.* (2011:148) also found micro-particles which existed as a small tail at the end of the distribution curve and it was concluded that these particles were thought of as aggregations of the nano-sized particles. The difference in the size of vesicles in a systems can be due to vesicle fusion by surface interactions of some vesicles (Bayindir & Yuksel, 2010:2055). All three readings of each sample were represented by a different coloured line and from most graphs, it was seen that all three readings were very close and similar to one another. This showed that the results were repeatable and reliable for each sample.

### C.3.4 Zeta-potential

The zeta-potential was measured to determine the surface charge of each vesicle system (Kurakula, *et al.*, 2012:36). The charge of vesicle surfaces has a significant effect on the system's stability (Kumar & Rajeshwarrao, 2011:213). If the surfaces are highly charged, there is avoidance of aggregation and fusion of vesicles which ensure integrity and uniformity of the formulation (Agarwal *et al.*, 2001:44). The zeta-potential of the vesicle systems were calculated using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). A 20x dilution with distilled water was made of a newly prepared sample and 2 ml was injected into cuvettes. The zeta-potential measurements were taken on the same day as the sample preparation, with three readings per sample. The entire experiment with fresh samples was repeated in triplicate for a good average determination.

#### C.3.4.1 Zeta-potential results

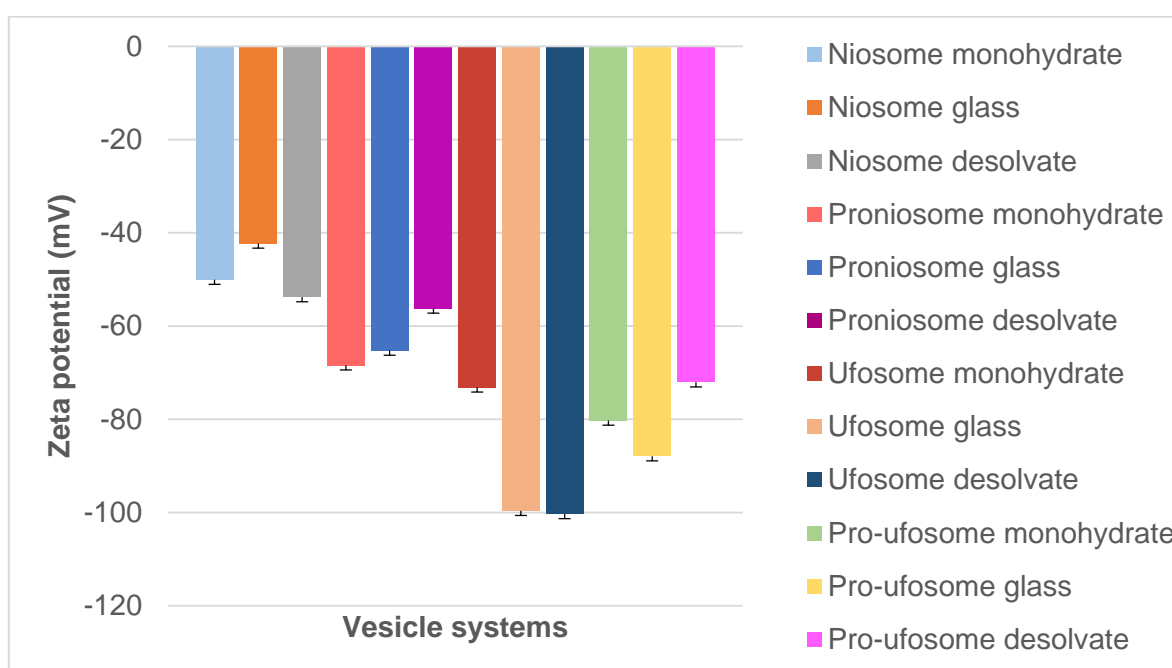


Figure C.8: Zeta-potential results of vesicle systems

From Figure C.8, it can be seen that the average zeta-potential for each vesicle system was highly negative. The values of all systems ranged from  $-42.28 \pm 13.70$  mV (niosomes glass) to  $-100.3 \pm 21.99$  mV (ufosomes desolvate), which showed good stability of all formulations. The results showed all the ufosomes and pro-ufosomes had higher negative zeta-potentials than the niosomes and proniosomes.

Zeta-potential results of vesicles, obtained by Manconi *et al.* (2011:40), compared well with the values obtained for the vesicle systems in this study. The zeta-potential values in their study were all highly negative (-47 to -69 mV). The more the negative charge of the vesicles increases, the more the particles repel one another which ensures minimal fusion of vesicles (Bayindir & Yuksel, 2010:2055). The highly negative values indicate good stability of vesicles with little chance of aggregation of particles (Manconi *et al.*, 2011:40). Even though all values show good stability, the zeta-potential values of the ufosomes and pro-ufosomes indicate it was less likely for them to aggregate, due to being more charged, than it was for the niosomes and proniosomes if any coagulation had to take place during studies.

### **C.3.5 pH determination**

The pH of the carrier system was measured in order to determine the lipid surface pH of the vesicles (Mahale *et al.*, 2012:52). The pH of the vesicular systems was measured with a Mettler Toledo Seven Compact pH meter (Mettler Toledo, Switzerland). The pH meter was calibrated at a pH of 4, 7 and 10 by submerging the probe into different buffer solutions. The pH of all the vesicle systems was measured by placing the probe in 5 ml of each of sample. Three readings were taken for each vesicle sample and the experiment was repeated with fresh samples twice more.

#### **C.3.5.1 pH determination results**

Table C.2 shows the average pH determined from all the samples of each vesicular system. From the table, it can be seen that the niosomes and proniosomes had a pH of about 7; the ufosomes and pro-ufosomes had an approximate pH value of 8.5. The pH of the ufosomes and pro-ufosomes was slightly higher than that of the niosomes and proniosomes and this may be due to the sodium oleate contained in the ufosomes instead of Span 60, as seen in the niosomes. The pH of all vesicles was in close range to the pH of the receptor phase (pH 7.4) during topical diffusion studies and this guaranteed no precipitation due to salt formation.

**Table C.2:** Average pH values of vesicle systems

| Vesicles     | API           | Average pH  |
|--------------|---------------|-------------|
| Niosomes     | Roxithromycin | 6.962 ±0.06 |
|              | Glass         | 7.156 ±0.11 |
|              | Desolvate     | 7.212 ±0.22 |
| Proniosomes  | Roxithromycin | 7.268 ±0.04 |
|              | Glass         | 7.262 ±0.06 |
|              | Desolvate     | 7.230 ±0.01 |
| Ufosomes     | Roxithromycin | 8.730 ±0.11 |
|              | Glass         | 8.516 ±0.23 |
|              | Desolvate     | 8.784 ±0.04 |
| Pro-ufosomes | Roxithromycin | 8.563 ±0.05 |
|              | Glass         | 8.458 ±0.39 |
|              | Desolvate     | 8.510 ±0.08 |

### C.3.6 Entrapment efficiency

The entrapment efficiency was used to determine the difference between the un-entrapped free drug and the total amount of drug in each sample, which yielded the amount of total drug entrapped (Jadon *et al.*, 2009:1188).

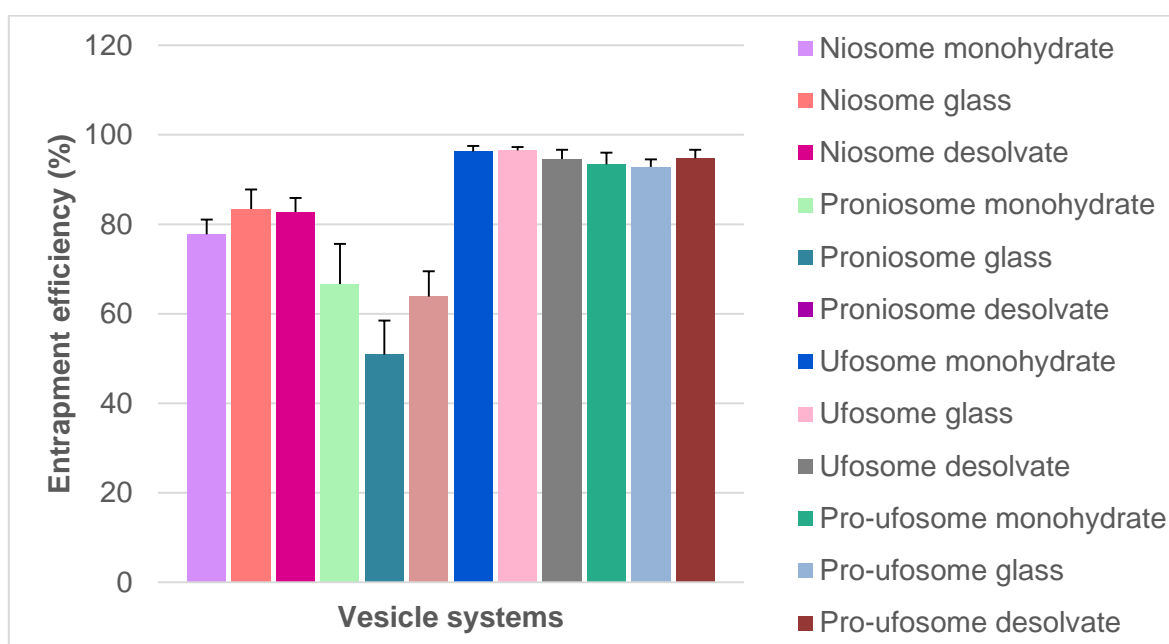
#### C.3.6.1 Entrapment efficiency using ultracentrifugation

Ultracentrifugation was used to test the EE% of the final vesicle formulas for this study (Mahale *et al.*, 2012:53). A Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) with a 50.2Ti fixed rotor was used to centrifuge the samples. Each sample was added to a suitable tube and diluted 25x with distilled water. They were loaded in the ultracentrifuge and left to run for 30 min at 25000 rpm to separate the free drug from the entrapped drug by the formation a pellet of vesicles at the bottom of each tube, leaving the free drug in the solution. When removed from the machine, 2 ml of the clear supernatant was removed from the tube and filtered through 0.45 µm PVDF filters into HPLC vials for HPLC analysis. Fresh samples were prepared for the experiment to be repeated in triplicate. A linear calibration curve was created with roxithromycin monohydrate standards. The entrapment efficiency was calculated using the same linear and entrapment efficiency equations as seen in Annexure B, Section B.4.4.2.

##### C.3.6.1.1 Entrapment efficiency results

Figure C.9 clearly shows the average entrapment efficiencies of all twelve vesicle systems in this study. Niosomes entrapping the different forms of API all had an average entrapment efficiency of approximately 80.00% with roxithromycin glass having the highest of

83.42 ± 4.37%. The proniosomes showed the lowest entrapment efficiency of all the vesicle systems with proniosome roxithromycin and desolvate having entrapments of 66.64 ± 8.99% and 63.83 ± 5.67%, while proniosome glass had the lowest entrapment of only 50.88 ± 7.61%. Ufosomes and pro-ufosomes showed the best entrapment efficiency, where averages were in very close range of each other with the lowest value of 92.81 ± 1.70% for pro-ufosome glass to the highest value of 96.51 ± 0.77% obtained by ufosomes containing the glassy amorphous form. The remainder of the ufosome and pro-ufosome EE% fall within these two values.



**Figure C.9:** Entrapment efficiencies of vesicular systems

The entrapment efficiencies for the ufosomes and the pro-ufosomes were considerably high. Lipid soluble drugs can have very high encapsulation of up to 100.00%, provided the drug content has not been overloaded to cause an overwhelming effect on the lipid membranes in the system (New, 1990:37). When comparing results with the entrapment efficiency determined for proniosomes by Ammar *et al.* (2011:149), it was seen that entrapment efficiencies ranged from 40.23% to 91.95%. The niosomes prepared by Bayindir & Yuksel (2010:2055), had entrapments of between 12.1% and 96.6% which proves that encapsulation values vary among studies and that very high entrapment efficiencies correlate with the high entrapment values for some of the formulations in this study.

To summarise, ufosomes had the best EE% followed closely by pro-ufosomes and then niosomes and lastly proniosomes, which showed the poorest entrapment of all the vesicles.

### C.3.6.2 Proof of identical methods

The minicolumn centrifugation method was used to determine the optimised vesicle formulations, as explained in Annexure B, Section B.4.4.2, adapted from the method used by New (1990:126-127). This method proved effective, but the ultracentrifugation method (Mahale *et al.*, 2012:52), later discovered in this study, was the preferred method to characterise the final vesicle formulas. The ultracentrifugation method had many advantages over the minicolumn centrifugation method, such as being faster in preparation before a run, more convenient to use and cheaper (Mahale *et al.*, 2012:53). The machinery was also more readily available in the laboratory and more samples could be loaded at one time.

A separate experiment was first done to prove that the ultracentrifugation method yielded the same results as the minicolumn centrifugation method when performed on the same samples, before it was used as the final method for characterisation. Three freshly prepared samples of niosomes and ufosomes were made and their entrapment efficiencies were tested according to the minicolumn centrifugation method used in Annexure B, Section B.4.4.2. Thereafter, the same three samples of each vesicle system were tested using the ultracentrifugation method discussed in Section C.3.6.1. The results of both methods were compared to determine if their results were the same and if the ultracentrifugation was accurate enough to continue using in the study.

#### C.3.6.2.1 Proof of identical methods results

The results of the entrapment efficiency of both methods were in very close range of each other, as seen in Table C.3.

**Table C.3:** Entrapment efficiency results comparing minicolumn centrifugation and ultracentrifugation methods

| Minicolumn centrifugation |              | Ultracentrifugation |              |
|---------------------------|--------------|---------------------|--------------|
| Vesicles                  | Average EE%  | Vesicles            | Average EE%  |
| Niosomes                  | 84.25 ± 4.95 | Niosomes            | 87.98 ± 3.49 |
| Ufosomes                  | 94.77 ± 3.57 | Ufosomes            | 96.60 ± 0.28 |

As illustrated in Table C.3, there is only about a 3% difference between the methods for the results obtained for average niosome EE% for the same sample. For the ufosomes, there was less than a 2% difference in average entrapment efficiency results. These results were adequate proof that the values were close and the new ultracentrifugation method would yield reliable results.

#### **C.4 Conclusion**

It is important to characterise vesicle systems in order to identify the properties each one has which may in turn affect their topical delivery through the skin. From the above results of microscopy, it can be seen all the formulations did form good spherical vesicles. The droplet size and distribution showed that all the systems were relatively stable and uniform in droplet size with ufosomes and pro-ufosomes having a smaller droplet size than niosomes and proniosomes. The very negative zeta-potentials gave evidence that there was no aggregation concern for any systems and the pH results showed that with the pH of the preparations, no unwanted interactions with the receptor phase during topical studies would occur. The entrapment efficiency gave a good indication of the amount of drug entrapped in each system, with ufosomes entrapping the most drug and proniosomes entrapping the least. These properties assisted in drawing conclusions in the further experimental results of this study.

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# Diffusion studies of vesicles containing roxithromycin and its amorphous forms

*Annexure*  
**D**

## D.1 Introduction

Topical delivery has great potential due to its non-invasive application of API delivery. Vesicles allow successful delivery into the layers of the skin and usually prevent systemic absorption of drugs (Varun *et al.*, 2012:632). The topical delivery route often results in very slow penetration of the API and in some cases, no delivery takes place at all. However, the rate and amount can, in some instances, be increased by incorporating the API into vesicles (Varun *et al.*, 2012:633). Due to the limited permeability of the skin, drugs are delivered topically by passive diffusion and the amount of API that reaches the target area is highly determined by the concentration of drug. It should also be noted that topical delivery is limited to drugs that require low doses for therapeutic effect, because only a small amount of API reaches the target area in the skin (Alexander *et al.*, 2012:28-29). The concentration of roxithromycin remained at 2% during all diffusion experiments in this study. The diffusion experiments performed were membrane release studies to determine the API release from the vesicles and transdermal diffusion studies followed by tape stripping to determine if roxithromycin was delivered transdermally and/or topically into the skin. The solubility and log D of the API was determined before the diffusion studies and HPLC was used to analyse all samples in this study.

## D.2 Methods

### D.2.1 Analysis of samples by high performance liquid chromatography

The concentration of the different roxithromycin forms in the receptor phase, the stratum corneum-epidermis and the dermis-epidermis samples were determined by HPLC analysis. It was done in a controlled environment at room temperature (25 °C). The method used in this study was developed by Dr Marique Aucamp and successfully validated for roxithromycin analysis, as discussed fully in Annexure A.

A Shimadzu UFLC (LC-20AD) chromatographic system (Kyoto, Japan) was used, with a Kinetex C<sub>18</sub> 150 × 4.6 mm column (5 µm particle size) (Separations, South Africa) as the stationary phase. The system is equipped with LabSolutions LC software for data analysis.

The mobile phase consisted of 0.06 M KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.4) and acetonitrile in a ratio of 1:1. The flow rate was 1 ml/min with an injection volume of 10 µl. The wavelength of detection was

205 nm and the retention time for roxithromycin was 4.1 min with an injection running time of 10.0 min. A new standard curve was produced for each analysis of new samples by injecting 1000 µg/ml of roxithromycin dissolved in PBS (KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) at different injection volumes.

### **D.2.2 Preparation of the donor phase**

The donor phase consisted of different vesicle systems encapsulating three different solid-state forms of roxithromycin for each experiment. The three solid-state forms used during this study included: the crystalline monohydrate, a glassy amorphous form and a chloroform desolvate amorphous form of roxithromycin. The three solid-state forms of roxithromycin were each encapsulated in either niosomes, proniosomes, ufosomes or pro-ufosomes. This resulted in a total of twelve formulations. The preparation of these formulations, used in the donor phases, were discussed in Annexure B, Section B.4.5. The preparation of a vesicle system sample was repeated three times and added together to achieve a 15 ml donor phase, which was needed to divide among twelve Franz cells in each experiment.

### **D.2.3 Preparation of the receptor phase**

The receptor phase for the membrane and skin diffusion studies consisted of the same PBS (KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) used to make the vesicles. A 0.06 M KH<sub>2</sub>PO<sub>4</sub> buffer dissolved in distilled water with a pH of 7.4 was made in a volume of 2.5 l for the membrane release studies and a fresh 2.5 l was made for all studies of the skin diffusion. The receptor phase was kept for a short time in the refrigerator (2-5 °C), between experiments, in order to remain fresh.

### **D.2.4 Aqueous solubility of roxithromycin and amorphous forms**

The solubility of roxithromycin in PBS (pH 7) was tested to determine how soluble the API was in a PBS solution. For the intended studies, it was considered important that roxithromycin had good solubility in PBS. An excess amount of roxithromycin monohydrate, the glassy amorphous form and the chloroform desolvated form was added to 5 ml of PBS (pH 7) in separate polytops to obtain saturated solutions of each. The temperature of the water bath was set at 32 °C (temperature of skin during a diffusion study) and the polytops containing the saturated solutions of each roxithromycin form and magnetic stirrers were placed into the water bath. The samples were agitated for a period of 24 h at 750 rpm. The solution was checked regularly for a precipitate which indicated a saturated solution still existed. When the samples were removed after 24 h, they were filtered through 0.45 µm PVDF filters into HPLC vials. Subsequently the resulting solutions were analysed by means of HPLC to determine the API concentration in each solubility sample. A standard solution of 1000 µg/ml roxithromycin monohydrate was prepared in order to obtain a standard curve for the analysis. The experiment was done in triplicate.

### D.2.5 *n*-Octanol-buffer partition coefficient of roxithromycin and amorphous forms

The log D of roxithromycin was determined in order to establish if the API was better soluble in an aqueous phase (PBS) or a lipophilic phase (octanol). Equal volumes of the PBS (pH 7) and *n*-octanol were added to a beaker and mixed vigorously for 24 h with a magnetic stirrer for co-saturation to occur in both phases. The mixture was then added to a separating funnel and allowed to separate for 24 h. Each volume was then tapped off into separate containers. Roxithromycin monohydrate (15 mg) and its two amorphous solid-state forms were added to 3 ml of the pre-saturated *n*-octanol phase in separate test tubes and rotated in a water bath for 10 min at 32 °C. 3 ml of the pre-saturated PBS (pH 7) was then added to each test tube and left to rotate for a further 45 min. Each test tube was then removed and centrifuged at 4000 rpm for 20 min. The octanol-phase and the PBS-phase of each test tube were separated into different polytops and the polytops containing the octanol-phases were diluted 10x with methanol. Each volume of *n*-octanol/methanol and PBS (pH 7) in the polytops was filtered through a 0.45 µm PVDF filter and placed in vials, after which HPLC was used to determine the concentration of API in each separated phase. A standard was prepared with a concentration of 500 µg/ml in a PBS:methanol (50:50) solution in order to produce an appropriate standard curve for analysis. The experiment was repeated in triplicate.

The distribution coefficient was calculated as the ratio of the concentration of drug in the *n*-octanol-phase to that in the buffer-phase, as seen in Equation D.1.

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

### D.2.6 Membrane release studies

Membrane release studies were done to determine if the monohydrate, glassy form of roxithromycin and desolvated amorphous form were released from the four vesicle preparations. Twelve vertical Franz cells were used in each release study, of which two were used as control cells. In total, twelve membrane release studies were performed, with each experiment having a different vesicle and form of roxithromycin encapsulated.

The process of the membrane release study took place as follows:

- The donor phase (vesicle preparation) was prepared and placed in a water bath at 32 °C to mimic the temperature of the skin's surface.
- The receptor phase (PBS at pH 7.4) was prepared and placed in the water bath at 37 °C in order to mimic the temperature of the blood in the body.

- Dow Corning® (Sigma-Aldrich, Germany) high vacuum grease was applied to the donor and receptor compartments of the Franz cells and a magnetic stirrer placed into the receptor compartment.
- PVDF membrane filters, with a pore size of 0.45 µm (Pall® Life Sciences, Michigan, USA), were cut into small circles of 15 mm in diameter and placed on the lower receptor compartment.
- The donor compartment was mounted onto the receptor compartment and sealed with vacuum grease.
- A horseshoe clamp was fitted to each cell to hold the two compartments together and prevent leakage of the cells.
- 2 ml of the PBS (pH 7.4) was injected (with the absence of air bubbles) in the receptor compartments of the cells and marked with a permanent marker at the 2 ml point.
- 1 ml of the API vesicle preparation was placed in the donor compartment and covered with Parafilm® to avoid loss of the API.
- The cells were placed on a Variomag® magnetic stirrer plate (Variomag, USA) in a Grant® water bath (Grant Instruments, UK) with the temperature constantly maintained at 37 °C. The receptor compartments were fully submerged in the water bath to maintain the controlled receptor phase temperature (37 °C), which resulted in the donor compartments retaining the skin's surface temperature (32 °C) (Williams, 2003:62). The magnetic stirrer was constantly in motion at 750 rpm.
- The receptor phase was extracted and refilled with new PBS (pH 7.4, 37 °C) every hour for 6 h, with extraction times at 1, 2, 3, 4, 5 and 6 h, respectively.
- HPLC analysis mentioned in Section D.2.1 was used to analyse the extracted samples after being filtered through 0.45 µm PVDF filters and the API concentration which permeated through the membrane into the receptor phase was determined.

### **D.2.7 Preparation of the skin for the diffusion studies**

The skin used for the transdermal diffusion studies was female Caucasian skin obtained after plastic surgery of the abdominal area. Approval for the use of the skin was given by the Research Ethics Committee of the North-West University (Reference: NWU-00114-11-A4). Before collection of the skin took place, willing patients signed a consent form in which their information remained confidential. Within 24 h of the surgery, the fresh skin was collected and transported in an icebox to the North-West University, Potchefstroom.

A Dermatome™ (Zimmer LTD, United Kingdom) was used to obtain strips of full-thickness skin (thickness of 400 µm) from the collected skin. Each strip was placed on Whatman® filter paper and left to dry. The filter paper holding the skin was then enclosed in tinfoil and stored in a freezer (- 20 °C) until used. The remaining subcutaneous fat layer of the skin was discarded appropriately. Before the use of the skin for the diffusion study, it was removed from the freezer and inspected for imperfections. Due to the short supply of skin, the same donor was not used for all twelve diffusion studies, but all the Franz cells in each diffusion experiment contained skin from the same donor. The skin samples were kept for no longer than six months.

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

The skin diffusion studies were done to determine the topical delivery of the roxithromycin and its amorphous forms. Each diffusion study consisted of twelve cells in total, two being control cells with no API and ten being test samples with API. The skin diffusion study was repeated twelve times because of the twelve formulas that were tested.

The same method discussed during the membrane release studies (Section D.2.6) was applied in the skin diffusion experiments, except circles of dermatomed abdominal skin were used instead of the PVDF membrane filters. Circles with a diameter of 15 mm were cut out of the previously prepared skin on the Whatman® filter paper and one circle was placed on the receptor compartment of the Franz diffusion cell with the stratum corneum facing upwards to the donor compartment. The Franz cells were assembled in the same way and kept in the same controlled conditions as discussed in the membrane release studies. The receptor phases of the skin diffusion study were only extracted once after 12 h based on the results obtained from a pilot study. Each sample's receptor phase solution was analysed by HPLC, as stated in Section D.2.1, to determine if roxithromycin had permeated through the skin.

#### **D.2.9 Tape stripping**

Tape stripping of skin samples is done for the purpose of removing the stratum corneum from the skin (Surber *et al.*, 1999:395). Tape stripping was done to determine how much of the API was present in the skin from the topical formulation (Ademola, 1997:532). As the study focused on the topical delivery of roxithromycin, tape stripping and testing API in the epidermis-dermis of the skin was a good indication of whether the API delivered topically after a 12 h diffusion study.

After the skin diffusion experiment, the skin was removed from the Franz cells. A circle of skin was pinned on wax paper fixed to a hardboard. The diffusion area of the skin was visible due to the indent made by the Franz cells. The skin was dabbed with tissue paper to remove any excess donor phase. 3 M Scotch® Magic™ tape was used to perform the tape stripping. One strip was placed on the diffusion area of the skin and discarded to ensure the surface of the skin

was clear of the donor phase. The next fifteen strips were used to remove the stratum corneum from the skin samples. A glistening appearance over the skin was an indication of complete removal. All tape strips were placed in a polytop containing 5 ml ethanol. The diffusion area of the skin was also cut into small pieces and placed in a polytop containing 5 ml of ethanol. The tape strips and the skin were left for 8 h in a fridge (2 – 5 °C) for the API to be extracted from the tape and epidermis-dermis skin. The process was repeated for all twelve Franz cells. The ethanol from all the polytops was removed and filtered into vials for HPLC analysis as discussed in Section D.2.1. The process was repeated after all twelve skin diffusion experiments for every vesicular system containing a solid-state form of the API.

#### **D.2.10 Data analysis of the diffusion studies and statistical analysis**

The data obtained from the skin diffusion studies was used to calculate the percentage of API which had diffused into the epidermis-dermis over a 12 h period and to make comparisons as to which vesicle system and form of roxithromycin had the best topical delivery into the epidermis-dermis. The data used for statistical evaluation was the results obtained from the epidermis-dermis, as it was the only phase in which the API had significant concentrations for all experiments.

The quantile-quantile graphs obtained from this study did not show normality of data and hence the decision was made to perform non-parametric tests instead of parametric tests (used for normally distributed data). The non-parametric tests performed were the Kruskal-Wallis analysis of variance (ANOVA) to compare the different formulations, followed by multiple comparisons amongst pairs of formulations. Formulations were said to have a significant difference if the p-value was smaller than 0.05 ( $p < 0.05$ ). These results showed the formulations were significantly different, but there was no indication of where the differences were. To determine where the significant differences lay, a multiple comparison test was performed on the groups of data. By use of the p-values, a significant difference between two formulas containing API could be seen.

### **D.3 Results and discussion**

#### **D.3.1 Aqueous solubility**

As mentioned previously, Aucamp *et al.* (2013:26) stated the experimental equilibrium aqueous solubility of roxithromycin monohydrate was 0.0335 mg/ml in water at 25 °C. In this study it was found that roxithromycin's experimental aqueous solubility in PBS (pH 7) at 32 °C was 1.98 mg/ml for roxithromycin monohydrate, 1.88 mg/ml for the glass amorphous form of roxithromycin and 1.92 mg/ml for the chloroform desolvate. The difference in solubility in comparison to Aucamp *et al.* may be attributed to the increased temperature used in this study

and the different mediums used, i.e. PBS (pH 7) instead of water. According to Williams (2003:37), these solubility values of above 1 mg/ml indicate that roxithromycin is a promising candidate for successful topical delivery.

When referring to the patents of Aucamp *et al.*, mentioned in Chapter 3, Section 3.4.2.3, it was expected that the two amorphous forms of roxithromycin would exhibit much higher aqueous solubilities than the roxithromycin monohydrate, but all values were more or less similar. Brittain & Grant (1999:281) stated that the solubility of drugs may differ when they are in different solid-state forms, but it is evident, from the close range in values above, that the two amorphous forms may have converted back to the more stable crystalline form while in solution and hence had similar aqueous solubility (Purohit & Venugopalan, 2009:891).

### **D.3.2 *n*-Octanol-buffer distribution coefficient**

Drugbank (2013) reported the predicted log P of roxithromycin was 2.9, but that the experimental log P was reported to be 1.7. In this study, the log D, rather than the log P, was determined for roxithromycin and its two amorphous forms. The results showed roxithromycin monohydrate, glassy amorphous form and chloroform desolvated amorphous form all gave the same log D value of 1.52. This draws the conclusion that a larger concentration of roxithromycin was found in the *n*-octanol phase, proving that roxithromycin is a lipophilic API. The value obtained in this study correlates well with the experimental value of 1.7 reported by Drugbank (2013). It is optimal for topical delivery if an API has a log P of 1 to 3 (Williams, 2003:36), therefore roxithromycin's log D (1.52) was within the optimal range.

The slight difference in the values obtained in this study, compared to the values of Drugbank, may be due to the differences in buffer compositions and the fact that log D was determined instead of log P.

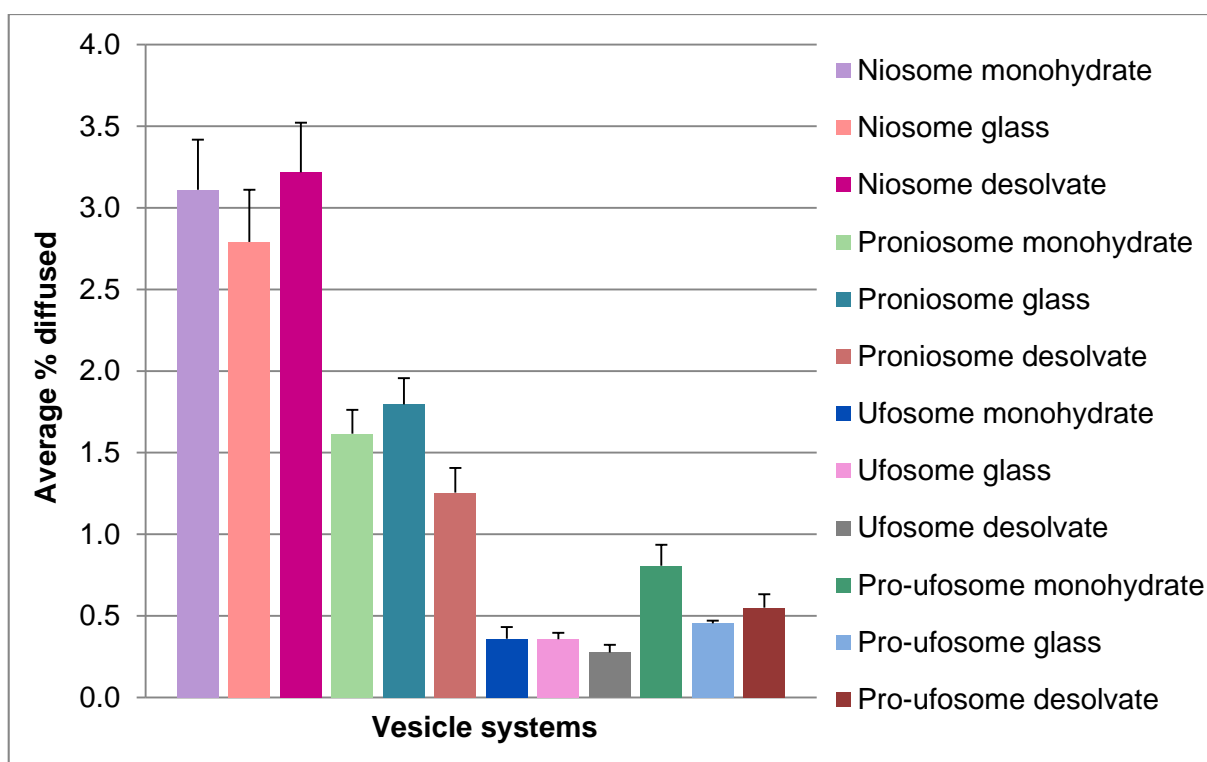
### **D.3.3 Membrane release studies**

The aim of performing the membrane release studies was to determine if all the API forms were released from their formulations. All twelve vesicle systems had API release through the PVDF membranes. Table D.1 shows the average flux of all twelve formulations. The flux indicates the concentration of API per area per hour which diffused through the membrane during the 6 h release studies. The values show the formulations with highest flux were the niosomes roxithromycin monohydrate, niosomes chloroform desolvate and niosomes glass. The proniosomes glass, proniosomes monohydrate and proniosomes desolvate followed, with only small amounts released with the pro-ufosome monohydrate, pro-ufosome chloroform desolvate, pro-ufosome glass, ufosome glass, ufosome monohydrate and ufosome desolvate. To

generalise, the niosomes had the leading flux, followed by the proniosomes, with the pro-fosomes and ufosomes having the least average amount released per hour for 6 h.

**Table D.1:** Comparative summary of the flux values ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) obtained for the different API forms released from their formulations during the membrane release after 6 h

| Formulation                           | Average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) |
|---------------------------------------|---|
| Niosome roxithromycin monohydrate     | $184.54 \pm 19.32$                                      |
| Niosome glass                         | $169.86 \pm 18.29$                                      |
| Niosome desolvate                     | $183.50 \pm 18.45$                                      |
| Proniosome roxithromycin monohydrate  | $90.14 \pm 8.47$  |
| Proniosome glass                      | $112.34 \pm 10.12$                                      |
| Proniosome desolvate                  | $75.62 \pm 10.42$                                       |
| Ufosome roxithromycin monohydrate     | $20.75 \pm 3.87$  |
| Ufosome glass                         | $21.29 \pm 2.36$  |
| Ufosome desolvate                     | $17.58 \pm 2.89$  |
| Pro-ufosome roxithromycin monohydrate | $56.29 \pm 10.64$                                       |
| Pro-ufosome glass                     | $25.72 \pm 1.39$  |
| Pro-ufosome desolvate                 | $29.22 \pm 4.12$  |



**Figure D.1:** Results of the %API released during release studies conducted on vesicle systems containing different forms of roxithromycin after 6 h.

Figure D.1 shows the average %API diffusion results obtained from the membrane release studies of the different forms of roxithromycin encapsulated in vesicles. The vesicle system with the best %release was, by far, the niosomes followed by the proniosomes, then the profosomes and lastly the ufosomes. It is evident from Figure D.1 that the type of vesicle had a larger impact on the release than the form of roxithromycin encapsulated by each vesicle. It can clearly be seen that there are four definite levels (vesicle systems) and the values within each vesicle system are fairly close to one another. The niosomes had the highest %API released with niosome desolvate ( $3.22 \pm 0.30\%$ ), followed by niosome monohydrate ( $3.11 \pm 0.31\%$ ) and niosome glass ( $2.79 \pm 0.32\%$ ). With proniosomes, the glass's release was the highest ( $1.80 \pm 0.16\%$ ), followed by the monohydrate ( $1.62 \pm 0.15\%$ ) and the desolvate ( $1.26 \pm 0.15\%$ ). The values of the pro-ufosomes fell considerably, with the monohydrate form ( $0.81 \pm 0.13\%$ ) being higher than the desolvate ( $0.55 \pm 0.08\%$ ), followed by the glassy form ( $0.45 \pm 0.02\%$ ). The ufosomes were the vesicle system with the least amount of API released through the PVDF membranes. They were all very close in range, with the monohydrate ( $0.36 \pm 0.07\%$ ) and glass ( $0.36 \pm 0.04\%$ ) similar, followed by the smallest value of the whole study, ufosome desolvate ( $0.28 \pm 0.05\%$ ).

It is important to keep in mind that the vesicle formulas with the best release through the PVDF membranes did not imply that those same formulas would also have the best skin diffusion results and there was no direct correlation between the results obtained from both studies. The membrane release results indicated that all vesicle systems had successful API release. Release from the formulation was thus ruled out as cause, if no topical/transdermal delivery of the API took place.

#### **D.3.4 Franz cell diffusion studies**

The aim of the diffusion studies and tape stripping was to determine if roxithromycin and its amorphous forms were delivered transdermally and/or topically by the vesicle systems. The aim was to achieve topical delivery, as roxithromycin targets the acne causing bacteria colonising in the dermis layer of the skin (Gollnick, 2003:1585). The study was able to distinguish if roxithromycin was more concentrated in the receptor phase (blood plasma), the stratum corneum-epidermis or the epidermis-dermis. The results of all three potential API targets are discussed below.

##### **D.3.4.1 Transdermal diffusion**

The results obtained from analysing the API concentration showed that the drug, in general, did not reach the receptor phase. This being said, four of the twelve vesicle systems did deliver very small amounts of roxithromycin to the receptor phase. Niosomes encapsulating glass ( $n = 7$ ) and niosomes with desolvate ( $n = 10$ ) had an average %API diffusion of  $0.0053 \pm 0.0003\%$  and  $0.0031 \pm 0.0007\%$ , respectively. Proniosomes containing roxithromycin

(n = 10) and pro-ufosomes encapsulating roxithromycin (n = 10) had an average % diffused of  $0.0133 \pm 0.0035\%$  and  $0.0104 \pm 0.0051\%$ , respectively. These four percentages were extremely small, but they were worth mentioning because their HPLC analysis peaks were large and above the LOD and LLOQ values mentioned in Annexure A, Section A.4.2. The reason for the API reaching the receptor phase for only four of the vesicle systems could be due to the fact that for each experiment performed, Caucasian skin from a different donor was used as there was not enough skin from one donor for all twelve diffusion studies. Different degrees of skin hydration, age or damaged skin (due to UV exposure or chemicals used on skin) from different skin donors would result in differences in API concentration in the receptor phases (Williams 2003:14, 22). Although all skin was dermatomed in the same way, as mentioned in Section D.2.7, no skin is the same and the physical integrity (affected by age, health conditions, skin care routine and environmental factors), strength, thickness of stratum corneum, stratum corneum damage and moisture content may have been factors which caused the slight differences in API delivery into the epidermis-dermis (Dayan, 2005:68; Hwa *et al.*, 2011:466-467). Variances can also result from potential freeze thaw damage to the extracellular lipids in the stratum corneum and epidermal layer while being stored in the freezer at  $-2 - 5\text{ }^{\circ}\text{C}$  before use (Menon, 2002:14).

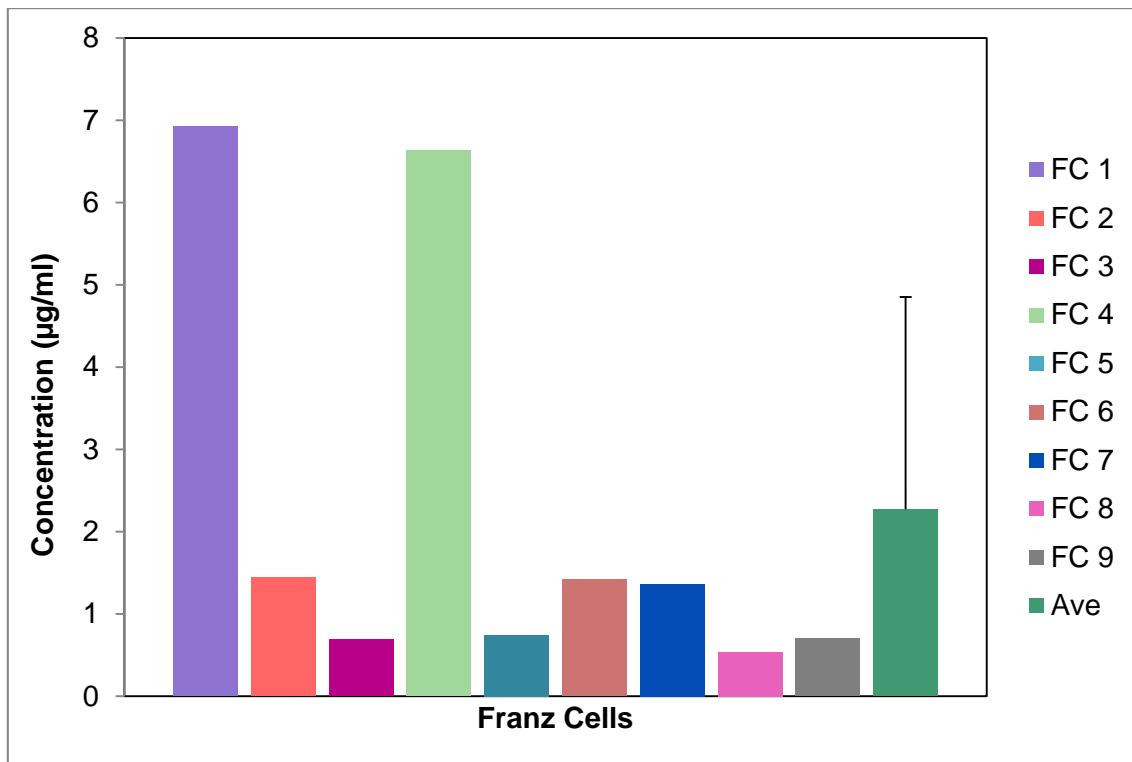
This study focused on the topical delivery of roxithromycin and not the transdermal delivery, hence, the target area for the API was the epidermis-dermis and not the blood stream and therefore the results obtained for the receptor phase was the preferred outcome.

#### **D.3.4.2 Tape stripping**

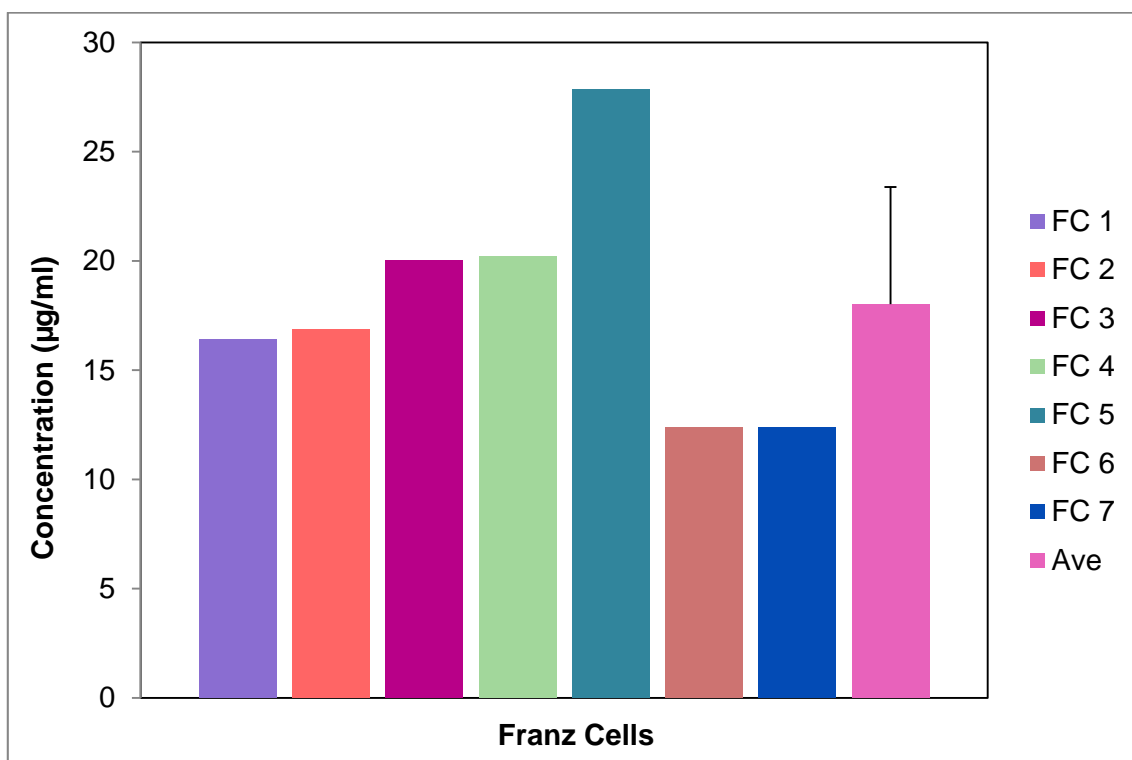
The results obtained from analysis of the tape strips concluded the API was not targeted in the stratum corneum-epidermis. All twelve experiments showed small traces of roxithromycin, but the peaks of each analysis were below the LOD and LLOQ calculated in Annexure A, Section A.4.2. This means that although small peaks were detected, the concentration of API was lower than the LLOQ, meaning the results could not be quantified to yield numerical values (ICH, 2005:5). These peaks were interrupted by noise and were ruled out of the study as unreliable results.

#### **D.3.4.3 Epidermis-dermis**

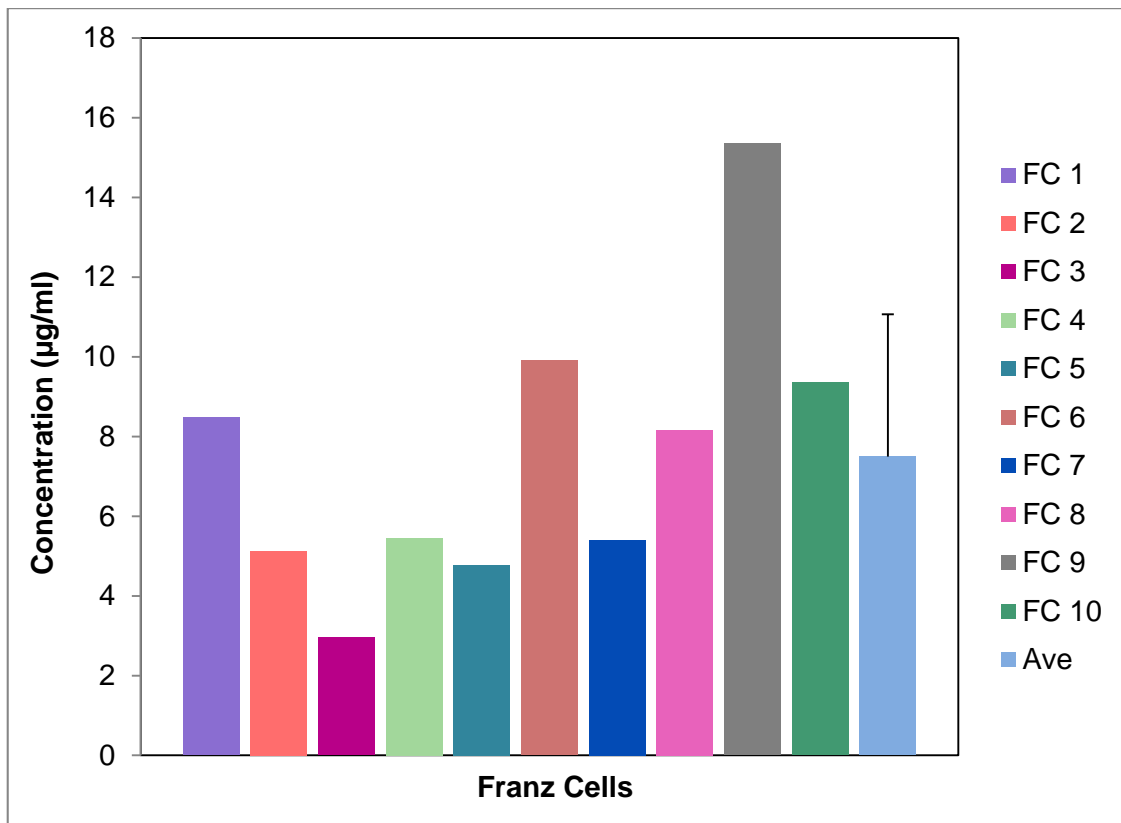
The intended and predicted target site for roxithromycin encapsulated in vesicles was the epidermis-dermis. The results obtained through HPLC analysis showed all twelve formulations successfully contained API in their epidermis-dermis layers. Figures D.2 - D.13 illustrates the results of the average API concentration obtained in the epidermis-dermis of each diffusion study, after a 12 h period, on the various vesicles containing the different forms of API. In each graph, FC is the abbreviation for Franz cell.



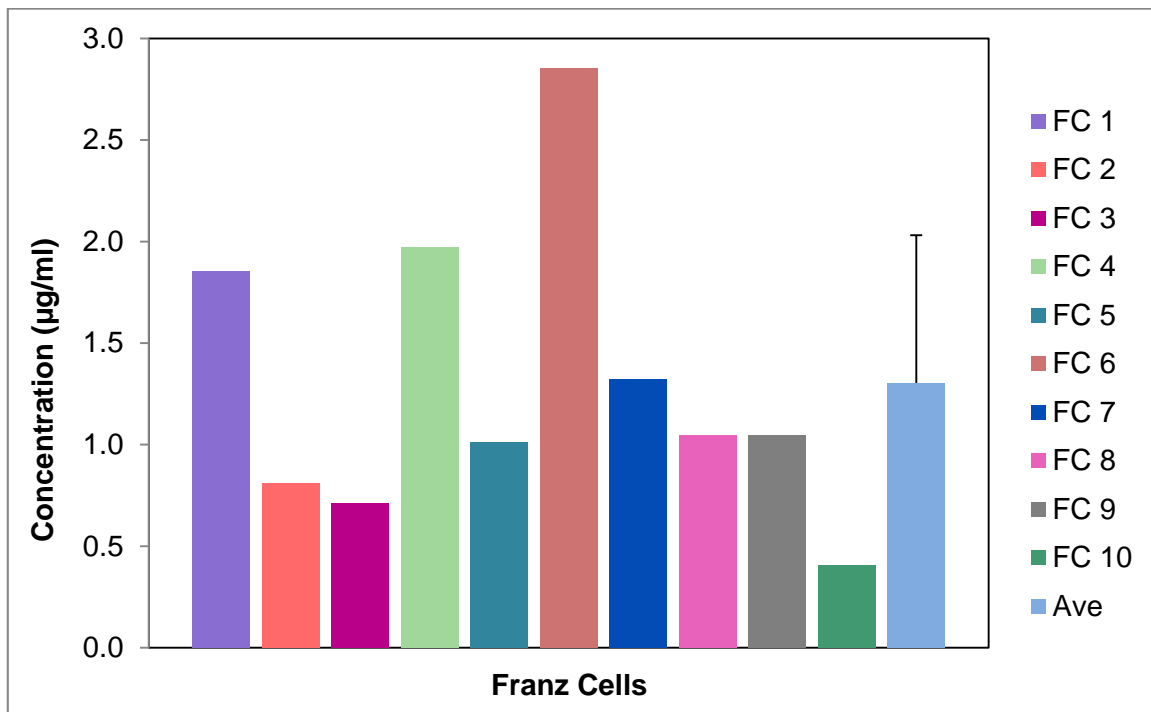
**Figure D.2:** Epidermis-dermis data of niosomes encapsulating roxithromycin monohydrate (n = 9)



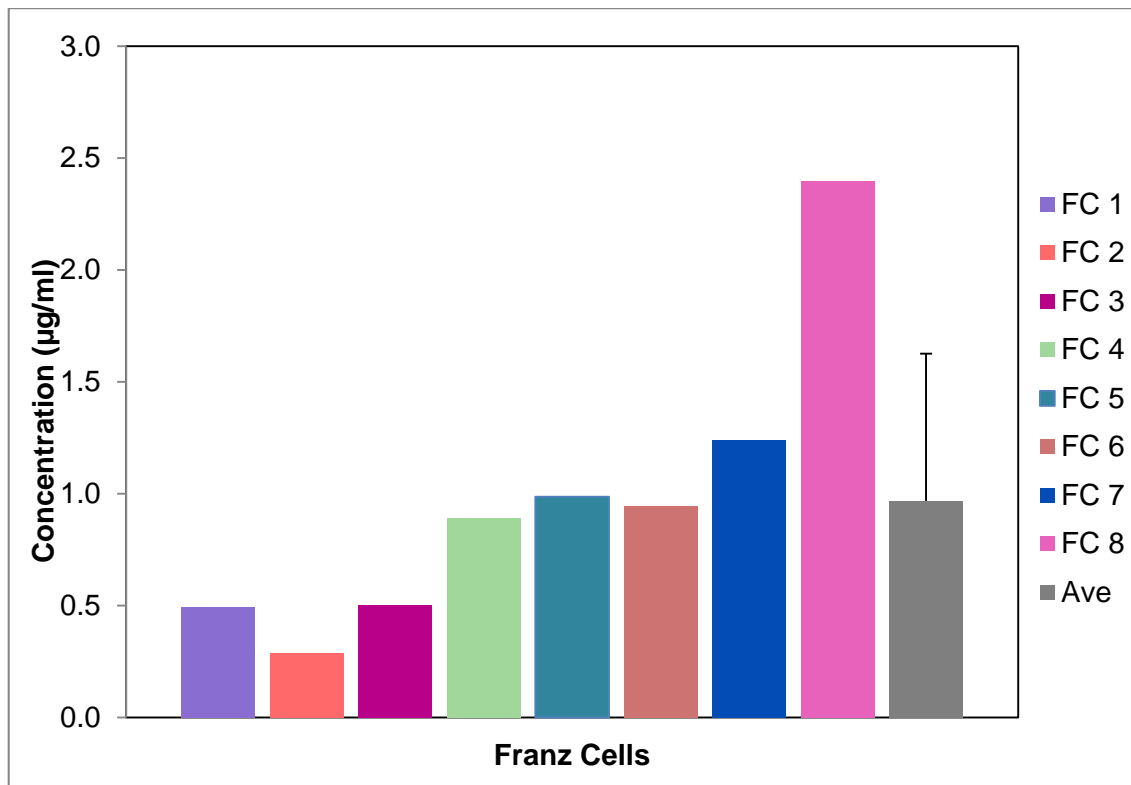
**Figure D.3:** Epidermis-dermis data of niosomes encapsulating the glassy amorphous form of roxithromycin (n = 7)



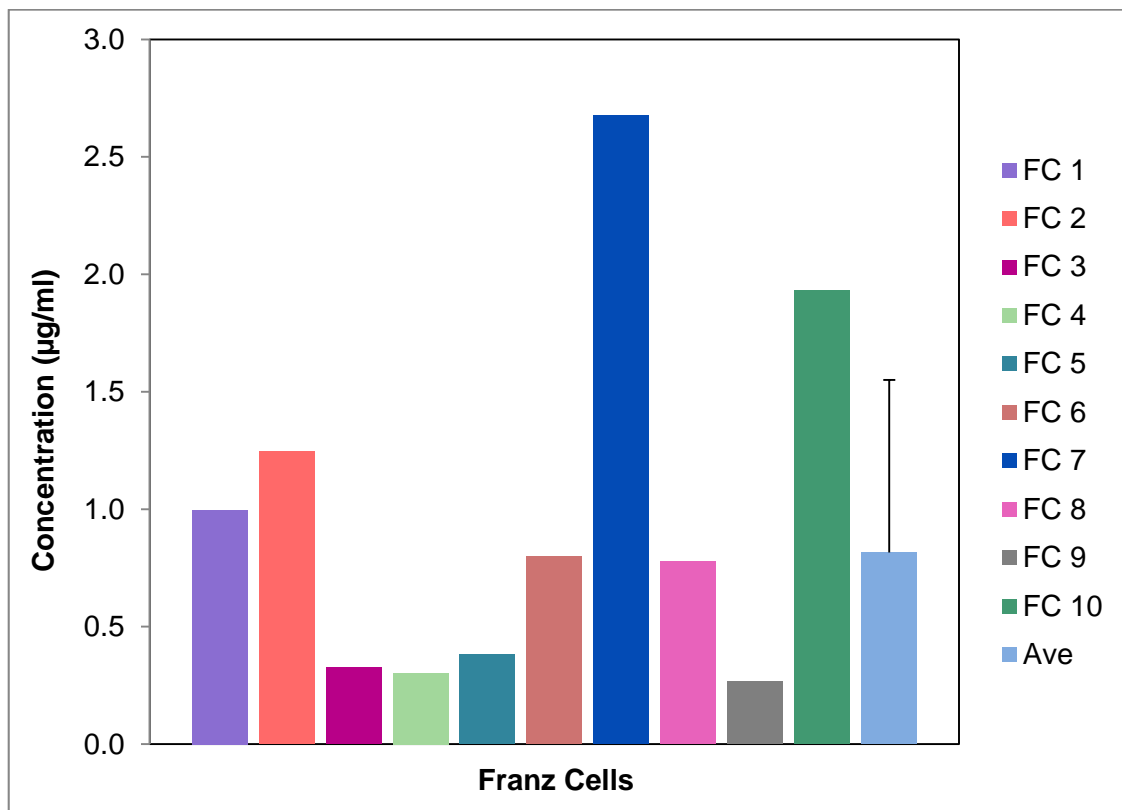
**Figure D.4:** Epidermis-dermis data of niosomes encapsulating the desolvated amorphous form of roxithromycin (n = 10)



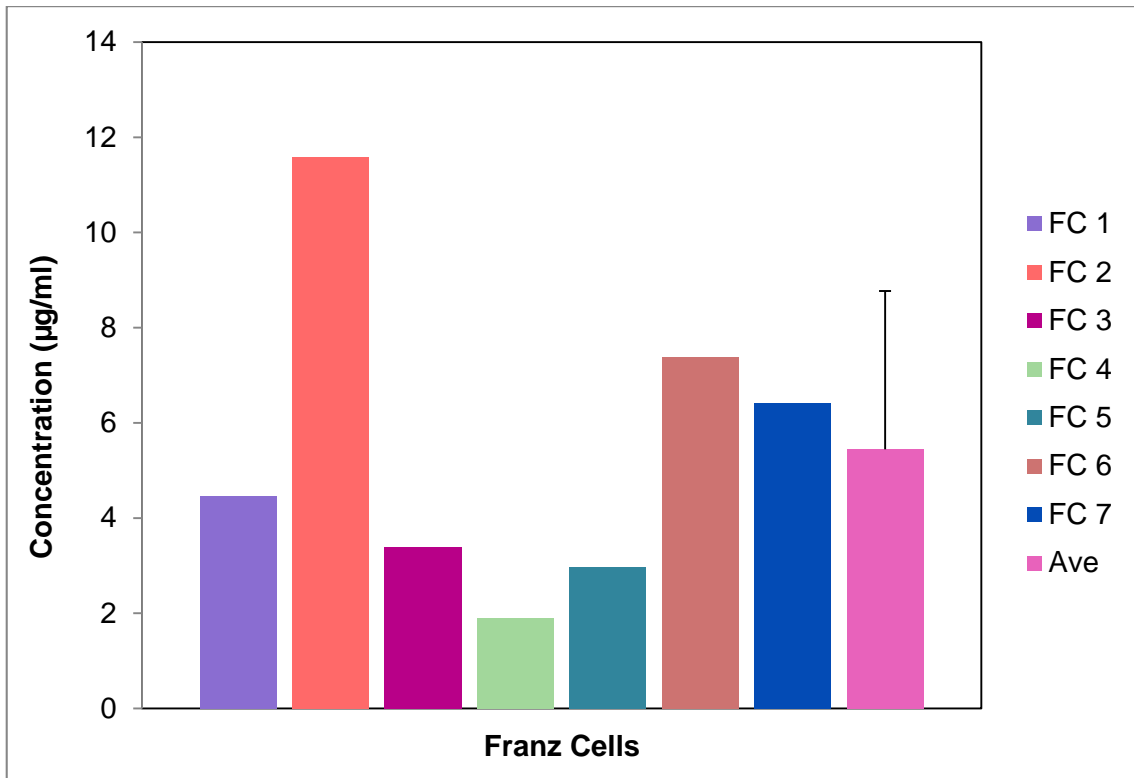
**Figure D.5:** Epidermis-dermis data of proniosomes encapsulating roxithromycin monohydrate (n = 10)



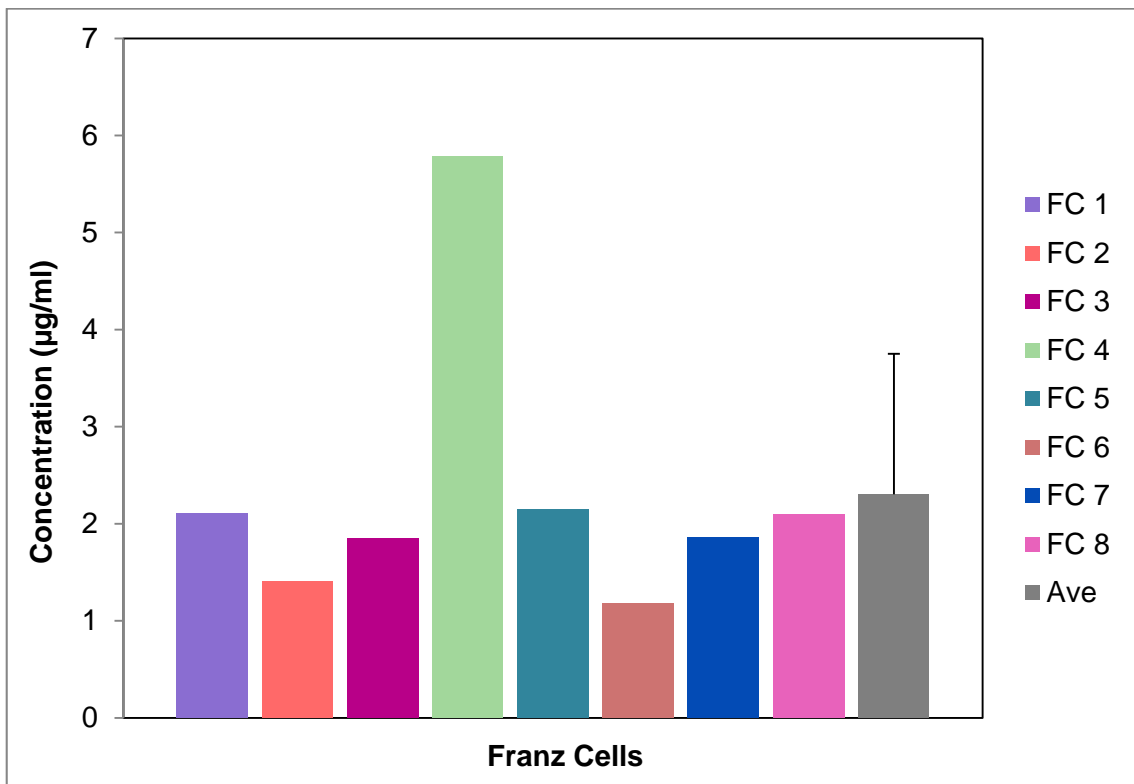
**Figure D.6:** Epidermis-dermis data of proniosomes encapsulating the glassy amorphous form of roxithromycin (n = 8)



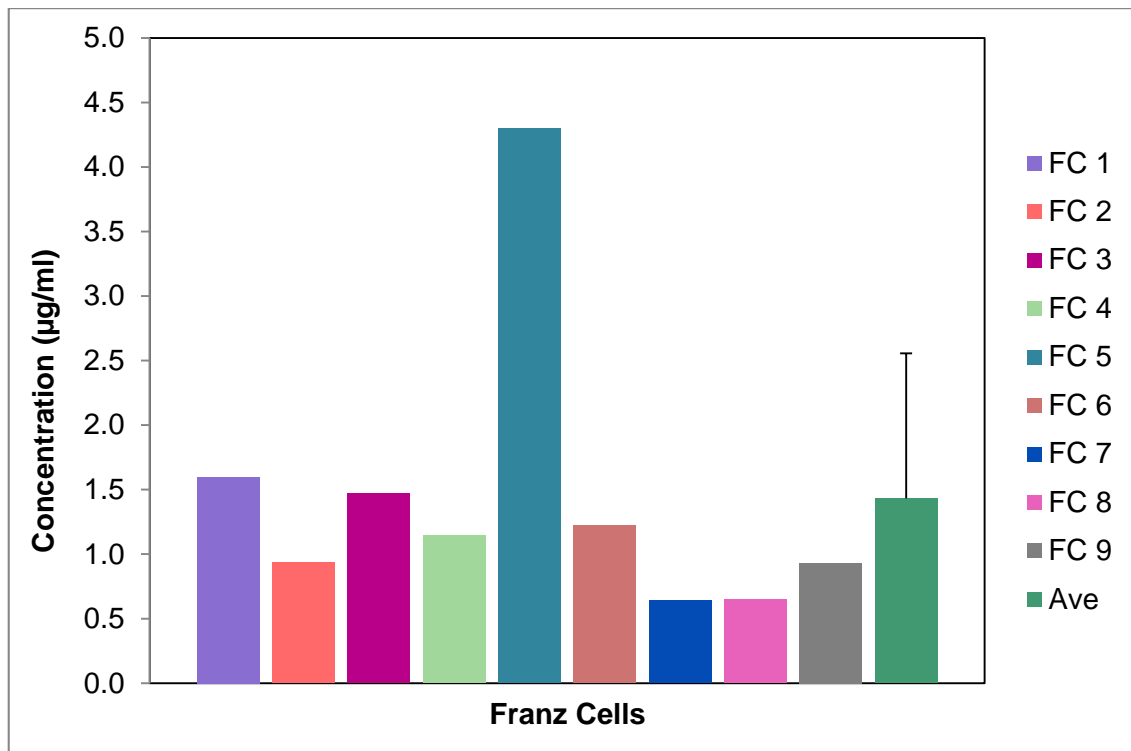
**Figure D.7:** Epidermis-dermis data of proniosomes encapsulating the desolvated amorphous form of roxithromycin (n = 10)



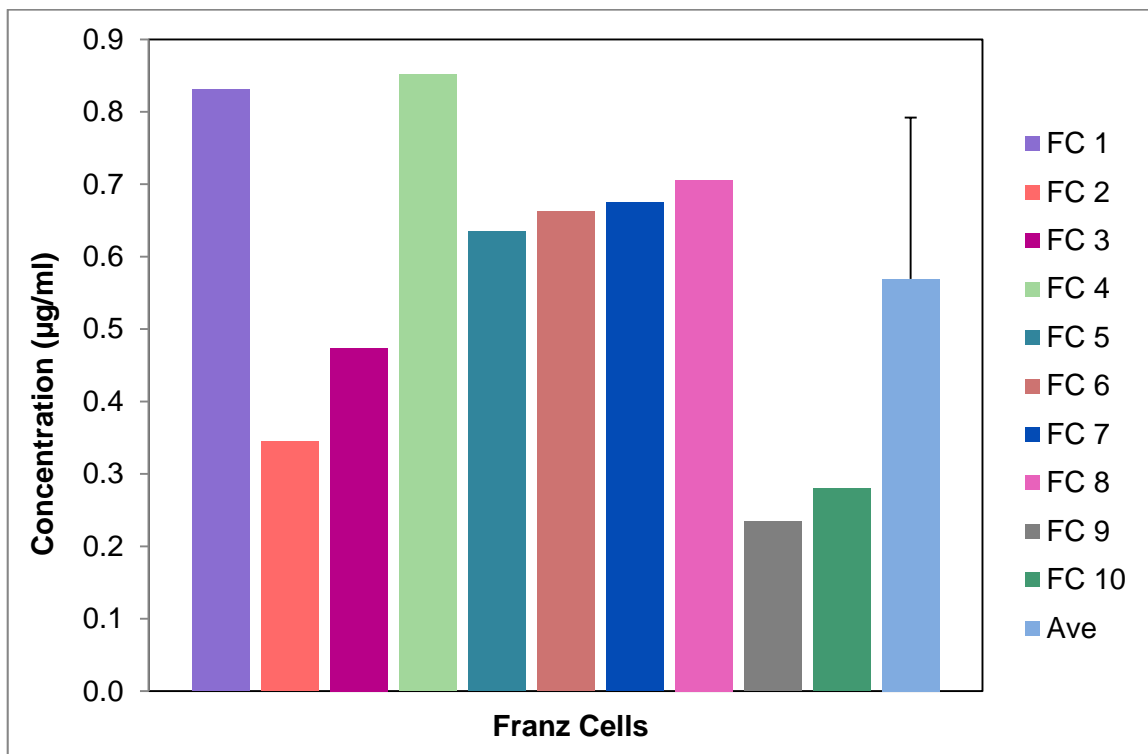
**Figure D.8:** Epidermis-dermis data of ufosomes encapsulating roxithromycin monohydrate (n = 7)



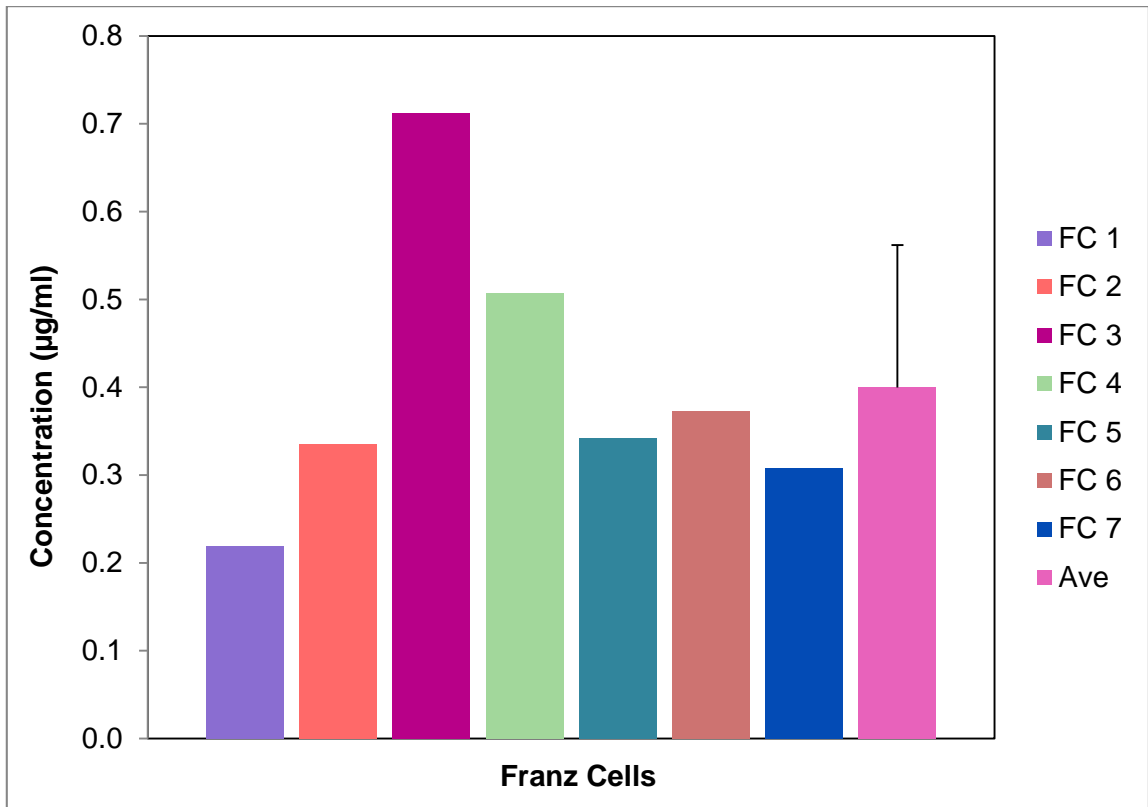
**Figure D.9:** Epidermis-dermis data of ufosomes encapsulating the glassy amorphous form of roxithromycin (n = 8)



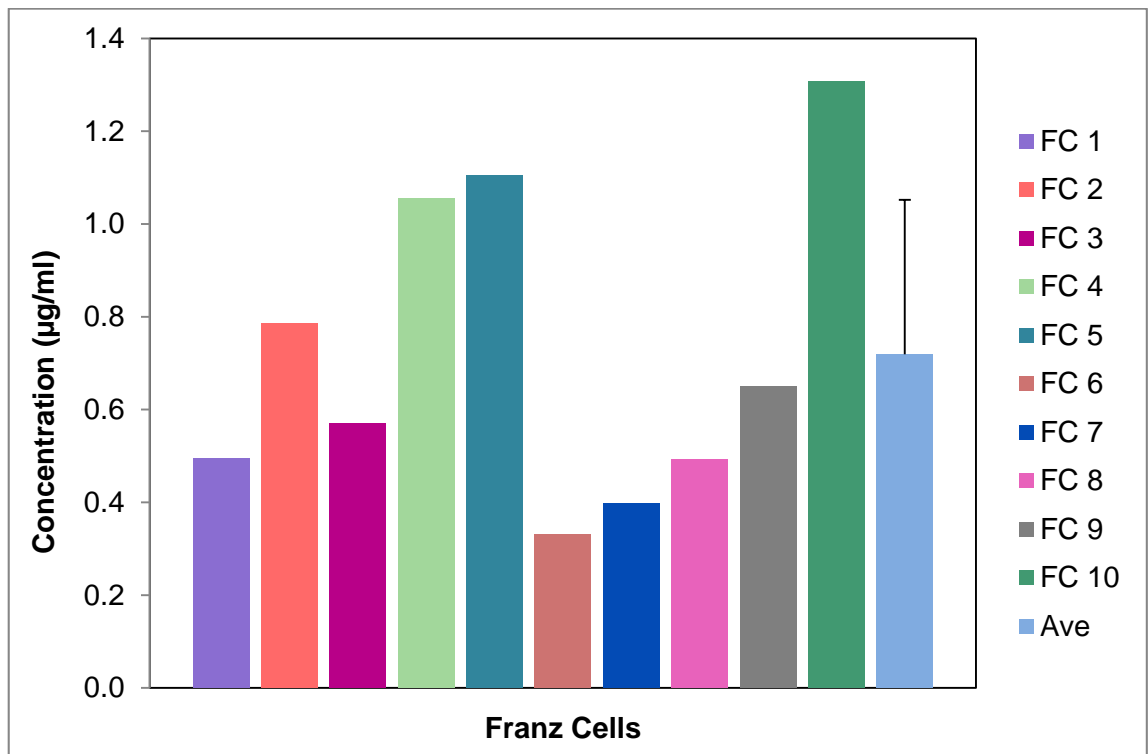
**Figure D.10:** Epidermis-dermis data of ufosomes encapsulating the desolvated amorphous form of roxithromycin (n = 9)



**Figure D.11:** Epidermis-dermis data of pro-ufosomes encapsulating roxithromycin monohydrate (n = 10)

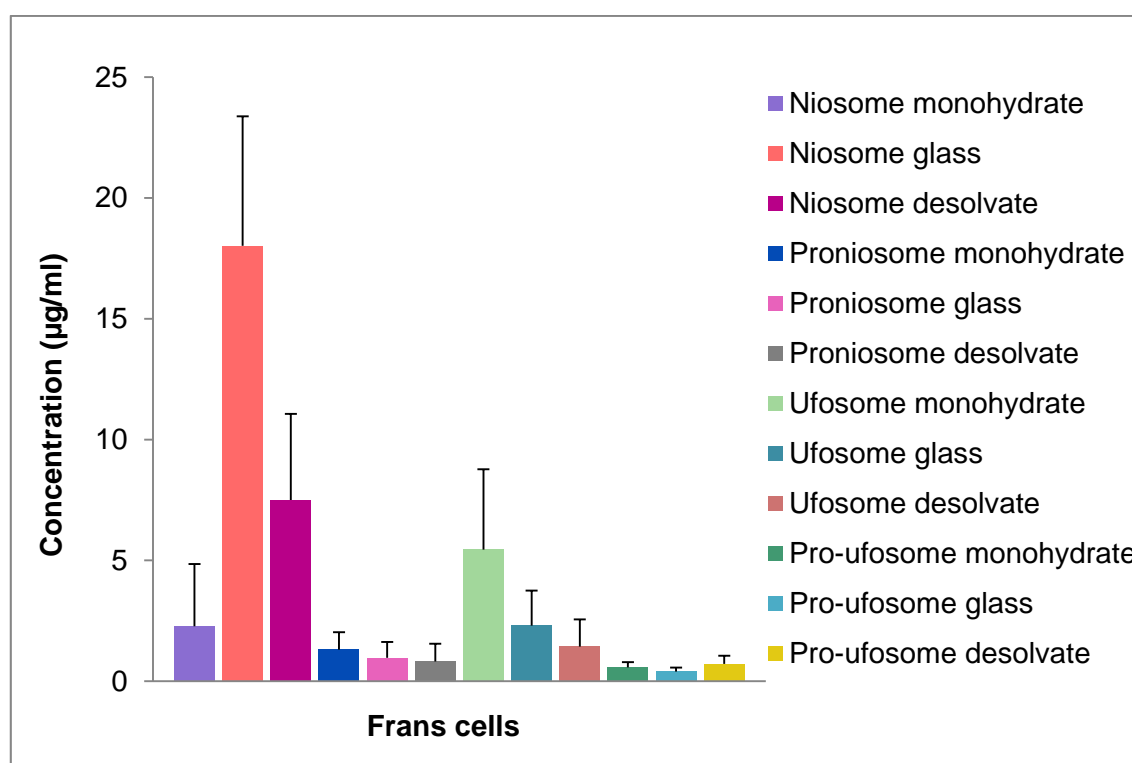


**Figure D.12:** Epidermis-dermis data of pro-ufosomes encapsulating the glassy amorphous form of roxithromycin (n = 7)



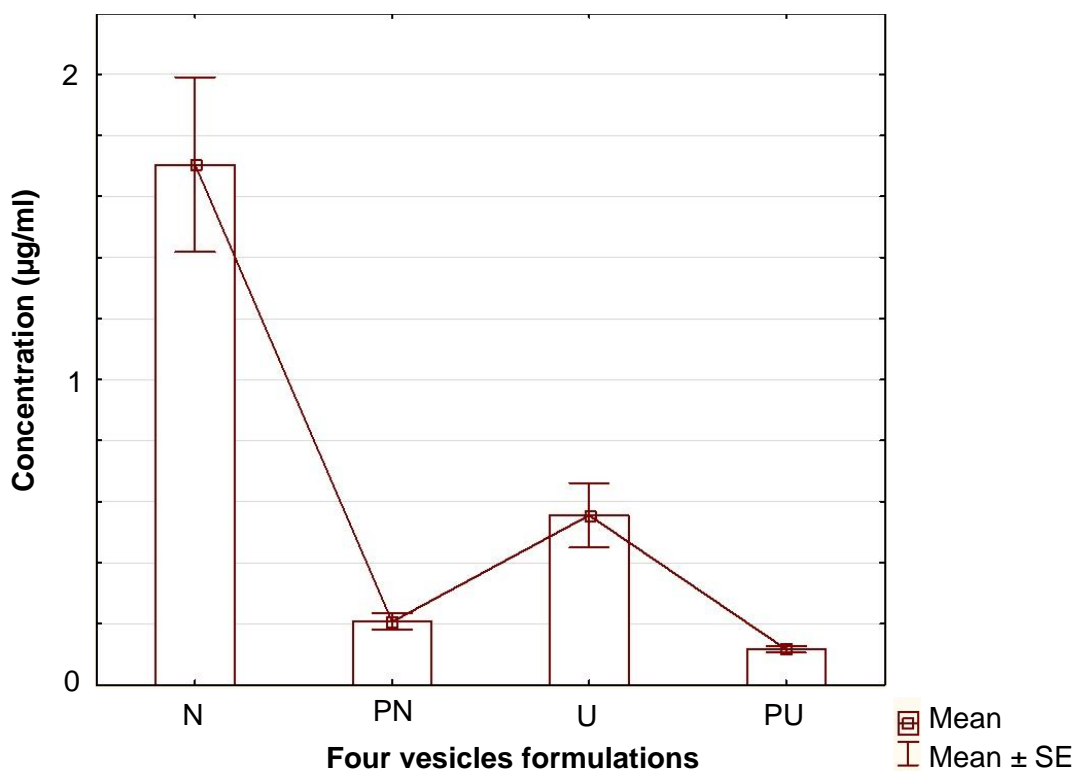
**Figure D.13:** Epidermis-dermis data of pro-ufosomes encapsulating the desolvated amorphous form of roxithromycin (n = 10)

The epidermis-dermis data in Figures D.2 - D.13 showed all the Franz cells contained roxithromycin, but the concentrations of the Franz cells in each experiment sometimes varied considerably. The reason this occurred may be due to the skin being a biologically variable material, known for erratic and unpredictable absorption which complicates the prediction of API penetration (Riviere, 1993:113). Only the Franz cells with noted leakages were removed from each experiment. With the API present in the epidermis-dermis, the conclusion drawn was that roxithromycin was successfully delivered to the intended target area (sebaceous glands in the dermis) where it will have a bactericidal effect on acne causing bacteria (Medsafe, 2014; Menon, 2002:4; Williams *et al.*, 2012:361).



**Figure D.14:** Comparative summary of the average API concentration (µg/ml) obtained in the epidermis-dermis after 12 h diffusion study

Figure D.14 shows a comparative summary of the average concentrations detected in the epidermis-dermis after the 12 h diffusion studies for all twelve vesicle systems. When comparing the vesicle systems containing the different forms of the API, the order from highest to lowest concentration are as follows: the niosomes glass ( $18.016 \pm 5.36 \mu\text{g/ml}$ ), niosomes desolvate ( $7.497 \pm 3.57 \mu\text{g/ml}$ ), ufosomes monohydrate ( $5.445 \pm 3.23 \mu\text{g/ml}$ ), ufosomes glass ( $2.303 \pm 1.45 \mu\text{g/ml}$ ), niosomes monohydrate ( $2.273 \pm 2.58 \mu\text{g/ml}$ ), ufosomes desolvate ( $1.432 \pm 1.12 \mu\text{g/ml}$ ), proniosomes monohydrate ( $1.304 \pm 0.73 \mu\text{g/ml}$ ), proniosomes glass ( $0.968 \pm 0.66 \mu\text{g/ml}$ ), proniosomes desolvate ( $0.816 \pm 0.73 \mu\text{g/ml}$ ), pro-ufosomes desolvate ( $0.719 \pm 0.33 \mu\text{g/ml}$ ), pro-ufosomes monohydrate ( $0.569 \pm 0.22 \mu\text{g/ml}$ ) and lastly, pro-ufosome glass ( $0.400 \pm 0.16 \mu\text{g/ml}$ ).



**Figure D.15:** Comparative view of the sum of all three forms of the API per vehicle system delivered topically in the epidermis-dermis after 12 h skin diffusion. N represents the niosomes, PN represents the proniosomes, U represents the ufosomes and PU represents the pro-ufosomes, all containing the sum of all three solid-state forms of roxithromycin.

From the group of niosomes, the roxithromycin glass had the best topical delivery of roxithromycin followed by the desolvate and then the monohydrate. For the proniosomes the values were fairly close with roxithromycin monohydrate having the best delivery proceeded by the glass and the desolvate. Examining the ufosomes, the monohydrate had the optimal delivery, the glass came in second with the desolvate which delivered the lowest amount of roxithromycin. The pro-ufosomes showed the desolvate was delivered more effectively than the monohydrate and the glass. It is evident from these results that there was no definite solid-state form of roxithromycin which had the leading topical delivery for all four vesicle systems. The delivery of the API formed a trend that was more dependent on what type of vesicle was used rather than the form of API. Looking at Figures D.14 and D.15 where the sum of all forms of API in one vesicle system is considered, a general trend follows with the niosomes (especially those containing glass) being the leading formulation, followed by ufosomes, then proniosomes and lastly pro-ufosomes.

By using ANOVO statistical analysis results, the aforementioned trend can be confirmed by plotting column graphs of the means and standard errors (SE) of all four vesicle systems containing the sum of all forms of roxithromycin in one vesicle system, as seen in Figure D.15. When comparing the total amount of API delivered topically by a vesicle system, regardless of which solid-state form it contained, it was evident the niosomes were by far the optimal vesicle formulation for topical delivery, followed by ufosomes, with proniosomes having a fairly smaller concentration and lastly the pro-ufosomes. The vesicular systems had better topical delivery than the pro-vesicular systems.

There are many factors which could have contributed to the specific results obtained from the topical data and it must be noted that all these factors influencing at different levels had to be taken into consideration for the final explanation of results. During comparison of the type of vesicle formula which yielded the best results (Figure D.15), the reason the niosomes had better topical delivery than any other system could be attributed to the fact that niosomes showed much greater release of the API than the other vesicles during the membrane release studies in Section D.3.3. Having a larger amount of API released from a formula at the stratum corneum, allowed for a greater available concentration of roxithromycin for topical diffusion to occur. Diffusion is dependent on the concentration gradient where larger concentrations have larger diffusion gradients and better diffusion (Barry, 2002:512). The same can be said for the proniosomes, which had much higher results for the membrane release and slightly higher topical delivery than the pro-ufosomes. If the API has a high affinity for the vehicle it is transported in, it will not be easily released from that vehicle as may be the case of the ufosomes and pro-ufosomes (Williams, 2003:18). Ufosomes showed the lowest membrane release from vesicles, but the API had higher topical delivery than that from proniosomes and pro-ufosomes. This shows that even though only a small amount of API was released from the ufosomes at the stratum corneum, their APIs still had better diffusion through the layers of the skin than the proniosomes and pro-ufosomes. The API of the proniosomes and pro-ufosomes had poor skin diffusion, as the results showed they had the lowest topical delivery even though large amounts of API (especially for proniosomes) were released during the membrane release studies. The reason for this may be due to the thickening of the pro-vesicular systems which slowly took place over the 12 h. The reason for the thickening is explained later when the difference in ingredients of the vesicles are discussed.

It is usually expected that vesicle formulations with the highest entrapment efficiency have the better transdermal delivery because they are able to carry more drug to the stratum corneum, but many other factors also play a role which may alter these expected results. The characterisation of vesicle systems in Annexure C, Section C.3.6.1.1 showed the entrapment efficiencies of all four vesicle systems and although ufosomes and pro-ufosomes had better entrapment efficiencies than niosomes and proniosomes, the API concentration from release

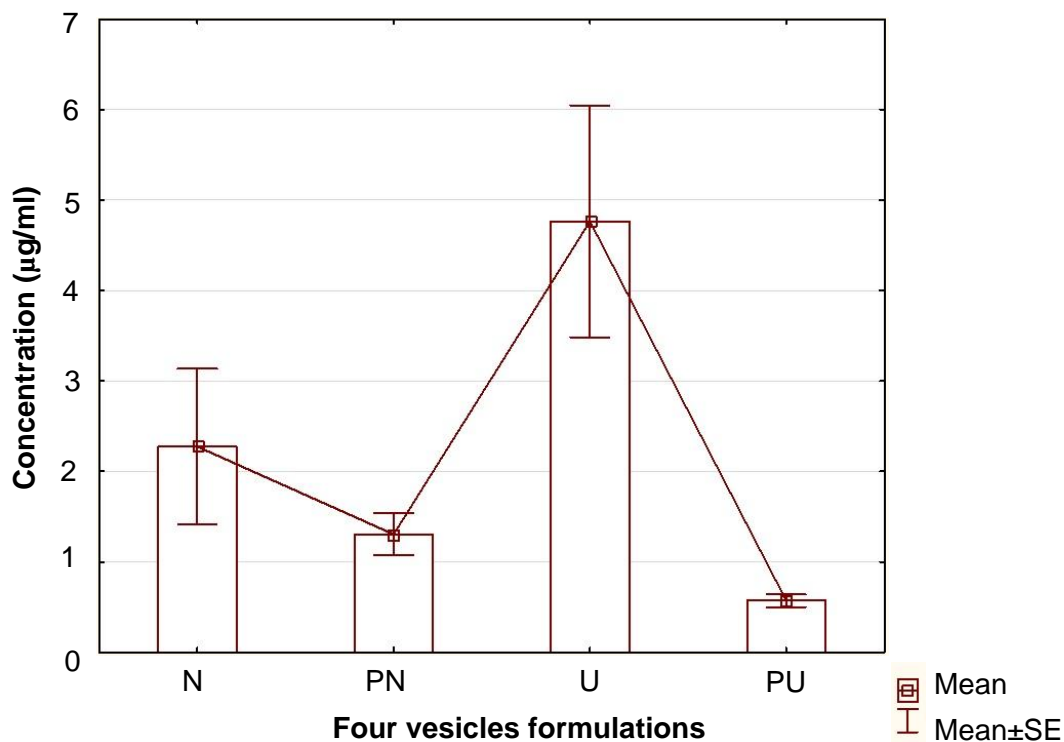
studies (Section D.3.3) were higher for niosomes and proniosomes. This illustrated that even though large amounts were entrapped for ufosomes and pro-ufosomes, if the release from the formulation was poor, then the topical delivery was poor. Niosomes and proniosomes released the API more readily and may be the reason why niosomes ( $\pm 80\%$  EE%) had better topical delivery than ufosomes ( $\pm 95\%$  EE%) and proniosomes ( $\pm 60\%$  EE%) had better delivery than the pro-ufosomes ( $\pm 93\%$  EE%). Similar results were found in a study by Agarwal *et al.* (2001:49), where liposomes had a better entrapment efficiency than niosomes which resulted because of the API having a higher affinity for the phospholipid bilayers than for the non-ionic surfactants. This higher affinity also resulted in a higher drug retention in vesicles for the liposomes than for niosomes.

The morphology and size of the vesicle systems could also contribute to the explanation of results obtained during the topical studies. The TEM micrographs for morphology were discussed in Annexure C, Section C.3.1.1. The niosomes appeared to have many “cracks” in its vesicles which may have resulted in easier release of the API than seen with the rest of the formulations. The ufosomes formed the largest amount (and most densely packed) of vesicles, where the majority were extremely small in size. The large amount of very small, well formed ufosome vesicles with fewer larger droplets than the rest of the formulations may have been the reason it resulted in good topical delivery, even though the API release from the vesicles were poor. The smaller the molecules, the better the API penetrates the skin for topical delivery (Williams, 2003:36). The vast amount of small molecules created a large membrane surface where API was entrapped and many of these tiny droplets were able to reach the stratum corneum at once to release API.

When comparing the better topical delivery of niosomes with ufosomes, it was of value to evaluate the properties of the ingredients used to make each vesicle as many of these ingredients enhanced penetration and some may have also penetrated the intact skin once the API was released from the vesicles. The only difference in ingredients was the Span 60, used for the niosomes lipid layer and the sodium oleate used for the ufosomes. Span 60 has a melting point of 53 - 57 °C (Chemical book, 2008b) and sodium oleate has a melting point of 232 – 235 °C (Chemical book, 2008a; Row *et al.*, 2009:678). As discussed earlier, components with lower melting points have better solubility and result in better diffusion across the skin than those with higher melting points (Barry, 2001:102; Williams, 2003:37). Compounds with melting points above 200 °C have less favourable transdermal diffusion than those with melting points lower than this value (Naik *et al.*, 2000:319). Row *et al.* (2009:676, 678) classified Span 60 as a solubilising agent for lipophilic drugs which has a melting point of only 53 – 57 °C along with better solubility. This was probably the reason the API released from the niosomes had better topical delivery than the ufosomes made from sodium oleate with an unfavourable melting point and poorer solubility. Span 60 is lipophilic and only partially soluble in water whereas sodium

oleate is very soluble in water (Row *et al.*, 2009:676; Chemical book, 2008b). This factor may suggest that after API release, components of the niosome membrane may have also topically penetrated through the lipophilic stratum corneum, as it was more lipophilic than the sodium oleate which was known to have good aqueous solubility when not in a mixture with the other vesicle ingredients. Span 60 and sodium oleate are also known as penetration enhancers which have different mechanisms for increasing penetration of the API and components of the lipid membrane may even have penetrated the stratum corneum themselves (Dayan, 2005:71,74). Span 60 and sodium oleate are also used as gelling agents (solid component of a gel), which could explain why the proniosomes and the pro-ufosomes formulations turned from liquid suspensions to gels after lying in the Franz cells for a 12 h diffusion study (Acme synthetic chemicals, 2015; Jibry *et al.*, 2004:1853). This factor may be the reason the proniosomes and pro-ufosomes always had the lowest topical delivery of all formulations, because they slowly thickened throughout the 12 h which increased their viscosity and decreased the diffusion of the API (Williams, 2003:18). The reason for it only happening to the two pro-vesicular systems could be that they initially contained an extra powder, namely sorbitol, which could contribute to the volume of the gel.

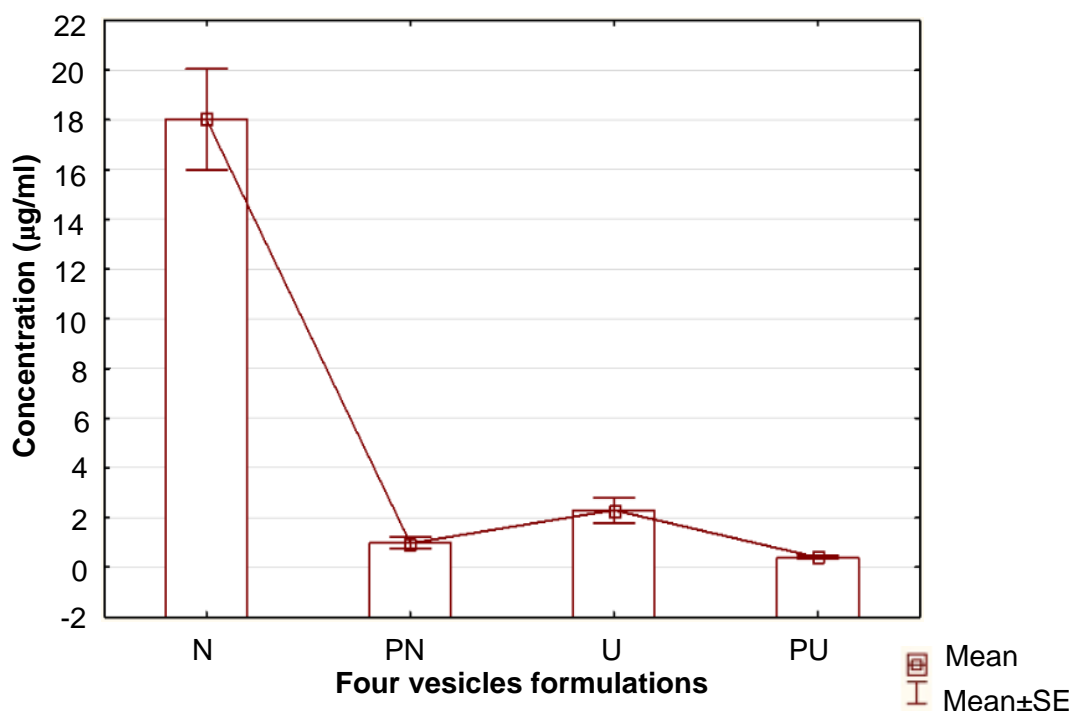
When statistically analysing the three solid-state forms of roxithromycin using ANOVA, Figures D.16 - D 18 illustrate the solid-state (roxithromycin monohydrate, roxithromycin glass or roxithromycin chloroform desolvate) concentration diffused in the epidermis-dermis from each of the four vesicle systems.



**Figure D.16:** Epidermis-dermis concentrations of vesicle systems containing only roxithromycin monohydrate after 12 h skin diffusion. N is the niosomes, PN the proniosomes, U the ufosomes and PU the pro-ufosomes all containing the monohydrate form of roxithromycin.

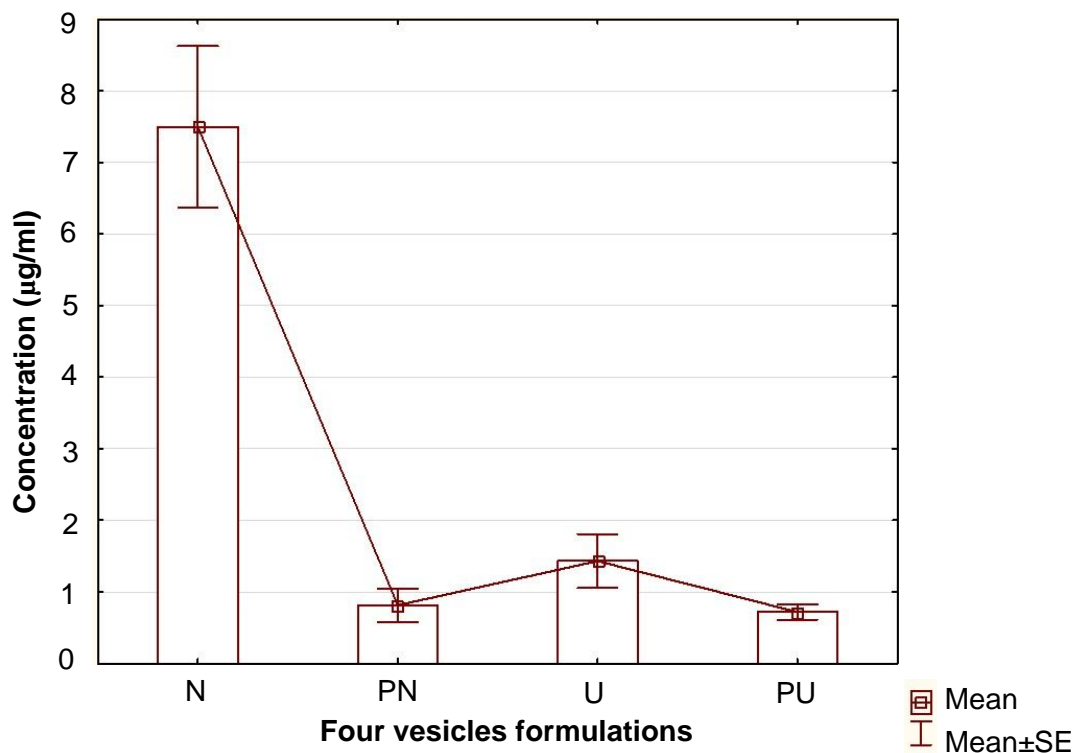
From Figure D.16 it can be seen that ufosomes had the best topical delivery for the roxithromycin monohydrate solid-state form. Niosomes also delivered the roxithromycin monohydrate fairly well, followed by the proniosomes, then the pro-ufosomes with the lowest amount of roxithromycin delivery.

It is evident from Figure D.17 that niosomes were the superior delivery system for the roxithromycin glass solid-state form after the 12 h diffusion studies. The roxithromycin glass had the second best delivery when encapsulated in the ufosomes. The proniosomes followed with the pro-ufosomes having the poorest roxithromycin glass delivery.



**Figure D.17:** Epidermis-dermis concentrations of vesicle systems containing only roxithromycin glass after 12 h skin diffusion. N is the niosomes, PN the proniosomes, U the ufosomes and PU the pro-ufosomes, all containing the monohydrate form of roxithromycin.

Figure D.18 shows the topical delivery of the chloroform desolvated form of roxithromycin entrapped in the four vesicles systems. Results seen in this figure are almost identical to those seen in Figure D.17 for roxithromycin glass. Niosomes were the major deliverer of the chloroform desolvate form to the stratum corneum for diffusion into the epidermis-dermis. Ufosomes were again the followers with proniosomes and pro-ufosomes trailing with the lowest amount of desolvate delivered topically.

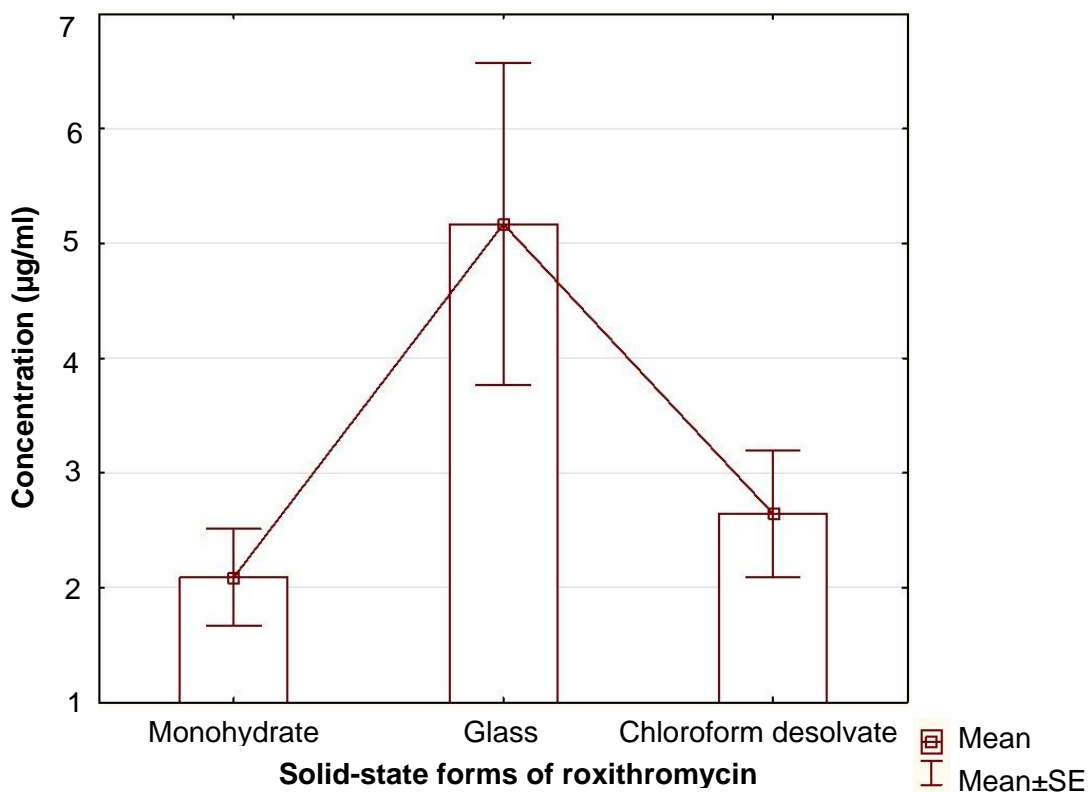


**Figure D.18:** Epidermis-dermis concentrations of vesicles encapsulating only the chloroform desolvate form of roxithromycin after 12 h skin diffusion studies. N is the niosomes, PN the proniosomes, U the ufosomes and PU the pro-ufosomes, all containing the monohydrate form of roxithromycin.

In conclusion, by referring back to the Figures D.16 to D.18, it is evident the best vesicle formulation for the roxithromycin monohydrate was the ufosomes and the optimal vesicle system for the topical delivery of roxithromycin glass and chloroform desolvate amorphous forms was, by far, the niosomes. The proniosomes followed by pro-ufosomes were always the two systems with lowest topical delivery for all three solid-state forms.

ANOVA column plots of means and standard errors were also used in Figure D.19 to show the total amount of each solid-state form delivered by all vesicle systems regardless of what vesicle system was used for the topical delivery. The roxithromycin glass totalled the highest concentration delivered for all four combined vesicles systems, resulting in almost double the roxithromycin chloroform desolvate concentration delivered into the epidermis-dermis. The form having the lowest combined concentration from all vesicles was the roxithromycin monohydrate. The reason for the roxithromycin glass and the roxithromycin desolvate having higher topical delivery than the roxithromycin monohydrate in this study may be due to the increased solubility of the amorphous forms as discussed in Chapter 3 (Section 3.4.2.3.1 and Section 3.4.2.3.2). The poor solubility of roxithromycin was discussed earlier (0.0335 mg/ml at 25 C in water)

referencing Aucamp *et al.* (2013:26). The solubility of roxithromycin glass in water increased by  $\pm 1800\%$  and the chloroform desolvate amorphous form had a 505.9% increase (Liebenberg & Aucamp, 2013; Liebenberg *et al.*, 2013). The optimal solubility for an API of topical delivery is 1 mg/ml (Naik *et al.*, 2000:319) and therefore the amorphous forms contained optimal solubility for delivery. The more soluble an API is, the better topical delivery it is capable of and this may explain why the glass had the highest topical delivery as it had the highest solubility of the three forms. The solubility of all roxithromycin forms were tested and reported in Section D.3.1. The solubility values in PBS (pH 7) were very similar for all three forms of roxithromycin due to predicted conversion back to the stable monohydrate form, which occurs often with amorphous solid-state forms as the metastable forms with higher free energy and higher solubility tend to spontaneously convert to the more stable form in selected solutions (Purohit & Venugopalan, 2009:891). It should be mentioned however that the solubility determinations in PBS were done over a 24 h period, therefore being a sufficiently long period for the amorphous solid-state forms to convert back to the poorly soluble crystalline solid-state form. During the skin diffusion studies, roxithromycin would have diffused to the epidermis-dermis before recrystallisation of the amorphous solid-state forms occurred, therefore resulting in higher concentrations of roxithromycin being obtained in the epidermis-dermis. This being said, it can be hypothesised that the solubility of the forms now encapsulated into the lipophilic membrane of the vesicles and not in a PBS (pH 7) solution may have retained their original amorphous forms with increased solubility and topical delivery even though there was no equipment available to verify this statement. It can be further hypothesised that the excipients used during the formulation of the niosomes acted as stabilisers of the amorphous solid-state forms, thereby inhibiting the recrystallisation process of the amorphous forms to the crystalline solid-state form. The aim of using amorphous forms, was to determine if there was an improvement in topical delivery of roxithromycin and these results draw the conclusion that using amorphous forms of roxithromycin which retained their increased solubility, resulted in better topical delivery of the API into the epidermis-dermis.



**Figure D.19:** Comparative view of the total sum of the epidermis-dermis concentrations of each solid-state form of roxithromycin after 12 h skin diffusion studies regardless of what vesicle system it was encapsulated in.

### D.3.5 Statistical data analysis of the diffusion studies

Non-parametric tests were performed on the data obtained from the epidermis-dermis after 12 h diffusion studies. It must be taken into account that the skin used in each experiment was from the same donor, but the donors varied for every new experiment performed. The biological tissue is erratic and can influence statistical results.

The Kruskal-Wallis ANOVA by ranks was performed on the type of vesicles to determine if there was a statistical significance between vesicle systems in the group containing different forms of API. The niosomes ( $p = 0.0001$ ) and ufosomes ( $p = 0.0128$ ) showed statistical significance, but there was no significant difference for the proniosomes ( $p = 0.965$ ) and pro-ufosomes ( $p = 0.1008$ ). Even though this test indicated there were statistical differences for niosomes and ufosomes, it was unsure where the difference was, therefore the multiple comparisons test was performed on these two groups to determine where the significant differences lay. The results of the multiple comparisons for niosomes showed there was a statistical difference between roxithromycin monohydrate and the glass ( $p = 0.00005$ ). The remaining forms had no statistical significance ( $p > 0.05$ ). With the ufosomes, there was only a statistical difference with the

roxithromycin monohydrate and the desolvate ( $p = 0.01035$ ). The rest of the formulation group had no significant difference.

The test was also performed on the forms of roxithromycin encapsulated in the vesicle systems in order to compare the vesicles. The Kruskal-Wallis ANOVA by ranks was performed on the roxithromycin monohydrate ( $p = 0.002$ ), roxithromycin glass ( $p = 0.000$ ) and roxithromycin desolvate ( $p = 0.0000$ ) and all three groups showed significant differences. For the multiple comparisons results of the roxithromycin API group, there was only a significant difference for ufosomes and pro-ufosomes ( $p = 0.000966$ ), while the rest of the vesicle systems showed no significant variance. The glass API group had a significant difference between the niosomes and the proniosomes ( $p = 0.003279$ ), niosomes and pro-ufosomes ( $p = 0.000027$ ) and ufosomes and pro-ufosomes ( $p = 0.024711$ ). In the chloroform desolvate group, niosomes and proniosomes ( $p = 0.000056$ ) and niosomes and pro-ufosomes ( $p = 0.000193$ ) had statistical differences. The groups of no mention showed no difference between the vesicle systems for each form of API.

The means and standard errors calculated during the statistics of the different groups were illustrated in the column graphs of Figures D.15 - D.19 in Section D.3.4.3 and were used to explain the differences in concentrations of formulas obtained from the epidermis-dermis skin diffusion studies.

#### **D.4 Conclusion**

As mentioned before, the epidermis-dermis was the intended target for roxithromycin to reach because this is where bacteria *P. acnes* accumulates in the sebaceous glands to cause acne (Gollnick, 2003:1581, 1585). Using a topical antibiotic has direct effect on the sebaceous glands found in the dermis and this causes a local decrease in inflammation of the glands infected with bacteria (Williams *et al.*, 2012:164). There was API release found with all the vesicles during the membrane release studies in order and from highest release to lowest were the niosomes, the proniosomes, the pro-ufosomes and the ufosomes. It was thought the API had higher affinity for the ufosomes and pro-ufosomes than with the niosomes and proniosomes, which resulted in less API being released from the vesicles (Williams, 2003:18). The targeted topical delivery into the epidermis-dermis was a success, as there was generally no API in the receptor phase and only small traces of API in the stratum corneum-epidermis during all experiments. Having the API in the epidermis-dermis skin layer proved the API released from vesicles reached the epidermis-dermis by successfully penetrating through the stratum corneum, which is usually a barrier which limits or prevents drug absorption (Foldvari, 2000:418). Vesicles (in nm range) improve drug penetration through the stratum corneum and also allow for drug targeting (in the epidermis-dermis during topical delivery) (Kumar &

Rajeshwarrao, 2011:209; Varun *et al.*, 2012:633). Many carrier system studies also reported a localised effect for vesicles, particularly in the stratum corneum-epidermis and epidermis-dermis (Barry, 2001:104).

Roxithromycin's optimal log D of 1.52, determined in this study, meant the API was lipophilic enough to cross the stratum corneum (Williams, 2003:36). The solubility of the API alone in PBS (pH 7) was also determined and it was found the amorphous forms (glass (1.88 mg/ml) and desolvate (1.92 mg/ml) had very close solubility values to that of the roxithromycin monohydrate (1.98 mg/ml). It can be deduced from the results of the solubility test that the amorphous forms of roxithromycin converted back to the stable monohydrate form whilst in solution, resulting in similar properties (Purohit & Venugopalan, 2009:891). When comparing the three forms of roxithromycin for the best topical delivery, the glass was by far the leading form with the chloroform desolvate in second place and the roxithromycin monohydrate having the lowest amount of topical diffusion. These results correlated well with the aim of this study, which was to improve topical delivery by using the amorphous forms of roxithromycin with their theoretical increased solubilities and the results proved that both amorphous forms had better topical delivery. The better topical delivery could be due to the amorphous forms retaining their increased solubility, as mentioned by Liebenberg & Aucamp (2013) and Liebenberg *et al.*, (2013), while being encapsulated in lipid layers of vesicle systems causing them to withhold their conversion back to the stable form as it did in normal PBS (pH 7).

The niosomes were the vesicle system with the best topical delivery into the epidermis-dermis during the skin diffusion studies, ufosomes were next, followed by the proniosomes and the pro-ufosomes. For each solid-state form of roxithromycin, this was a trend which showed that the topical delivery of the API was highly dependent on the vesicle type, as the values of all three forms of one vesicle type lay fairly close to one another. Niosomes may have had the better delivery due to it having the highest release during the membrane release studies. Proniosomes may also have had better delivery than pro-ufosomes because of the better release. Having a high release at the stratum corneum resulted in a large drug concentration available for diffusion into the epidermis-dermis and diffusion is directly proportional to the drug concentration gradient (Barry, 2002:512; Williams, 2003:41-42). Even though the ufosomes and pro-ufosomes had better entrapment efficiencies than the niosomes and proniosomes, the API had a very high affinity for these two vehicles as the release from their carriers was rather poor. This result was confirmed in similar results obtained by Agarwal *et al.*, (2001:49). The topical delivery of components into the skin is highly dependent on the size of the particles, the affinity for the carrier vehicle or skin and the compatibility with the lipids in the skin (Dayan, 2005:74). Ufosomes had very poor release during membrane studies, but the API had good epidermis-dermis penetration once released from the vesicle where it was seen to have the second best API delivery. Ufosomes had very good morphology with the majority of tiny, very

densely packed vesicles containing API, which may have been the reason for its fairly good topical diffusion despite its poor release. According to Dayan (2005:74), the smaller the average droplet size of a formulation, the easier it is for the penetration of the API through the stratum corneum layers of the skin as many smaller droplets have a larger surface area and a vast quantity of them can line up against the stratum corneum for a good amount of API to be released for diffusion. The fatty acids (oleate acid) used to produce the ufosomes and pro-ufosomes also acted as penetration enhancers by increasing epidermal permeability through a perturbation mechanism acting on the stratum corneum lipid layers (Dayan, 2005:74). The non-ionic surfactant (Span 60) used in preparation of niosomes and proniosomes also acted as a penetration enhancer by causing a fluidising effect on the stratum corneum which resulted in the disruption of its lipids (Dayan, 2005:71) Niosomes had cracks in its lipid membrane which may also contribute to its great delivery. The Span 60 used in the niosomes and proniosomes had a lower melting point and better solubility than the sodium oleate used in the ufosomes and pro-ufosomes, which resulted in niosomes having better topical delivery than ufosomes and the proniosomes' delivery was better than the pro-ufosomes. This result in better diffusion was most likely attributed to the optimal low melting points and good solubility of the lipid membranes of niosomes and proniosomes, which may have resulted in some components also penetrating the stratum corneum on release of API in comparison to their counterparts (Barry, 2001:102; Williams, 2003:37). The proniosomes and pro-ufosomes always had the lowest diffused amounts of API in the epidermis-dermis. These two formulations always started as good suspensions but after the 12 h study they had formed gels, as Span 60 and sodium oleate are also gelling agents (Acme synthetic chemicals, 2015; Jibry *et al.*, 2004:1853). The more viscous they became, the slower topical diffusion took place which resulted in a lower concentration reaching the epidermis-dermis (Williams, 2003:18).

In conclusion, the aims and the objectives of this study were met as the targeted delivery to the epidermis-dermis was successful, with higher concentrations of the roxithromycin amorphous forms reaching the epidermis-dermis than the roxithromycin monohydrate.

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## **E.3 Manuscript Submission**

### **E.3.1 Manuscript Submission**

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

### **E.3.2 Permissions**

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

### **E.3.3 Online Submission**

Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

**Important note:** The approval document has to be submitted with the manuscript for all studies requiring review by the responsible local or national ethical committee.

### **E.3.4 Proposal of Expert Reviewer**

To shorten the time of the editorial process and to increase the quality of manuscripts published in ADR at least 3 reviewers must be proposed. Of these three at least two must be from outside of the country of the corresponding author. Members of the Board of Associate Editors cannot be proposed as a reviewer. It is advisable to suggest experts who are active in the respective field and have recently published articles related to topic of the submission. Suggestion of people who are well known in global dermatology but are no longer active as scientist and/or clinician is not advisable.

### **E.3.5 General Remark**

Please provide text and figures of your manuscript as separate electronic files (one file for each figure). The text file should include references, figure legends and tables; it should not include figures. Tables and figure legends are placed within the text file at the end of the main text. Depending on the type of paper the text file should contain the following sections:

- Manuscript sections (pdf, 50 kB) [http://static.springer.com/sgw/documents/559698/application/pdf/ManuscriptSections\\_403.080628.pdf](http://static.springer.com/sgw/documents/559698/application/pdf/ManuscriptSections_403.080628.pdf)
- Further useful information

The Springer Author Academy is a set of comprehensive online training pages mainly geared towards first-time authors. At this point, more than 50 pages offer advice to authors on how to write and publish a journal article.

- Springer Author Academy

Scientific style: Generic names of drugs and pesticides are preferred; if trade names are used, the generic name should be given at first mention.

## **E.4 Title page**

### **E.4.1 Title Page**

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

### **E.4.2 Abstract**

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

### **E.4.3 Keywords**

Please provide 4 to 6 keywords which can be used for indexing purposes.

## **E.5 Text**

### **E.5.1 Text Formatting**

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 182 kB)

## **E.5.2 Headings**

Please use no more than three levels of displayed headings.

## **E.5.3 Abbreviations**

Abbreviations should be defined at first mention and used consistently thereafter.

## **E.5.4 Footnotes**

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables. Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols. Always use footnotes instead of endnotes.

## **E.5.5 Acknowledgments**

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

## **E.6 References**

### **E.6.1 Citation**

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

### **E.6.2 Reference list**

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference

list. Reference list entries should be alphabetized by the last names of the first author of each work and numbered consecutively.

### **Journal article**

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol* 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. *N Engl J Med* 341:325–329

### **Article by DOI**

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. *J Mol Med*. Doi:10.1007/s001090000086

### **Book**

South J, Blass B (2001) *The future of modern genomics*. Blackwell, London

### **Book chapter**

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) *The rise of modern genomics*, 3rd edn. Wiley, New York, pp 230-257

### **Online document**

Doe J (1999) Title of subordinate document. In: *The dictionary of substances and their effects*. Royal Society of Chemistry. Available via DIALOG. [http://www.rsc.org/dose/title of subordinate document](http://www.rsc.org/dose/title%20of%20subordinate%20document). Accessed 15 Jan 1999

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see [ISSN.org](http://www.issn.org) LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- Endnote style (zip, 2 kB)

**Please note:** References should be in alphabetical order using the first author's name and should be numbered consecutively. Please note that this numbering is not chronologically – it does not reflect the citation order within the text.

## **E.7 Tables**

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

**Please note:** A table should have a title and a legend explaining any abbreviation used in the table. Tables should be placed within the manuscript text file at the end of the main text.

## **E.8 Artwork and Illustrations Guidelines**

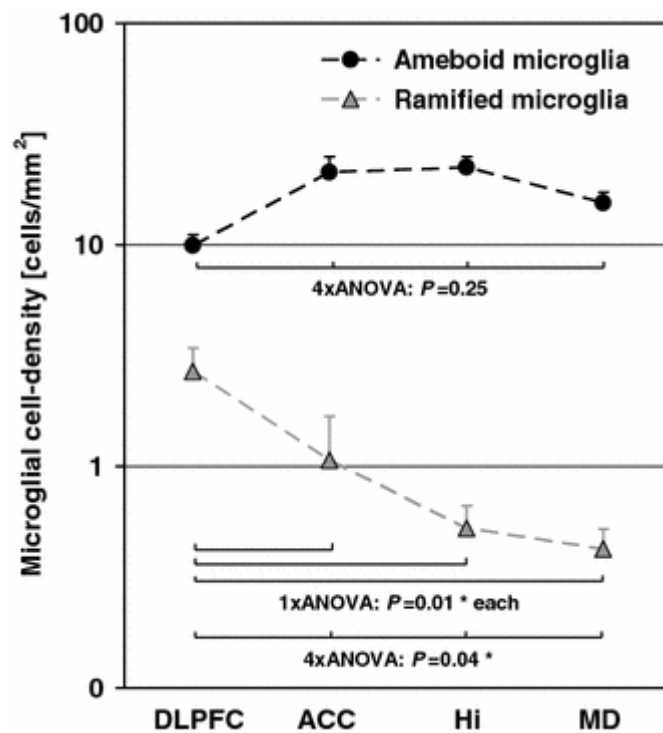
### **E.8.1 Electronic Figure Submission**

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig 1.eps.

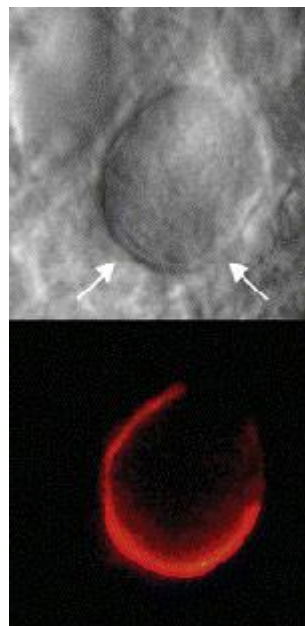
### **E.8.2 Line Art**

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

- Vector graphics containing fonts must have the fonts embedded in the files.

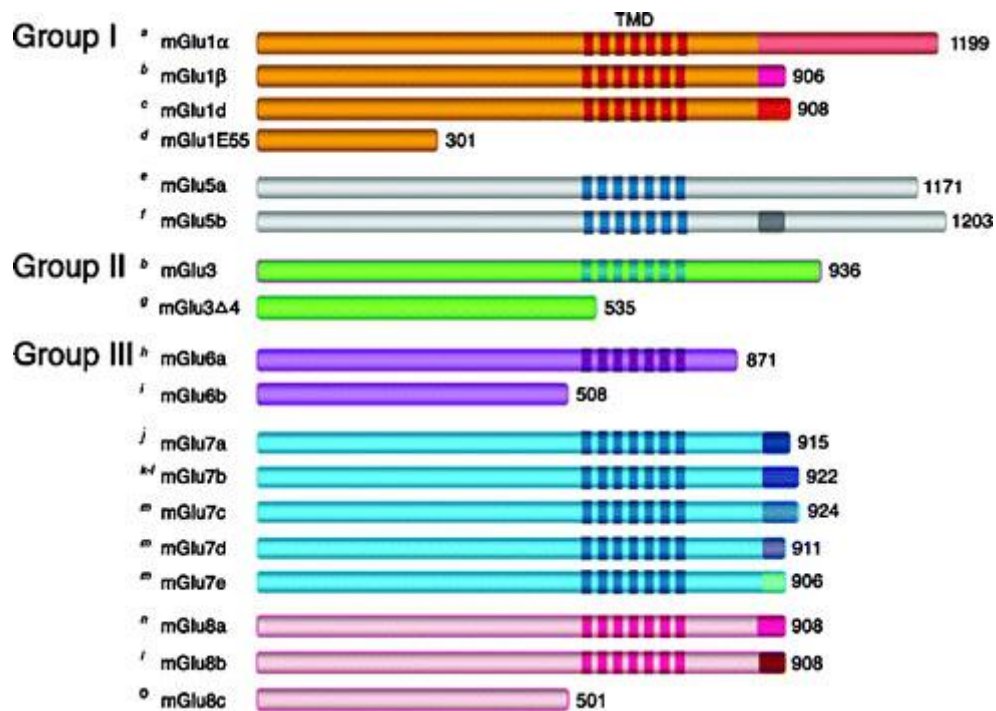


### E.8.3 Halftone Art



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

## E.8.4 Combination Art



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

## E.8.5 Color Art

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

## E.8.6 Figure Lettering

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

- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

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

### **E.8.8 Figure Captions**

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

### **E.8.9 Figure Placement and Size**

- Figures should be submitted separately from the text, if possible.
- When preparing your figures, size figures to fit in the column width.
- For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.
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- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

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- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

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- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

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- Name the files consecutively, e.g. “ESM\_3.mpg”, “ESM\_4.pdf”.

### **E.9.8 Captions**

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- Research involving Human Participants and/or Animals
- Informed consent

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See below examples of disclosures:

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- “This article does not contain any studies with human participants or animals performed by any of the authors.”

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All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. Hence it is important that all participants gave their informed consent in writing prior to inclusion in the study. Identifying details (names, dates of birth, identity numbers and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scientific purposes and the participant (or parent or guardian if the participant is incapable) gave written informed consent for publication. Complete anonymity is difficult to achieve in some cases, and informed consent should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort scientific meaning.

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## F.1 Aim

The journal Die **Pharmazie** publishes reviews, experimental studies, letters to the editor, as well as book reviews. The following fields of pharmacy are covered: Pharmaceutical and medicinal chemistry, pharmaceutical analysis and drug control, pharmaceutical technology, biopharmacy (biopharmaceutics, pharmacokinetics, biotransformation), experimental and clinical pharmacology, pharmaceutical biology (pharmacognosy), history of pharmacy. Articles are published in English (preferred) or German and are classified as:

- Reviews
- Original articles
- Short communications
- Book reviews
- Letters to the Editor

**Reviews:** A summarizing presentation encompassing the current state of our knowledge and providing comprehensive interpretation with citation of the literature.

**Original articles:** Publications from all fields mentioned above.

**Short communications:** Brief publications about the fields mentioned above.

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1. For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be determined and classified in a suitable way, problems must be formulated in view of the data, hypotheses should be suggested an/or the author should give possible explanations for any inconsistencies.

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3. To achieve clarity and brevity of the presentation, original contributions should be subdivided after the abstract (see 2.) as follows:

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Sample structured abstract:

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**Methods:** The abstract is organized into four distinct sections: Purpose, Methods, Results, and Conclusions.

Results: A more informative abstract results.

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1. Kohane DS, Anderson DG, Yu C, Langer R. pH triggered release of macromolecules from spraydried polymethacrylate microparticles. *Pharm Res.* 2003;20(10):1533-1538. [Journal Article]
2. Swarbrick J, Boylan JC. *Encyclopedia of pharmaceutical technology.* New York: Marcel Dekker; 2001. [Book]
3. Lee VHL. Ocular epithelial models. In: Borchardt RT, Smith PL, Wilson G, editors. *Models for assessing drug absorption and metabolism.* New York: Plenum; 1996. p. 425-436. [Contribution to a Book]
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