Topical delivery of artemether, encapsulated in niosome and proniosome carriers

C van der Merwe

22775374

Dissertation submitted in fulfilment of the requirements for the degree *Master of Science* in Pharmaceutics at the Potchefstroom Campus of the North-West University

Supervisor: Prof J du Plessis
Co-Supervisor: Prof JL du Preez
Co-Supervisor: Dr M Gerber

November 2016

This dissertation is presented in article format, which include three subchapters, one article for publication in a pharmaceutical journal (Chapter 3) and four annexures (Appendix A – D) enclosing experimental results and discussions. The article for publications has an author's guide for publishing (Appendix E).

Trust in the LORD with all your heart, and lean not on your own understanding; In all your ways acknowledge Him, and He shall direct your paths. ~ Proverbs 3:5~

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
ARM	Artemether
ATL	Analytical Technology Laboratory
CTB	Cutaneous tuberculosis
DHA	Dihydroartemisinin
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ED	Epidermis-dermis
EE%	Encapsulation efficiency
EIM	Ether injection method
FAT	Freeze and thaw method
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HaCaT	Cultured Human Keratinocyte
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HSM	Hand shaking method
$K_2H_2PO_4$	Dipotassium phosphate

K_a	Ionisation/dissociation constants
K_m	Partition coefficient
LDH	Lactase dehydrogenase
LOD	Limit of detection
Log D	Octanol-buffer distribution coefficient
Log P	Octanol-water partition coefficient
LOQ	Limit of quantification
MeOH	Methanol
MDR-TB	Multidrug - resistant TB
MR	Mycothiol reductase
MSH	Mycothiol
MTB	*Mycobacterium tuberculosis*
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	NADH phosphate-oxidase
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
PBS	Phosphate buffer solution
PDI	Polydispersity index
pK_a	Negative logarithm of K_a
PVDF	Polyvinylidene difluoride
RPE	Reverse phase evaporation method

RPM	Revolutions per minute
ROS	Reactive oxygen species
%RSD	%Relative standard deviation
SCE	Stratum corneum-epidermis
SD	Standard deviation
TB	Tuberculosis
TEM	Transmission electron microscopy
TEWL	Transepidermal water loss
THF	Thin-film hydration method
THF	Tetrahydrofuran (Organic solvent)
UGT	Uridine diphosphate glucuronosyltransferase
USP	United States Pharmacopeia
UV	Ultraviolet
WHO	World Health Organization

LIST OF EQUATIONS

LIST OF FIGURES

APPENDIX B

APPENDIX C

APPENDIX D

LIST OF TABLES

CHAPTER 3

APPENDIX A

APPENDIX B

APPENDIX C

APPENDIX D

ACKNOWLEDGEMENTS

Firstly, I want to thank God Almighty for the opportunity to study and finish my Masters. He gave me strength to carry on and keep my head high when I felt I could not. He was there every step of the way, guiding me and giving me the human support systems I needed.

I would like to express my gratitude towards the following people:

- **My parents**, I could not have asked for a better support system. You were always understanding, there to listen and ready with wisdom and help in any way you could. Through your examples of how one should do everything for God and your education as Christians, these two years with all the obstacles were overcome, but also the good in every opportunity were recognised and savoured.

- My sisters. **Jannika** it was an unbelievable privilege to spend the last two years of my university time with you so near. Thank you for always listening to my stories, even if you did not understand. **Denè**, these last two years living with you was so much fun and I am grateful for the friendship we gained from it. Thank you for supporting me in everything I do.

- To my friends, **Tanja** and **Esmari**. God knew I would need you these past two years. Thank you for your constant support, all the help and working together in the laboratory, the figuring out of the data processing and making the hours spent typing in the office a ball. Not only were you there as colleagues, you also made our time outside the studies fun and gave me many precious memories I will always treasure. I gained two best friends these last two years for whom I am truly grateful and blessed. Thank you for your unconditional friendship.

- **Ewald** and **Zenobia**, at first the three of us were total strangers in this faculty and had to rely on each other. I am glad I had people understanding the change and embracing it with me. Thank you for all your support.

- To my other **colleagues**, thank you for all the help and advice these past two years. **Johan**, thank you for all your assistance, as well as your advice.

- My supervisor, **Professor Jeanetta du Plessis**, thank you for always making the time when your help or advice were needed. Thank you for putting our needs first.

- To my co-supervisor, **Professor Jan du Preez**, thank you for all your help regarding the HPLC and our validation. Prof, thank you for your friendliness and patience with all the questions I continually asked. Thank you for your willingness to help with everything, even if it was not HPLC related.

- To my assistant-supervisor, **Dr Minja Gerber**, thank you for all your proofreading and thank you for allowing me to always pop in with questions, even with just the smallest things.

- **Mrs Alicia Brümmer**, thank you for all your assistance regarding the laboratory work as well as your trouble with our cytotoxicity studies and your constant friendly smile.

- **Dr Anine Jordaan**, thank you for all your help with the size determination of our vesicles using the TEM microscope.

- **Prof Lissinda du Plessis**, thank you very much for the effort you made to help us with our cytotoxicity studies and the appendix concerning it.

- **Mrs Hester de Beer**, thank you very much for always caring about how we were doing; your smile, friendliness and conversations were always a bright point in my day.

- **Mrs Anina van der Walt**, thank you for always being friendly, listening, sharing your wisdom and keeping our spirits high.

The work of this dissertation was carried out with the financial support of the South African **National Research Foundation (NRF)** (Grants no. IFRR81178 and CPRR**90569**), the **South African Medical Research Council (MRC)** for the Flagship Project MALTB-Redox, and the **Centre of Excellence for Pharmaceutical Sciences (Pharmacen)** of the North-West University, Potchefstroom Campus, South Africa

.

UITTREKSEL

Artemisia annua is ’n Chinese krui wat in 1971 deur Chinese wetenskaplikes ontdek is. Artemisinien is uit die krui geïsoleer en as ’n Chinese medikasie genaamd qinghaosu gebruik. Artemeter (ARM) is ’n middel van die artemisinienfamilie en is die aktiewe bestanddeel van die plant *Artemisia annua* (Ansari *et al.*, 2010:901; Ansari *et al.*, 2015:2; White, 2008:330). Navorsing van ’n nuwe kombinasie as behandeling vir tuberkulose (TB) word tans gedoen. Artemeter en die artemisinienfamilie besit sekere eienskappe wat hulle die vermoë gee om as behandeling teen die bakteriële stam *M. tuberculosis* op te tree. In hierdie kombinasieterapie sal artemeter as die oksidant optree (Haynes, 2015:3; Miller *et al.*, 201:2076).

Die voorkoms van infeksie deur *M. tuberculosis* het aansienlik toegeneem en veroorsaak elke jaar ’n groot aantal sterftes (Hershkovitz *et al*., 2008:1). Kutane tuberkulose (KTB) veroorsaak velletsels en is die gevolg van ’n primêre infeksie, meestal veroorsaak deur *M. tuberculosis.* ’n Persoon moet dus tuberkulose (TB) hê om KTB te kan kry (Van Zyl *et al.*, 2015:629). Die sielkundige effek van KTB (Dos Santos *et al*, 2014:219) lei saam met die ernstige newe-effekte van medikasie vir die behandeling van TB tot ’n lang behandelingstydperk. Behandeling van KTB met die topikale aflewering van artemeter, saam met die huidige middels vir TB, verkort die algehele behandelingstydperk vir albei hierdie siektes.

Die vel is die grootste selfhernuwende orgaan van die menslike liggaam. Die belangrikste funksie van hierdie orgaan is om die liggaam teen die eksterne penetrasie van enige skadelike eksterne komponente te beskerm. Die vel bestaan uit drie verskillende lae, naamlik 1) epidermis, 2) dermis en 3) hipodermis. Die belangrikste fisiese versperring van die vel is die stratum corneum, die boonste laag van die epidermis, wat verantwoordelik is vir die beweging van water en elektroliete. Die versperringseienskappe van die vel maak geneesmiddelaflewering van ’n aktiewe bestanddeel moeilik (Baroni *et al*., 2012:257; Bolzinger *et al*., 2012:156; Feingold & Denda, 2012:263; Foldvari, 2000:417).

Die hoeveelheid navorsing oor transdermale geneesmiddelafleweringsmetodes saam met die fisiese uitvoering van topikale behandeling het oor die afgelope 50 jaar grootliks toegeneem. Hierdie toename is as gevolg van die voordele wat hierdie roete bo die tradisionele afleweringsmetodes bied (Moss *et al.*, 2012:166). Tydens die topikale aflewering van ’n geneesmiddel penetreer die geneesmiddel die stratum corneum waarna dit deur die verskillende vellae na die geteikende posisie diffundeer met ’n minimum tot geen sistemiese absorpsie (Rahimpour & Hamishehkar, 2012:141). Die suksesvolle diffusie van ’n aktiewe bestanddeel deur die vel is direk eweredig aan die fisies-chemiese eienskappe van daardie aktiewe bestanddeel (Hadgraft, 2001:291). Volgens Naik *et al.* (2000:319) moet ’n aktiewe bestanddeel ’n

wateroplosbaarheid groter as 1 mg/ml besit om ideale resultate vir transdermale geneesmiddelaflewering te lewer. Die wateroplosbaarheid van artemeter is 0.46 mg/ml (Human metabolome database, 2013:2) en dus nie ideaal vir die topikale aflewering nie, maar dit kan dalk verbeter word as dit in ’n vesikel draersisteem omsluit word.

Vesikel geneesmiddelafleweringsisteme funksioneer as draerstelsels tydens die topikale aflewering van ’n middel na ’n geteikende posisie. Niosome is nie-lipied biodraers bestaande uit nie-ioniese oppervlakaktiewe stowwe en lipiede, en dit kan dus beide lipofiele en hidrofiele geneesmiddels omsluit. Die gebruik van niosome tydens topikale aflewering het verskeie voordele, naamlik 1) beheerde vrystelling van die middel, 2) beter geneesmiddelstabiliteit en 3) verbeterde velpenetrasie van ’n geneesmiddel (Jain *et al.*, 2014:1-2; Kamboj *et al*., 2013:125; Shilakari *et al.*, 2013:77).

Die doel van hierdie studie was om te bepaal of die topikale aflewering van artemeter omsluit in ’n vesikelsisteem moontlik is. Vir die analise van artemeter is 'n hoëdruk vloeistofchromatografiese (HDVC) analisemetode vir artemeter ontwikkel. Drie verskillende niosoomdispersies (1%, 2% en 3%) wat almal artemeter omsluit, is geformuleer en gekarakteriseer om die beste dispersie vir verdere analises te identifiseer. Die 3%-niosoom en -proniosoom vesikeldispersies is gekies, gebaseer op hulle algehele eienskappe, naamlik viskositeit, pH, omsluitingseffektiwiteit, zetapotensiaal, deeltjiegrootte en polidispersiteitsindeks (PdI). Die morfologie van die 3%-niosome en -proniosome plasebodispersies is met behulp van transmissie-elektronmikroskopie (TEM) bepaal.

Die fosfaatbufferoplossing (PBS, pH 7.4) en wateroplosbaarheid van artemeter is as 0.09 ± 0.003 mg/ml en 0.11 ± 0.002 mg/ml, onderskeidelik bepaal. Die oktanol-water verdelingskoëffisiënt (log P) en die oktanol-buffer verspreidingskoëffisiënt (log D) van artemeter is 2.26 ± 0.117 en 2.35 ± 0.067, onderskeidelik. Dus kan daar gesê word dat artemeter van nature meer lipofiel is en nie wateroplosbaar nie. Studies van diffusie deur membrane en vel is met albei artemeter omsluite vesikeldispersies gedoen. Studies van vrylating uit membrane het getoon dat beide vesikels die artemeter wat omsluit is vrygestel het. Die resultate van die veldiffusiestudies het lae artemeterkonsentrasies in van die epidermis-dermis monsters aangedui, maar geen artemeter in die stratum corneum-epidermis en die reseptorfase nie; die hoeveelhede wat wel aanwesig was, het ’n gemiddelde konsentrasie laer as die limiet van kwantifisering van artemeter gehad. Die konsentrasie artemeter in die stratum corneum-epidermis en epidermis-dermis was nie kwantifiseerbaar nie, maar die feit dat artemeter in die epidermis-dermis gekry is dui daarop dat geteikende geneesmiddelaflewering plaasgevind het (Karim *et al*., 2010:374). Die topikale aflewering van artemeter kan verbeter word deur ’n ander draer te oorweeg of deur die konsentrasie artemeter wat by die formulering gevoeg word te verhoog (Herkenne *et al*., 2008).

Die toksisiteit van artemeter, die leë niosome en die artemeter gelaaide niosome is met die laktasedehidrogenase (LDH)-toets bepaal. Die LDH-toets het getoon dat die ongelaaide vesikels nie sitotoksies is nie, terwyl die gelaaide vesikels en die aktiewe bestanddeel, artemeter, vanaf artemeterkonsentrasies van 300 mg/ml en 150 mg/ml onderskeidelik sitotoksies vir die HaCaT-selkulture was. Ongelukkig kan *in vivo* en *in vitro* toksiese ontledings nie met mekaar vergelyk nie word as gevolg van die invloed van ander faktore teenwoordig tydens *in vivo*-blootstellings (López-García *et al*., 2014:45; Yoon *et al*., 2012:634).

Die eienskappe van die vesikels het aangedui dat hulle optimaal vir topikale geneesmiddelaflewering was en tydens hierdie studie is opgemerk dat die vesikels geteikende aflewering van artemeter in die epidermis-dermis verseker het. Die sitotoksisiteit van artemeter deur middel van die LDH-toets het aangedui dat artemeter en die gelaaide niosome sitotoksies vir die selkulture was, terwyl die leë niosome nie sitotoksies was nie. Hierdie sitotoksiese resultate kan ongelukkig nie met *in vivo*-omstandighede soos tydens topikale geneesmiddelaflewering vergelyk word nie.

Ten slotte kan gesê word dat die topikale aflewering van artemeter bereik is. Artemeter is geïdentifiseer (hoewel nie kwantifiseerbaar nie) in die doelgebied (epidermis-dermis), maar die biobeskikbaarheid van artemether in die epidermis-dermis kan verbeter word met verskeie metodes, soos die toevoeging van penetrasiebevorderaars tot die formulering (Herkenne *et al*., 2008:8; Morrow *et al*., 2007:39), die vesikelsisteem te verander, of deur die konsentrasie artemeter wat by die formulering gevoeg word te verhoog (Herkenne *et al*., 2008:87).

Sleutelwoorde: Artemether, Diffusiestudies, Tuberkulose, Vel, Vesikels

ABSTRACT

Artemisia annua is a Chinese herb discovered by Chinese scientists in 1971. Artemisinin was extracted from this herb and used as a Chinese medicine named Qinghaosu. Artemether (ARM) is a derivative from the artemisinin family and also the active ingredient in the herb *Artemisia annua* (Ansari *et al*., 2010:901; Ansari *et al*., 2015:2; White, 2008:330). Recently a new combination therapy acting as treatment against tuberculosis (TB) has been researched. Artemether and its artemisinin family have certain characteristics which gives these compounds the ability to act as treatment against the *M. tuberculosis* strain. During this combination therapy, artemether would represent the oxidant drug (Haynes, 2015:3; Miller *et al*., 201:2076).

Infection rates caused by the bacterial species, *M. tuberculosis,* have increased significantly and cause a lot of deaths each year (Hershkovitz *et al*., 2008:1). Cutaneous tuberculosis (CTB) is an illness presenting with skin manifestations due to a main infection, generally caused by this bacterium strain, *M. tuberculosis*, therefore a person must have TB in order to have CTB (Van Zyl *et al*., 2015:629). The psychological effect of being infected with CTB (Dos Santos *et al*., 2014:219), along with the severe side effects of TB treatment medication, leads to prolonged treatment periods. The aim for CTB treatments, by means of the topical drug delivery of artemether in conjunction with current TB treatments, is to decrease the overall treatment period.

The largest self-renewing organ of the human body is the skin, and the main function of this organ is to protect the body against the intrusion of any harmful external agents. The skin consists of three different layers, i.e. 1) epidermis, 2) dermis and 3) hypodermis, with the main physical barrier lying within the stratum corneum, which is the top layer of the epidermis responsible for the movement of water and electrolytes. The barrier properties of the skin makes the drug delivery of an active pharmaceutical ingredient (API) difficult (Baroni *et al*., 2012:257; Bolzinger *et al*., 2012:156; Feingold & Denda, 2012:263; Foldvari, 2000:417).

The amount of research conducted on, as well as the execution of topical and transdermal drug delivery methods have increased over the last 50 years. This increased usage is due to the large improvements on the topical and transdermal drug delivery methods over other traditional delivery methods (Moss *et al*., 2012:166). During topical drug delivery, the drug penetrates the stratum corneum and then permeates into the different skin layers to a

targeted site with minimum to no systemic absorption (Rahimpour & Hamishehkar, 2012:141). Successful permeation of an API across the skin is directly proportional to the physicochemical properties of the API (Hadgraft, 2001:291). According to Naik *et al*. (2000:319), an API should have an aqueous solubility larger than 1 mg/ml to deliver ideal results regarding transdermal drug delivery. The aqueous solubility of artemether is 0.46 mg/ml (Human Metabolome database, 2013:2) and therefore not ideal for topical drug delivery, but might be improved when encapsulated in a vesicle carrier system.

Vesicular drug delivery systems function as carrier systems during the topical deliverance of drugs to the targeted sites. Niosomes are non-lipoidal biocarriers consisting of non-ionic surfactants and lipids, therefore these vesicles can encapsulate both lipophilic and hydrophilic drugs. Using niosomes during topical delivery has many advantages, i.e. 1) controlled release of the drug, 2) better drug stability and 3) enhancing the skin permeation of a drug (Jain *et al*., 2014:1-2; Kamboj *et al*., 2013:125; Shilakari *et al*., 2013:77).

The aim of this study was to determine if the topical delivery of artemether was possible after being encapsulated in a vesicle. In order to analyse artemether, a high performance liquid chromatography (HPLC) method was developed. Three different niosome dispersions, (1%, 2% and 3%), each encapsulating artemether, were formulated and then characterised to determine the best dispersion for further analysis. The 3% niosome and 3% proniosome vesicle dispersions were chosen, based upon their overall characteristics, i.e. viscosity, pH, entrapment efficiency, zeta-potential, particle size and polydispersity index (PdI). The morphology of the 3% niosome and proniosome placebo dispersions were determined using transmission electron microscopy (TEM).

The phosphate buffer solution (PBS; pH 7.4) and water solubility of artemether was determined as 0.09 ± 0.003 mg/ml and 0.11 ± 0.002 mg/ml, respectively. The octanol-water partition coefficient (log P) and the octanol-buffer distribution coefficient (log D) of artemether was 2.26 ± 0.117 and 2.35 ± 0.067, respectively. Hence, it can be said that artemether is more lipophilic in nature and not water-soluble. Membrane and skin diffusion studies were executed with both artemether entrapped vesicle dispersions. During the membrane release studies it was acquired that both vesicles do release the entrapped artemether. The results of the skin diffusion studies revealed low artemether concentrations in some of the epidermis-dermis samples, with no artemether present in the stratum corneum-epidermis and the receptor phase; those concentrations that were present gave an average concentration lower than the limit of quantification (LOQ) of artemether. These results

indicated that the stratum corneum-epidermis and epidermis-dermis results were not quantifiable, but the fact that artemether concentrations were acquired in the epidermis-dermis indicates that targeted drug delivery occurred (Karim *et al*., 2010:374). The topical delivery of artemether can be improved by considering a different carrier or by increasing the concentration of artemether added to the formulation (Herkenne *et al*., 2008).

The toxicity determination of artemether, the empty niosomes and the artemether loaded niosomes were determined by means of the lactase dehydrogenase (LDH) assay. During the LDH assay, it was determined that the unloaded vesicle dispersion was not cytotoxic, while the loaded vesicles and the API artemether were strongly cytotoxic to the Cultured Human Keratinocyte (HaCaT) cell cultures from the concentrations 300 µg/ml. Unfortunately *in vivo* and *in vitro* toxicity analysis cannot be compared due to other influencing factors present during *in vivo* exposure (López-García *et al*., 2014:45; Yoon *et al*., 2012:634).

The characteristics of the vesicles indicated they were optimal for topical drug delivery and during this study, it was observed that the vesicles did ensure targeted delivery of artemether into the epidermis-dermis. The cytotoxicity of artemether using the LDH assay indicated artemether and the loaded niosomes were cytotoxic to the cell cultures, while the empty niosomes were not cytotoxic. These cytotoxicity results unfortunately cannot be compared to *in viv*o circumstances such as topical drug delivery.

In conclusion, it can be said that the topical delivery of artemether was reached. Artemether was identified (although unquantifiable) in the target area (epidermis-dermis), but the bioavailability of artemether in the epidermis-dermis can be improved through various methods, such as adding permeation enhancers to the formulation (Herkenne *et al*., 2008:8; Morrow *et al*., 2007:39), changing the vesicle system, or by increasing the concentration artemether added to the formulation (Herkenne *et al*., 2008:87).

Keywords: Artemether, Diffusion studies, Skin, Tuberculosis, Vesicles

References

Ansari, M.T., Karim, S., Ranjha, N.M., Shah, N.H. & Muhammad, S. 2010. Physicochemical characterization of artemether solid dispersions with hydrophilic carriers by freeze dried and melt methods. *Archives of Pharmacal Research*, 3(6):901-910.

Ansari, M.T., Hussain, A., Nadeem, S., Majeed, H., Saeed-Ul-Hussan, S., Tariq, I., Mahmood, Q., Khan, A.K. & Murtaza, G. 2015. Preparation and characterization of solid dispersions of artemether by freeze-dried method. *BioMed Research International*, 2015:1-11.

Baroni, A., Buommino, E., De Gregorio, V., Ruocco, E., Ruocco, V. & Wolf. R. 2012. Structure and function of the epidermis related to barrier properties. *Clinics in Dermatology*, 30(3):257-262.

Dos Santos, J.B., Figueiredo, A.R., Ferraz, C.E., De Oliveira, M.H., Da Silva, P.G. & De Medeiros, V.L.S. 2014. Cutaneous tuberculosis: epidemiologic etiopathogenic and clinical aspects – part 1. *Anais Brasileiros de Dermatologia*, 89(2):219-228.

Feingold, K.R. & Denda, M. 2012. Regulation of permeability barrier homeostasis. *Clinics in Dermatology*, 30(3):263-268.

Foldvari, M. 2000. Non-invasive administration of drugs through the skin: challenges in delivery system design. *Pharmaceutical Science & Technology today*, 3(12):417-425.

Hadgraft, J. 2004. Skin deep. *European Journal of Pharmaceutics and Biopharmaceutics*, 58(2):291-299.

Hershkovitz, I., Donoghue, H.D., Minnikin, D.E., Besra, G.S., Lee, O.Y., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Bar-Gal, G.K. & Spigelman, M. 2008. Detection and molecular characterization of 9000-year old Mycobacterium tuberculosis from a Neolithic settlement in the eastern mediterranean. *PLoS ONE*, 3(10):1-6.

Human metabolome 2016. Showing metabocard for artemether (HMDB15643). http://www.hmdb.ca/metabolites/HMDB15643 Date of access: 27 Sept. 2016.

Jain, S., Jain, V. & Mahajan, S.C. 2014. Lipid based vesicular drug delivery systems. *Advances in Pharmaceutics*, 2014:1-12.

Kamboj, S., Saini, V., Magon, N., Bala, S. & Jhawat, V. 2013. Vesicular drug delivery systems: a novel approach for drug targeting. *International Journal of Drug Delivery*, 5(2):121-130.

Moss, G.P., Wilkinson, S.C. & Sun, Y. 2012. Mathematical modeling of percutaneous absorption. *Current Opinion in Colloid & Interface Science*, 17(3):166-172.

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical Science & Technology today,* 3(9):318-326.

Rahimpour, Y. & Hamishehkar, H. 2012. Niosomes as carrier in dermal drug delivery. (*In* Sezer, A.D., *ed*. Recent advances in novel drug carrier systems. Rijeka: InTech, p. 141-164).

Shilakari, G., Singh, D. & Asthana, A. 2013. Novel vesicular carriers for topical drug delivery and their application's. *International Journal of Pharmaceutical Sciences Review and Research*, 21(1):77-86.

Stockert, J.C., Blázquez-Castro, A., Cañete, M. & Horobin, R.W. 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica*, 114(8):785-796.

Van Zyl, L., Du Plessis, J. & Viljoen J. 2015. Cutaneous tuberculosis overview and current treatment regimens. *Tuberculosis*, 95(6):629-638.

White, N.J. 2008. Qinghaosu (Artemisinin): the price of success. *Science*, 320(5874):330-334.

CHAPTER 1

INTRODUCTION, AIM AND OBJECTIVES

Artemether, also called alpha-dihydroartemisinin methyl ether, is a synthetic derivative of the artemisinin family and is lipophilic in nature (Ansari *et al*., 2010:901; Silamut *et al*., 2003:3798; Tayade & Nagarsenker, 2010:637). Upon oral administration of artemether, the absorption is very rapid, but incomplete, resulting in a decrease in its bioavailability (Ansari *et al*., 2014:1). The oral administration of artemether, as treatment against *Mycobacterium tuberculosis* (MTB), has many side effects. The topical application of artemether would serve as treatment against the lesions caused by cutaneous tuberculosis (CTB), in conjunction with systemic treatment against TB. The two worst side effects of TB and CTB are that the TB treatments cause great discomfort for the patient, while the lesions caused by CTB increases patients' self-consciousness. The current approach in the treatment of CTB is to shorten the treatment period for CTB, resulting in less discomfort for the patient.

Topical drug delivery is a method used for drug administration into the skin. The skin is the largest organ of the human body, consisting of four layers, i.e. the stratum corneum, the viable epidermis, the dermis and the hypodermis (Hadgraft, 2004:291; Jepps *et al*., 2012:153-154). All the skin layers function as effective barriers against the outside penetration of drugs, with the stratum corneum being the main barrier. The stratum corneum mainly consists of lipids, therefore preventing the diffusion of aqueous compounds. In contrast, the diffusion through the epidermis and dermis is regarded as diffusion through an aqueous medium, hence these layers are effective barriers against lipophilic compounds (Jepps *et al*., 2012:153-156; Menon *et al*., 2012:6; Prausnitz & Langer, 2008:2). The transport of an active pharmaceutical ingredient (API), during topical delivery, across the stratum corneum is mainly facilitated through passive diffusion (Jepps *et al*., 2012:252-253). For a drug to penetrate the stratum corneum it must have specific physicochemical properties (Prausnitz & Langer, 2008:2), i.e. an aqueous solubility above 1 mg/ml (Naik *et al*., 2000:319) and a log P (octanol-water partition coefficient) value between 1 and 4 (Mbah *et al*., 2011:68; Williams, 2013:680). The solubility of artemether is 0.11 mg/ml and the log P value 3.48 (DrugBank, 2013:5), which does not meet the criteria therefore it would make diffusion through the stratum corneum very difficult.

Low aqueous soluble molecules, such as artemether, result in low or no diffusion into the skin layers, however by encapsulating artemether into a vesicle system topical drug delivery should be possible. A vesicular system is a pharmaceutical drug carrier used to deliver the API for targeted drug action. These vesicle systems, i.e. niosomes and proniosomes, have hydrophilic, amphiphilic and lipophilic characteristics. Therefore, these carriers are capable of carrying APIs

with the aforementioned characteristic. The niosomes and proniosomes change the physicochemical characteristics of the dispersion yielding increased drug permeability (Karim *et al.,* 2010:374; Rahimpour & Hamishehkar, 2012:141).

The research problem for this study was that currently there are no topical drugs clinically used for the treatment against CTB, causing a need for research in this field (Van Zyl *et al*., 2014:2). The *Mycobacterium tuberculosis* bacterium causes CTB, as well as TB (Van Zyl *et al*., 2014:1). Artemether possesses good properties against the targeting of the *Mycobacterium tuberculosis* bacterium in general (Haynes, 2013:1-2; Haynes, 2015:3; Miller *et al*., 2011:2076), therefore, it should deliver good results as a topical treatment against CTB. The topical delivery of artemether would be a challenge, due to the stratum corneum acting as a barrier and also because the physicochemical properties of artemether are not ideal for penetration through the skin (Hadgraft, 2004:291; Prausnitz & Langer, 2008:2).

The aim of this study was to determine the possible topical delivery of artemether by formulating it into vesicle systems, i.e. niosomes and proniosomes.

The objectives of this study were as follows:

- Validate a HPLC method that would obtain adequate concentrations of artemether during analysis.
- Determine the aqueous solubility along with the log D (octanol-buffer distribution coefficient) and log P of artemether.
- Formulate optimum artemether (as API) entrapped in niosomes.
- Characterize three different niosome dispersions for the purpose of selecting the optimum dispersion based upon the overall best characteristics regarding topical drug delivery.
- Formulate and characterise the chosen percentage proniosome dispersion.
- Perform membrane release studies for the purpose of determining whether the entrapped artemether is released from the vesicle systems.
- Determine whether topically applied artemether, entrapped inside the vesicles, can permeate through the different skin layers to the target-site (dermis), by making use of tape stripping and diffusion studies.
- Determine whether the application of artemether or any vesicle systems results in cytotoxicity towards cell cultures.

References

Ansari, M.T., Karim, S., Ranjha, N.M., Shah, N.S. & Muhammed, S. 2010. Physicochemical characterization of artemether solid dispersions with hydrophilic carriers by freeze dried and melt methods. *Archives of Pharmacology Research*, 33(6):901-910.

Ansari, M.T., Hussain, A., Nadeem, A., Majeed, H., Saeed-Ul-Hassan, S., Tariq, I., Mahmood, Q., Khan, A.K. & Murtaza, G. 2014. Preparation and characterization of solid dispersions of artemether by freeze-dried method. *BioMed Research International*, 2015:1-11.

DrugBank. 2013. Artemether (DB06697). http://www.drugbank.ca/drugs/DB06697 Date of access: 27 Oct. 2016.

Haynes, R. 2013. SA MRC flagship proposal executive summary. October 2013. [Correspondence]. 29 Jul. 2015, Potchefstroom.

Haynes, R.K. 2015. Development of oxidant and redox drug combinations for treatment of malaria, TB and related diseases. [PowerPoint presentation].

Hadgraft, J. 2004. Skin deep. *European Journal of Pharmaceutics and Biopharmaceutics*, 58(2):291-299.

Jepps, O.G., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. 2013. Modelling the human skin barrier: towards a better understanding of dermal absorption. *Advanced Drug Delivery Reviews*, 65(2):152-168.

Karim, K.M., Mandal, A.S., Biswas, N., Guha, A., Chatterjee, S., Behera, M. & Kuotsu, K. 2010. Niosome: a future of targeted drug delivery systems. *Journal of Advanced Pharmaceutical Technology and Research*, 1(4):374-380.

Mbah, C.J., Uzor, P.F. & Omeje, E.O. 2011. Perspectives on transdermal drug delivery. Journal of Chemical and Pharmaceutical Research, 3(3):680-700.

Menon, G.K., Cleary, G.W. & Lane, M.E. 2012. The structure and function of the stratum corneum. *International Journal of Pharmaceutics*, 435(1):3-9.

Miller, M.J., Walz, A.J., Zhu, H., Wu, C., Moraski, G., Möllman, U., Tristani, E.M., Crumbliss, A.L., Ferdig, M.T., Checkley, L., Edwards, R.L. & Boshoff, H.I. 2011. Design, synthesis and study of a mycobactin-artemisinin conjugate that has selective and potent activity against tuberculosis and malaria. *Journal of the American Chemical Society*,133(7):2076-2079.

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical Science and Technology Today,* 3(9):318-326.

Nnamani, P.O., Hansen, S., Windbergs, M. & Lehr, C. 2014. Development of artemether-loaded nanostructured lipid carrier (NLC) formulation for topical application. *International Journal of Pharmaceutics*, 477(1-2):208-217.

Prausnitz, M.R. & Langer, R. 2008. Transdermal drug delivery. *Nature Biotechnology*, 26(11):1261-1268.

Rahimpour, Y. & Hamishehkar, H. 2012. Niosomes as carrier in dermal drug delivery. (*In* Sezer, A.D., *ed*. Recent advances in novel drug carrier systems. Rijeka:InTech. p. 141-164).

Silamut, K., Newton, P.N., Teja-Isavadharm, P., Suputtamongkol, Y., Siriyanonda, D., Rasameesoraj, M., Pukrittayakamee, S. & White, N.J. 2003. Artemether bioavailability after oral or intramuscular administration in uncomplicated *Falciparum* malaria. *Antimicrobial Agents and Chemotherapy*, 4(12):3795-3798.

Tayade, N.G. & Nagarsenker, M.S. 2010. Development and evaluation of artemether parental microemulsion. *Indian Journal of Pharmaceutical Sciences*, 72(5):637-640.

Van Zyl, L., Du Plessis, J. & Viljoen, J. 2015. Cutaneous tuberculosis overview and current treatment regimens. *Tuberculosis*, 95(6):629-638.

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London, UK. p. 675-697).

CHAPTER 2

TOPICAL DELIVERY OF ARTEMETHER, ENCAPSULATED IN NIOSOME AND PRONIOSOME CARRIERS

2.1 Introduction

Tuberculosis (TB) is an infectious disease caused by the species *Mycobacterium tuberculosis*, mainly in regions undergoing severe poverty. This disease poses a huge public health threat with approximately 20 - 40% of the world's population being affected by it (Perrin, 2015:112; Van Zyl *et al*., 2014:1). There is a well-established resistance of TB against the treatment of various drugs, making treatment almost impossible. Due to the resistance against the drugs, combination therapies are being evaluated as a new method for TB treatment. The artemisinin family works well as a combination therapy against TB. Artemether is part of the artemisinin family and a topical delivery of this drug for use against cutaneous tuberculosis (CTB) will be investigated. It must be stressed that the aim is not to ever replace oral treatment of TB and even CTB, but as an add-on to the oral dosage form to decrease, potentially, the total time of treatment needed.

There are two different types of drug delivery systems using the skin: 1) transdermal drug delivery and 2) topical drug delivery.

1) The administration of drugs through the skin and into the blood stream as a means of long-term drug delivery is called the transdermal drug delivery system. Drugs administered via transdermal drug delivery systems enter the skin by the action of permeation through diffusion. Transdermal drug delivery systems provide constant drug administration, controlled drug release, predictable drug activity, extended duration of activity, direct entering of the drug into the systemic circulation (therefore no first-pass metabolism) and lastly, it is a less invasive method (Bolzinger *et al*., 2012:156; Cai *et al.,* 2012:1; Sachan & Bajpai, 2013:748–750; Vandana *et al*., 2012:1&5).

2) Topical delivery is when the skin is targeted for the purpose of drug delivery, while avoiding systemic absorption to the best of its abilities, therefore it is for local use (Rahimpour & Hamishehkar, 2012:141). Topical application of drugs was developed to overcome most of the disadvantages experienced with conventional administration methods. However, this study aims at topical delivery and no transdermal delivery should take place.

The skin has very good barrier properties, making topical and transdermal delivery very difficult. Artemether has a very low aqueous solubility, making it hydrophobic. The first layer of the skin,

known as the stratum corneum, is also hydrophobic. Due to the outer skin layer and artemether both being lipophilic (totally dissolving in or having an affinity for fats or lipids), passive diffusion of artemether through the stratum corneum should be successful, but the other skin layers are hydrophilic (dissolving in water or mixing with water), which will cause the API to be stuck in the stratum corneum and not be able to diffuse through the dermis and hypodermis (Jepps *et al*., 2013:155-156). Hence, for topical delivery of artemether to succeed, encapsulation into a vesicular system used as a colloidal carrier, might be of great help. Topical application of artemether using a vesicle system can lead to fewer side effects than obtained during conventional administration methods and can result in more effective drug delivery (Bolzinger *et al.*, 2012:156; Human metabolome database, 2013:2; Menon *et al*., 2012:3; Tavano *et al*., 2014:7).

2.2 Tuberculosis

The *Mycobacterium* genus contains multiple pathogens of which *M. tuberculosis* is one of the more important. TB in humans is one of the oldest infectious diseases, caused by a closely related group of bacterial species, i.e. *M. tuberculosis*, which is mostly associated with poverty conditions such as overcrowding, low-nutrition food intake, addictions (alcohol, smoking, marijuana and narcotics) and co-infection with human immunodeficiency virus (HIV). Using comparative genomic and molecular marker analysis (deletion analysis), the findings of ancient cases of paleopathological changes and execution of ancient DNA (deoxyribonucleic acid) analysis indicated that the human TB lineage, *M. Tuberculosis,* is the oldest strain and exists as far back as 9 000 years ago (Hershkovitz *et al*., 2008:2; Perrin, 2015:112; Rivero-Lezcano, 2013:123).

The number of active *M. tuberculosis* infected cases has decreased, with an average of 1.3% per year since 2002, but there is still approximately 8 - 9 million new cases of infected human TB added to the already infected total per year. These statistics still cause a major health concern, which makes the aim of the World Health Organization (WHO), to totally eliminate TB by 2050, very difficult. Host resistance against TB depends on the differences in genetic factors and immune system between individuals. The innate and adaptive responses are involved in the defence action of the body fighting the TB virus invasion, but the ultimate outcome depends on the balance between *M. tuberculosis* and the individuals immune system. TB is such a major problem due to the spread of drug-resistant and multidrug-resistant TB (MDR-TB), which makes the prevention, cure and control of this disease very difficult (Brennan & Young, 2008:85; Perrin, 2015:113; Schito & Dolinger, 2015:1262; Van Zyl *et al*., 2015:629). No definite statistical information could be found regarding CTB, other than that approximately 1 - 2% of TB infected patients has CTB (Van Zyl *et al*., 2015:629).

CTB is a skin illness obtained from a chronic infection, mainly caused by *Mycobacterium tuberculosis* and although it can be caused by other species from the *Mycobacterium* genus, it only occurs in patients infected with TB. CTB is a disfiguring illness causing unwanted skin appearances that have a huge psychological effect on patients (Dos Santos *et al*., 2014:219; Van Zyl *et al*., 2015:629). Through topical treatment of CTB we aim to shorten the treatment period needed, thereby adding to the wellness of these patients.

2.3 Artemether

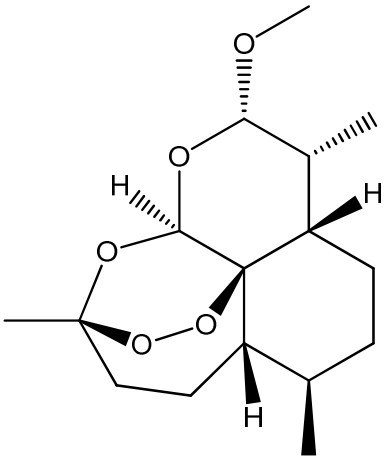


Figure 2.1: Structure of artemether

Artemether (also called dihydroartemisinin methyl ether) is a lipid-soluble compound which is an artemisinin derivative. The peroxide lactone artemisinin is isolated from the Chinese herb Qing Hao (*Artemisia annua*) as the active component (Ansari *et al*., 2010:901; Ansari *et al*., 2014:2; WHO, 1995:51). During liver metabolism, artemether is metabolised to its demethylated derivative known as dihydroartemisinin (DHA). Artemether is mainly metabolised to DHA by cytochrome P450 3A4 (CYP3A4), known as the hepatic and intestinal cytochrome, but it can also be metabolised by P450 2B6 (CYP2B6), P450 2C9 (CYP2C9) and P450 2C19 (CYP2C19). The pharmacologically active DHA is then inactivated by the enzyme uridine diphosphate glucuronosyltransferase (UGT) 1A9 and 2B7. Artemether has two epimers, α-artemether and β-artemether (Ansari *et al*, 2010:901; Lamorde *et al*., 2012:962; Silamut *et al*., 2003:3795; Wang *et al*., 2015:61).

2.3.1 Toxicity of artemether

Artemether is not hazardous, has a low toxicity and is only harmful to the human body when in contact with the eyes, is swallowed or inhaled. Therefore artemether must first be metabolised to dihydroartemisinin (Gao *et al*., 2013:134; Sigma-Aldrich, 2013:1-2).

2.4 Artemether acting as anti-TB medication

The resistance of TB against most of the anti-TB medication is increasing significant, which in turn leads to an increase in the occurrence of CTB. To date, no topical medication has been used clinically for the treatment of CTB (Almaguer-Chávez *et al*., 2009:562; Van Zyl *et al*., 2015:1-2).

The modern aim for anti-TB medication is to develop a combination therapy. During this combination therapy, an oxidant and redox drug, which will overwhelm the natural defences the TB bacteria possess against oxidative stress, will be used. The process of the TB bacteria's natural defences against oxidative stress begins with the fact the TB bacterium has different stages. One of these stages is called the alveolar macrophage stage, where the bacterium is exposed to high concentrations of oxygen in the lungs. The lungs contain various intracellular enzymes, which have the function of maintaining the reducing environment for the bacterium, in turn counteracting the production of reactive oxygen species (ROS) and other oxidants, such as reactive oxygen-nitrogen species. One of these enzymes maintaining the reducing environment is flavoenzyme disulphide reductase, mycothiol reductase (MR). During an electron transfer from reduced flavin, MR generates mycothiol (MSH), which in turn deactivates ROS. MSH is the TB equivalent of reduced glutathione (GSH) and this process is how the TB bacterium controls oxidative stress. The main electron source for this reaction is NAD(P)H (nicotinamide adenine dinucleotide phosphate-oxidase), which is the reduced form of NAD^+ (nicotinamide adenine dinucleotide) and a coenzyme (Haynes, 2013:1-2; Haynes, 2015:2-8).

During the development of an anti-TB drug, artemisinin and its derivatives (specifically artemether in this study) will act as the oxidant drug. A redox drug that will counteract the resistance against artemether will also be included. All the drugs that are part of the artemisinin family act as oxidants by reducing the flavin cofactors of the flavoenzyme disulphide reductase, consequently generating reduced thiol. The artemisinins are called the oxidant drugs, because they are irreversibly reduced and therefore has an oxidative capacity. The artemisinins have a negative influence on the MR action, leading to a build-up of ROS until cytotoxic levels are reached (Haynes, 2013:1-2; Haynes, 2015:3-8).

2.5 The human skin: structure and function

2.5.1 Structure and function of the human skin

The human skin is the largest continuously self-renewing organ of the body. The human skin is approximately 0.5 mm thick, has a surface area of approximately 1.8 m^2 and rounds up to approximately 16% of the total body weight. The skin has continuous mucous membranes that

line the surface of the body (Baroni *et al*., 2012:257; Foldvari, 2002:417; Kolarsick *et al*., 2011:203; Wickett & Visscher, 2006:98).

The skin has many functions, the main one being to protect the body against the penetration of any foreign external agents such as heat, infection, water and electromagnetic radiation. The skin, mostly the epidermis, also prevents the loss of water from the body to the outside environment, by retaining water inside the body. The barrier the skin provides against the movement of water and electrolytes is essential for survival in the terrestrial environment (Baroni *et al.*, 2012:257; Bolzinger *et al.*, 2012:156). The skin functions as an immune and sensory organ by regulating the temperature and synthesising vitamin D (Feingold & Denda; 2012:263; Wickett & Visscher, 2006:98). Under drastic conditions, the absence of these functions could lead to death. The skin has a very acidic surface that prevents infections and contributes to the barrier function (Baroni *et al*., 2012:259).

The skin is compiled of three main layers, which stretch from the skin surface to the deepest part and differ in structure, composition and function. The three layers are the epidermis, dermis and hypodermis (subcutaneous tissue). The combination of the different structures and compositions of the layers provide strength and flexibility to the skin (Bolzinger *et al.*, 2012:156; Kolarsick *et al.*, 2011:203; Wickett & Visscher, 2006:98).

2.5.2 The epidermis

The viable epidermis is the outer most layer of the skin, with a continuously renewing epithelium that has sensory and immunological functions; it is mainly hydrophilic of nature (Baroni *et al.*, 2012:257; Foldvari, 2002:417). This layer is responsible for producing derivative structures such as nails, pilosebaceous apparatuses and sweat glands. The epidermis and dermis layers are separated via a very thin layer called the basement membrane, consisting of extracellular matrix to which the epidermis adheres (Kolarsick *et al.*, 2011:204; Mikesh, 2013:191).

The epidermis is composed of two different types of cells, i.e. keratinocytes and dendritic cells, of which the keratinocytes are the predominant cell type. Keratinocytes are produced when cells migrate from the basal layer to the skin surface, passing the synthetic and degradative phases during a process called keratinisation. Keratinocytes are nucleated and viable from the granular- to the basal layer and contain neurotransmitters, which can be responsible for the regulation of the skin's permeability barrier function. The dendritic cells are subdivided into four types of cells, i.e. melanocytes, lymphocytes, Langerhans and Merkel cells. Melanocytes originate from neural crest, which are found in the basal layer of the epidermis, and produce melanosomes that contain the pigment melanin. The main function of melanocytes is to protect the skin from ultraviolet (UV) radiation and to give it its colour. Lymphocytes are only found in the epidermis under abnormal conditions when the immune system of the body is compromised,

causing the activation of specific immune responses. The Langerhans cells are derived from bone marrow and due to their immunocompetency, they play a very important role in the barrier function of the epidermis. Their main function is during allergic contact dermatitis. The function of Merkel cells is being part of the composition of the sensory nerves in the skin (Baroni *et al.*, 2012:258; Kolarsick *et al.*, 2011:2).

To protect the body from the penetration of any potentially hazardous environmental threats the epidermis consists of four different barriers, namely: the physical, chemical, biochemical and immunologic barriers. These four barriers each consist of different components. The physical barrier mainly consists of the stratum corneum, but cell-cell junctions and cytoskeletal proteins also make up part of the structure. The chemical and biochemical barriers consist of lipids, acids macrophages, hydrolytic enzymes and antimicrobial peptides. Lastly, the immunological barrier consists of the humoral and cellular components of the immune system (Baroni *et al.*, 2012:258; Kolarsick *et al.*, 2011:204).

The epidermis, compiled from four sub-layers/strata, is differentiated based on the two qualities, the keratinocyte morphology and the position of their division into horny cells. The four sub-layers (outer to inner), as shown in Figure 2.2, are the stratum corneum, stratum granulosum (granular layer), stratum spinosum (spinous layer) and stratum germinativum (basal layer) (Baroni *et al.*, 2012:257; Kolarsick *et al.*, 2011:204).

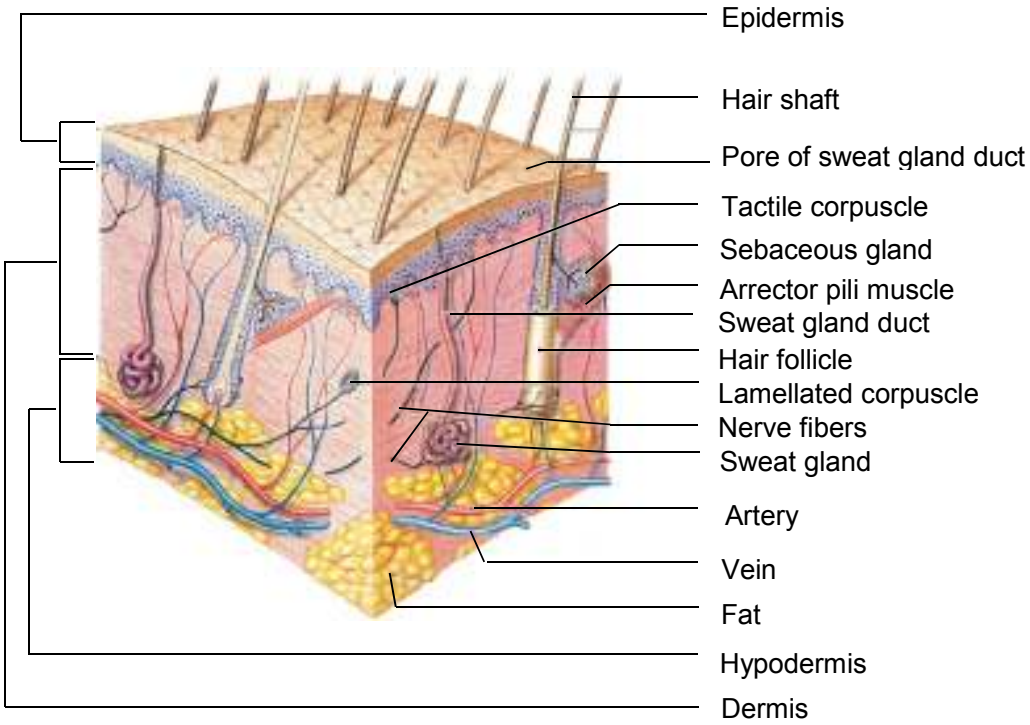


Figure 2.2: Epidermal differentiation in the skin (Martini *et al.*, 2012:145).

2.5.2.1 The stratum corneum

The stratum corneum is known as the outer layer of the viable epidermis and functions as a permeability barrier that protects the body against the penetration of foreign intruders and prevents desiccation. This layer has a two compartment structural organisation, consisting of corneocytes and a lipid matrix (Baroni *et al*., 2012:259).

The epidermal lipids or lipid matrix (synthesised by keratinocytes) in between the cells, function as the glue between the stratum corneum cells and therefore holds the corneocytes together, forming a brick-and-mortar complex (Baroni *et al.,* 2012:259; Bolzinger *et al.*, 2012:157; Feingold & Denda, 2012:263; Menon *et al.*, 2012:4); the corneocytes are flattened, dead cell bodies of keratinocytes forming the bricks and the intercellular lipid lamellae are the mortar. These flattened keratinocytes form as the last step in the keratinocytes differentiation process, where their structures undergo dramatic changes leading to flat and anucleated squamous cells and cover from 15 - 20 rows in the skin. The matrix consists of lipid membranous sheets and these sheets have unique compositions of ceramides, free fatty acids and cholesterol (Baroni *et al.*, 2012:258; Bhowmick & Sengodan, 2013:636; Menon *et al.*, 2012:6; Vandana *et al*., 2012:1).

The specific mixture of lipids in the intercellular space of the stratum corneum regulates the fluxes of chemicals and water between the skin and the outside environment leading to the counteracting of the loss of salt and water, as well as the penetration of foreign water-soluble compounds (Baroni *et al.*, 2012:258-259; Bolzinger *et al.*, 2012:156; Kolarsick *et al.*, 2011:203). The loss of water from the body to the atmosphere through the skin is called the transepidermal water loss (TEWL). To determine the type of damage caused physically, by chemicals or pathologically, the TEWL can be measured, which will increase proportional to the degree of damage (Baroni *et al*., 2012:259; Bolzinger *et al.*, 2012:159).

Due to the composition of the stratum corneum (mainly lipids) it is a hydrophobic layer with only 13% of water, meaning only hydrophobic compounds can penetrate the first layer of the skin. The layers (viable epidermis) underneath the stratum corneum are hydrophilic, containing 70% of water. These properties of the layers make penetration through the skin difficult, which leads to the conclusion that for a substance to penetrate all of the layers, it must be both hydrophilic and hydrophobic (Bolzinger *et al.*, 2012:156).

2.5.2.2 The stratum spinosum

The stratum spinosum, also called squamous cell layer, is located on top of the basal layer and underneath the stratum corneum layer. The cells, of which this layer is composed, have different shapes, subcellular properties and structures determined by their location. One of the cells found in this layer is lamellar granules, which are active in the interface, are also involved

in the deliverance of precursors from the stratum corneum to the lipids inside the intercellular space (Kolarsick *et al.*, 2011:204-205).

2.5.2.3 The stratum granulosum

This layer (granular layer) is composed of flattened cells containing a liberal amount of keratohyalin granules in their cytoplasm. The proteins involved in keratinisation are further synthesised and modified by these flattened cells. The keratohyalin granules found in the cytoplasm are basophilic, have an irregular shape and size and function by forming the interfibrillary matrix, holding the keratin filaments and the inner lining of the horny cells together (Kolarsick *et al.*, 2011:205).

2.5.2.4 The stratum germinativum

Another name for this layer is the basal layer, which consists of column-shaped keratinocytes attached to the dermis. The basal cells this layer consists of adhere to one another and superficial squamous cells and then form a single layer. The function of these basal cells is to undergo proliferation that renews the outer epidermis, but only 15% of these cells are involved in this process, the rest is in a quiescent state until drastic and fast proliferation is required. Melanocytes make up 5 - 10% of the cell population in the basal layer (Kolarsick *et al.*, 2011:204; Venus *et al.,* 2010:469).

These different epidermis layers are connected to one another, and one of the ways they are connected is via gap junctions. These gap junctions form an intercellular pore and are responsible for physiological communication using chemical signals. This communication is very important for cell metabolism, growth and differentiation during cell metabolism regulation (Kolarsick *et al.*, 2011:205).

2.5.3 The dermis

The second skin layer, just below the epidermis, is the dermis and these two layers are separated by the basement membrane. The dermis layer contains numerous cells, the most important being fibroblasts and macrophages. The functions of the fibroblasts are to synthesise and renew the extracellular matrix, while the macrophages help with the elimination of foreign material, as well as tissue portions that have been damaged (Baroni *et al.,* 2012:260). This second layer is compiled of collagen (70% of dry weight of skin) and elastic fibres functioning as connective tissues and providing elasticity, pliability and tensile strength. The principal component of the dermis is collagen, of which ± 15 genetically distinct types are found in the tendons, ligaments, dermis and the lining of bones (Kolarsick *et al.*, 2011:209). The dermis has a good network of lymphatic vessels, is highly vascularised and hosts sweat and sebaceous

glands along with hair follicles. This composition of the epidermis enables it to provide a mechanical, compact and supple support. The epidermis and dermis collaborate with one another during the wound healing process (Baroni *et al.*, 2012:206; Kolarsick *et al.*, 2011:209).

2.5.4 The hypodermis

The hypodermis, also called subcutaneous tissue/fat, is the innermost layer of the skin. This layer has three main functions: 1) protecting the body against mechanical shock, 2) insulating the body against externally applied heat or cold and 3) being active in energy metabolism and functioning as an energy storehouse (Baroni *et al*., 2012:260; Kolarsick *et al*., 2011:211).

2.6 Drug delivery

Conventional drug administration routes, such as oral or parenteral delivery, are complicated when optimum results are required. During oral administration, drugs undergo hepatic first-pass metabolism, i.e. the drug is absorbed via the gastric intestinal tract and then metabolised by the liver, resulting in a decrease in the bioavailability of the drug when reaching the site of action. During this first-pass metabolism the drug can be deactivated, resulting in the drug not being able to perform its function. Oral administration can be very difficult when a patient experiences diarrhoea, vomiting, or when he/she is unconscious. Oral administration also has a number of side effects, where in turn parenteral administration of drugs damages the skin and is less pleasant for the person receiving it (Gaikwad, 2013:1; Vandana *et al*., 2012:5).

During topical drug delivery, the drug is transported into the different skin layers i.e. stratum corneum, viable epidermis and dermis in order to obtain a local therapeutic effect (Vandana *et al*., 2012:5). In order to obtain local therapeutic effects, topical formulations are formulated in such a way that during the transportation to the skin layers, small lipophilic drugs stay inside the stratum corneum. These lipophilic drugs staying in the stratum corneum are constantly being released into the viable epidermis over an extended period of time (Sachan & Bajpai, 2013:761).

2.6.1 Advantages of topical drug delivery

Applying topical treatment to cutaneous diseases, will lead to less adverse effects in the rest of the body (Prausnitz *et al*., 2012:270). Should the topical drug cause negative side effects, it can easily be stopped by wiping the medication off from the skin. In this case the treatment of CTB, using topical treatment alongside oral treatment, will help the patient recover faster than when only using the oral treatment. This will also lead to less time experiencing the side effects of the orally used treatment.

2.6.2 Disadvantages of topical drug delivery

There are not a lot of disadvantages to using topical drug delivery systems for drug administration, but one major problem is the barrier of the skin, mainly the stratum corneum. The stratum corneum is hydrophobic, therefore only small lipophilic drugs can penetrate this layer, limiting the amount of drugs that can be administered. The rest of the skin's layers are hydrophilic, concluding that for any drug to pass through the stratum corneum into the dermis or epidermis it has to have both hydrophilic and lipophilic characteristics. There are very few, if any, drugs that contain both of these characteristics. The drug delivered must be potent, because only a small amount can be administered through the patches; in turn a too high dose drug will lead to skin irritations and sensitivity reactions (Barliya, 2013:1; Jepps *et al*., 2013:155-156; Vandana *et al.*, 2012:5).

2.6.3 Pathways for topical delivery

There are three pathway routes for transdermal permeation: the transcellular route and the paracellular route falls under the epidermal routes and the third route is the appendageal route. The two main routes are the appendageal and transcellular, with the less important route being the paracellular route. The optimum route for a specific drug will depend on the physicochemical properties of the drug and the nature of the formulation (Barliya, 2013:3; Bhowmick & Sengodan, 2013:636; Vandana *et al.*, 2012:5-6).

2.6.3.1 Transcellular route (Intracellular route)

Undergoing this route, the drugs pass the cytoplasm of the dead keratinocytes in the stratum corneum, then go across the phospholipid membranes (intercellular lipids) while crossing the skin. This is the shortest route, but not the easiest as the drugs passing through this route encounters permeation resistance from the lipophilic nature of each cell. The drugs must pass the lipophilic membrane, hydrophilic cellular contents and the phospholipid bilayer of each cell (Barliya, 2013:3; Prasanthi & Lakshmi, 2012:18). This route contains corneocytes which consist of highly hydrated keratin, leading to an aqueous environment. Due to the aqueous environment, the drug must be hydrophilic to travel this route through the corneocytes, as well as undergoing partition and diffusion. Very few drugs have the properties to cross the skin via this route (Barliya, 2013:3; Vandana *et al.*, 2012:5-6).

2.6.3.2 Paracellular route (Intercellular route)

This route is a continuous route where the drug diffuses through the lipid matrix. This route has two big disadvantages. The first obstacle is that this route's pathway is twisty due to the brick and mortar model of the stratum corneum, unlike the straight route of the transcellular pathway

(Prasanthi & Lakshmi, 2012:18; Vandana *et al*., 2012:6). The second obstacle is the composition of this route. This route consists of alternating structured bilayers making it only hydrophobic in nature. In order for a drug to permeate the skin it must partition into and diffuse through hydrophilic, as well as hydrophobic domains. The twisting route, however, reduces the permeation rate greatly (Barliya, 2013:3; Vandana *et al.*, 2012:6).

2.6.3.3 Appendageal route (follicular route)

This route consists of skin appendages that create a continuous channel across the stratum corneum barrier, which leads to direct contact with the dermal microcirculation. The appendages are hair follicles, hair shafts and sebaceous glands that create a pilosebaceous unit (Choi & Maibach, 2005:210; Vandana *et al*., 2012:5). This sebaceous gland cells are more permeable than the corneocytes, which the transcellular and intercellular routes consists of (Choi & Maibach, 2005:210). The disadvantage of this route is that there is only a restrictive amount of these skin appendages (1/1000 of skin surface area) leading to a limited amount of drug penetrating the skin. This route is mostly used by vesicular systems (Barliya, 2013:3; Choi & Maibach, 2005:210; Vandana *et al.*, 2012:5).

2.6.4 Physicochemical factors influencing topical drug delivery

2.6.4.1 Partition coefficient

The partition coefficient is determined as the octanol-water (log P) distribution, where the distribution coefficient is determined as the octanol-buffer (log D) distribution. In order for a drug to be able to penetrate the stratum corneum, which is the hydrophobic outer layer of the skin, the drug must be soluble in lipids (Bolzinger *et al.*, 2012:156). During the partition and distribution coefficient techniques, the partition of the compound in the two-phases will determine the affinity the compound has for the aqueous media. Therefore, the lipophilicity of a drug can be determined via these techniques (Gulyaeva *et al*., 2003:391). The ratio of the partition is expressed as the logarithm of the constant P and the ratio of the distribution as the logarithm of the constant D. The most common non-aqueous solvent used for these measurements is *n*-octanol, due to its many similarities to a biological membrane (Aulton & Taylor, 2013:323). For ideal penetration of the drug through the stratum corneum it must obtain a log P value between 1 and 4 (Mbah *et al*., 2011:68; Williams, 2013:680).

Artemether has a log P value of 3.48 (DrugBank, 2013:5), which falls in the range needed for optimum absorption and solubility, therefore, theoretically it will give good results.

2.6.4.2 Molecular size and shape

Due to the skin acting as a barrier against the penetration of harmful external chemicals, large molecules cannot enter the skin (Williams, 2013:680). When the drug follows the paracellular route it can have a molecular weight of up to 400 Da, but for optimum permeation it must be less than 200 Da. The shape of the molecule also plays an important role in the paracellular permeation. In order to obtain absorption via the transcellular route, the molecule must have a molecular weight of less than 500 Da, though there are a few molecules with a molecular weight larger than 700 Da which get absorbed (Ashford, 2013:324). Overall, for effective drug delivery the molecular weight of the molecule must be less than 1000 Da; with larger molecules the drug dosage must be very low (Gaikwad, 2013:5; Williams, 2013:680).

For a drug to qualify using the paracellular (less than 400 Da) or transcellular (less than 500 Da) drug delivery routes it must have a molecular size of less than 400 –and 500 Da respectively. The molecular size of artemether is 298.3746 g/mol, which falls within this range (DrugBank, 2013:2; Human Metabolome Database, 2013:1).

2.6.4.3 pH, pKa and ionisation

Many of the drugs used for topical drug delivery are weak bases or acids. Weak bases and acids have ionisation/dissociation constants (K_a), of which the negative logarithm is the pK_a. When the dielectric constant of a molecule is strong enough to separate the attractive forces between two oppositely charged ions, the molecule dissociates into ions. These ions are called electrolytes and their dissociation or ionisation leads to a specific K_a value for each molecule. In turn, the pH has an effect on the degree of ionisation of the drug (Aulton, 2013:41; Martin & Lansley, 2013:681).

The pH generally indicates the stability of the drug, but a pH that favours unionised molecules will increase the absorption of the drug. The pH of the place of application, in this case the skin, must be taken into consideration (Martin and Lansley, 2013:681). The pH of the skin is between 4.2 and 5.6, thus solutions with a pH between this range need to be used in order to minimise damage to the skin (Gaikwad, 2013:5). The relationship between the pH, pK_a and solubility of the drug is determined by the Henderson-Hasselbalch equation (York, 2013:32). The pH, as well as the pK_a, of the drug determines its degree of absorption and solubility (Martin & Lansley, 2013:681;York, 2013:14).

The pK_a of the strongest basic of artemether is between - 3.9 and - 4.1 and the pK_a of the strongest acidic is 12.11 (DrugBank, 2013; Human Metabolome Database, 2013:2).

2.6.4.4 Drug concentration

The daily dose for effective drug permeation is derived from the knowledge that a good permeant has a flux of 1 $mg.cm^{-2}.day^{-1}$, in collaboration with a patch size of 10 cm^2 leading to the concluding dose of 10 mg/day (Williams, 2013:680).

The concentration of a drug in a solution must be below the saturation solubility of the drug in order to avoid drug precipitation out of the solvent, which can be caused by temperature fluctuations during storage (Murdan, 2013:396).

During this study, 3% (3 g/100 ml) artemether will be formulated into niosomes and proniosomes.

2.6.4.5 Hydrogen bonding

Permeation through the skin is influenced by the number of hydrogen bonds within the molecule. An increase in hydrogen bonds will increase the degree of damage to the drug absorption (Aulton & Taylor, 2013:324). For optimum absorption results, the molecule must contain no more than five hydrogen bond donors and 10 hydrogen bond acceptors (Ashford, 2013:324). The hydrogen bonding potential can also control the extent to which the molecule binds to skin constituents during transport, which in turn will influence the bioavailability (Williams, 2013:680).

Artemether contains five hydrogen bond acceptors and no hydrogen bond donors, therefore, it falls in the range for optimum absorption results (Human Metabolome database, 2013:3).

2.6.4.6 Drug solubility and solubility parameter

The solubility of a drug means the extent of the transfer of a molecule/ion (for the drug) from a solid-state into a solution-state under as specific set of experimental conditions (Aulton, 2002:16). Over-solubilisation of the drug would lead to a decline in the penetration of the drug through the skin. When the melting point of a drug is low, it will have a high solubility in the stratum corneum, which will cause increased penetration through the skin, therefore the melting point and solubility of a drug is inversely proportional (N'Da, 2014:20788).

For optimal results regarding the transdermal delivery of a drug, the aqueous solubility of the drug must be larger than 1 mg/ml (Naik *et al*., 2000:319). Artemether has an aqueous solubility of 0.457 g/L (Human Metabolome database, 2013:2), which states it is not very soluble in an aqueous medium or water. When the aqueous solubility of artemether is compared to the standard value needed for optimal results, the conclusion can be drawn that penetration of artemether through the stratum corneum will be difficult.

2.6.4.7 Melting point

A low drug melting point will give optimal results, where a higher melting point will cause decreased chemical stability (Gaisford, 2013:382). Artemether has a melting point of 86 - 90 °C (DrugBank, 2013), which is quite high and shows the inversely proportional relation of the melting point to the solubility of artemether.

2.7 Vesicle systems

2.7.1 Niosomes

Vesicular systems are used as colloidal carriers in drug delivery systems. Niosomes are a type of vesicle system, used during drug delivery, initiated by the cosmetic industry in the 1970s. Niosomes are lipid based carriers composed of hydrated, non-ionic amphiphiles (surfactants), i.e. sorbitan fatty acid esters, polysorbates and polyethoxy fatty esters (Jadon *et al*., 2009:1186; Kazi *et al*., 2010:374; Moghassemi & Hadjizadeh, 2014:23; Prasanthi & Lakshmi, 2012:19; Yeom *et al*., 2014:83). The non-ionic surfactants have no charge and are relatively nontoxic. These vesicles are uni- or multilamellar spheroidal structures, which are formed when the non-ionic surfactants assemble in aqueous media forming closed bilayer structures (Bayindir & Yuksel, 2010:2050; Rahimpour & Hamishehkar, 2012:143; Tavano *et al*., 2014:7).

Niosomes can encapsulate lipophilic as well as hydrophilic molecules, which is made possible by the unique structure composition of the niosome containing hydrophilic and lipophilic moieties. During the encapsulation, the hydrophilic molecules either get entrapped in the aqueous core of the vesicle or get adsorbed in the bilayer surfaces, while the lipophilic molecules partition into the lipophilic domain of the bilayers (Moghassemi & Hadjizadeh, 2014:23).

The absorption of drug molecules across the stratum corneum, the rate limiting step, is a passive process. The physicochemical properties, i.e. partition and diffusion coefficient of the drug, plays an important role in the capability of the drug to penetrate through the stratum corneum and diffuse across the membrane (Rahimpour & Hamishehkar, 2012:141). The physicochemical properties of the vesicle provide the optimum penetration where the drugs cannot always change their properties. The physiological properties of the niosomes also play a big role in their effectiveness as colloidal carriers, i.e. their composition, size, charge, lamellarity and their application conditions (Choi & Maibach, 2005:210). The above mentioned properties which niosomes possess, enhances the penetration of drugs through the stratum corneum, in turn solving the biggest problem regarding the topical delivery of drugs (Mali, 2013:587).

2.7.2 Proniosomes

Proniosomes are solid colloidal carrier particles coated with surfactants. This vesicle system is a free-flowing, granular product that disperses, forming a multilamellar niosome suspension once it comes into contact with water (Hu & Rhodes, 2000:111; Kazi *et al*., 2010:374-375). Proniosomes are an improvement on the stability of niosomes in terms of aggregation, fusion and leaking, but they are also an improvement on the transportation, distribution, measuring, storage and dosing of niosomes (Hu & Rhodes, 2000:111; Malakar *et al*., 2011:5120).

2.7.3 Advantages of vesicle systems

A vesicular system has numerous advantages over a conventional dosage form and micelles, i.e. vesicle systems enhance:

- Skin permeation.
- Bioavailability.
- The delivery through the outer layer barrier of the skin, namely the stratum corneum, which is particularly impermeable.
- The time the drug is occupied in the skin.
- The penetration of the trapped substances across the skin.

Vesicle systems reduce:

- The degree of drug degradation.
- The loss of drug.
- The systemic absorption of the drug.
- The harmful side effects (Mali *et al.,* 2013:587; Tavano *et al*., 2014:7).

Liposomes were the first lipid based vesicular systems used; niosomes are the improvement on the disadvantages of liposomes. Niosomes are preferred above other vesicular systems because they have better chemical stability, lower costs, are biodegradable, biocompatible, non-immunogenic, have larger membrane flexibility, better drug entrapment efficiency, low toxicity (depending on the surfactants structure) and are an improvement on variable impurity of phospholipids (liposomes) (Jadon *et al*., 2009:1186, Tavano *et al.,* 2014:7; Yeom *et al*., 2014:83).

2.7.4 Disadvantages of vesicle systems

Niosomes share a few disadvantages with liposomes, i.e. 1) long storage of aqueous dispersed

niosomes leads to degradation and hydrolysis and 2) sedimentation, aggregation, fusion and drug leakage occurs often (Moghassemi & Hadjizadeh, 2014:28; Yeom *et al*., 2014:83).

2.7.5 Drug solubility in vesicle systems

The solubility of the drug encapsulated in the vesicle will influence the absorption of the drug, thus the bioavailability. To obtain optimum bioavailability, the solubility of the drug inside the vesicle and the movement of the drug from the vesicle to the stratum corneum must be maximised. The partition coefficient (K_m) describes the ability/willingness of the drug to leave the vesicle and partition into the stratum corneum. This partition coefficient is determined by the solubility of the drug inside the vesicle (C_v) versus the solubility of the drug inside the stratum corneum (C_{sc}) using Equation 2.1 (Prausnitz *et al*., 2012:2069).

$$K_m = C_{sc}/C_v \qquad \textbf{Equation 2.1}$$

2.7.6 Effectiveness of using niosomes in topical drug delivery systems

During topical application, the vesicle adsorbs and fuses to the surface of the skin causing a high concentration gradient of the drug at the interface of the skin and niosome. This high concentration gradient is the driving force needed for the permeation of the lipophilic drug (Mali *et al.,* 2012:587). The rate of drug release, when using niosomes, can be controlled by changing the composition of the niosome. The vesicles can change the stratum corneum structure, which will lead to the intercellular lipid barrier of the stratum corneum being more permeable and loose. The non-ionic surfactants, which are the main materials used in niosome preparations, act as a permeation enhancer which can help with the permeation of the drug (Rahimpour & Hamishehkar, 2012:141&146).

2.8 Conclusion

The infectious disease TB, caused by the bacterium *M. Tuberculosis,* poses a great health risk for the entire world due to the increasing infection numbers and resistance of the TB bacterium against treatment. This fact increases the percentage of CTB infected patients.

A combination therapy of artemisinin (and its derivatives) is suggested for the treatment of TB in humans. Topical application of artemether for CTB should help shorten the length of treatment needed for CTB. As topical delivery is for local effects, the CTB can be treated locally and this will cause a quicker cure/relief of the unwanted skin appearances caused by CTB.

The human skin functions as a barrier to protect the body from the penetration of any foreign external agents. The hydrophobicity of the stratum corneum, along with the other barrier characteristics of the skin, will make the topical delivery of lipophilic artemether very difficult.

References

Agubata, C.O. & Onunkwo, G.C. 2012. Some physical characteristics of artemether and piroxicam solid lipid microparticles prepared with dika fat. *Journal of Chemical and Pharmaceutical Research*, 4(6):2955-2958.

Almaguer-Chávez, J., Ocampo-Candiani, J. & Rendón, A. 2009. Current panorama in the diagnosis of cutaneous tuberculosis. *Actas Dermo-Sifiliográficas*, 100(7):562-570.

Ansari, M.T., Karim, S., Ranjha, N.M., Shah, N.H. & Muhammed, S. 2010. Physicochemical characterization of artemether solid dispersions with hydrophilic carriers by freeze dried and melt methods. *Archives of Pharmacal Research*, 33(6):901-910.

Ansari, M.T., Hussain, A., Nadeem, A., Majeed, H., Saeed-Ul-Hassan, S., Tariq, I., Mahmood, Q., Khan, A.K. & Murtaza, G. 2014. Preparation and characterization of solid dispersions of artemether by freeze-dried method. *BioMed Research International*, 2015:11.

Ashford, M. 2013. Bioavailability-physicochemical and dosage form factors. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London, Elsevier. p. 314-333).

Assefa, A., Kassa, M., Tadese, G., Mohammed, H., Animut, A. & Mengesha, T. 2010. Therapeutic efficacy of artemether/lumefantrine (Coartem®) against *Plasmodium falciparum* in Kersa, South West Ethiopia. *Parasites and Vectors*, 3(1):9.

Aulton, M.E. & Taylor, K.M.G. 2013. Aulton's pharmaceutics: the design and manufacture of medicines. 4th ed. London, UK: Churchill Livingston Elsevier Ltd.

Barliya, T. 2013. Introduction to transdermal drug delivery (TDD) system and nanotechnology. http://pharmaceuticalintelligence.com/2013/01/28/introduction-to-transdermal-delivery-tdd-system-and-nanotechnology/. Pdf Date of access: 4 Feb. 2016.

Baroni, A., Buommino, E., De Gregorio, V., Ruocco, E., Ruocco, V. & Wolf, R. 2012. Structure and function of the epidermis related to barrier properties. *Clinics in Dermatology*, 30(3):257-262.

Bayinder, Z.S. &Yuksel, A. 2009. Characterization of niosomes prepared with various non-ionic surfactants for paclitaxel oral delivery. *Journal of Pharmaceutical Sciences*, 99(4):2049-2060.

Bhowmick, M. & Sengodan, T. 2013. Mechanism, kinetics and mathematical modelling of transdermal permeation - an updated preview. *International Journal of Research and Development in Pharmacy and Life Sciences*, 2(6):636-641.

Bolzinger, M., Briançon, S., Pelletier, J. & Chevalier, Y. 2012. Penetration of drugs through skin, a complex rate-controlling membrane. *Current Opinion in Colloid & Interface Science*, 17(3):156-165.

Brennan, P.J. & Young, D.B. 2008. Handbook of anti-tuberculosis agents. 2nd ed. (vol 88). NY:Elsevier.

Cai, B., Söderkvist, K., Engqvist, H. & Bredenberg, S. 2012. A new drug release method in early development of transdermal drug delivery systems. *Pain Research and Treatment*, 2012:1-6.

Choi, M.J. & Maibach, H.I. 2005. Liposomes and niosomes as topical drug delivery system. *Skin Pharmacology and Physiology*, 18(5):209-219.

Dos Santos, J.B., De Oliveira, M.H., Figueiredo, A.R., Da Silva, P.G., Ferraz, C.E. & De Medeiros, V.L.S. 2014. Cutaneous tuberculosis: epidemiologic , etiopathogenic and clinical aspects – Part 1. *Anais Brasileiros de Dermatologia*, 89(2):219-228.

DrugBank. 2013. Artemether (DB06697). http://www.drugbank.ca/drugs/DB06697 Date of access: 8 Jul. 2015.

Feingold, K.R. & Denda, M. 2012. Regulation of permeability barrier homeostasis. *Clinics in Dermatology*, 30(3):263-268.

Foldvari, M. 2000. Non-invasive administration of drugs through the skin: challenges in delivery system design. *Pharmaceutical science & technology today*, 3(12):417-425.

Fore-Pfliger, J. 2004. The epidermal skin barrier: implications for the wound care practitional part I. *Advances in skin & wound care*, 17(8):417-425.

Gaikwad, A.K. 2013. Transdermal drug delivery system: formulation aspects and evaluation. *Comprehensive Journal of Pharmaceutical Sciences*, 14(1):1-10.

Gaisford, S. 2013. Pharmaceutical preformulation. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 366-394).

Gao, H., Chen, W. & Bao, S. 2013. Thermal stability evaluation of β-artemether by DSC and ARC. *Thermochimica Acta*, 569:134-138.

Gulyaeva, N., Zaslavsky, A., Lechner, P., Chlenov, M., McConnell, O., Chait, A., Kipnis, V. & Zaslavsky, B. 2003. Relative hydrophobicity and lipophilicity of drugs measured by aqueous two-phase partitioning, octanol-buffer partitioning and HPLC. A simple model for predicting blood-brain distribution. *European Journal of Medical Chemistry*, 38(4):391-396.

Hafeez, A., Jain, U., Singh, J., Maurya, A. & Rana, L. 2013. Recent advances in transdermal drug delivery system (TDDS): an overview. *Journal of Scientific and Innovative Research*, 2(3):695-709.

Haynes, R. 2013. SA MRC flagship proposal executive summary October 2013. [Correspondence]. 29 Jul. 2015, Potchefstroom.

Haynes, R.K. 2015. Development of oxidant and redox drug combinations for treatment of malaria, TB and related diseases. [PowerPoint presentation].

Hershkovitz, I., Donoghue, H.D., Minnikin, D.E., Besra, G.S., Lee, O.Y-C., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Bar-Gal, G.K. & Spigelman, M. 2008. Detection and Molecular Characterization of 9000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the eastern Mediterranean. *Public Library of Science ONE*, 3(10):1-6.

Hu, C. & Rhodes, D.G. 2000. Proniosomes: a novel drug carrier preparation. *International Journal of Pharmaceutics*, 206(1-2):110-122.

Human metabolome database. 2013. Showing metabocard for artemether (HMDB15643). http://www.hmdb.ca/metabolites/HMDB15643 Date of access: 23 Feb. 2016.

Jadon, P.S., Gajbhiye, V., Jadon, R.S., Gajbhiye, K.R. & Ganesh, N. 2009. Enhanced oral bioavailability of Griseofulvin via niosomes. *American Association of Pharmaceutical Scientists*, 10(4):1186-1192.

Jepps, O.G., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. 2013. Modeling the human skin barrier – towards a better understanding of dermal absorption. *Advanced Drug Delivery Reviews*, 65(2):152-168.

Kazi, K.M., Mandal, A.S., Biswas, N., Guha, A., Chatterjee, S., Behera, M. & Kuotsu, K. 2010. Niosome: a future of targeted drug delivery systems. *Journal of Advanced Pharmaceutical Technology & Research*, 1(4):374-380.

Kolarsick, P.A.J., Kolarsick. M.A. & Goodwin, C. 2011. Anatomy and physiology of the skin (*In* Journal of the Dermatology nurses' association 3(4):203-213).

Lamorde, M., Byakika-Kibwika, P., Mayito, J., Nabukeera, L., Ryan, M., Hanpithakpong, W., Lefèvre, G., Back, D.J., Khoo, S.H. & Merry, C. 2012. Lower artemether, dihydroartemisinin and lumefantrine concentrations during rifampicin-based tuberculosis treatment. *Official Journal of the international AIDS society*, 27(6):961-965.

Malakar, J., Datta, P.K., Dey, S., Gangopadhyay, A. & Nayak, A.K. 2011. Proniosomes: a preferable carrier for drug delivery system. *Pharmacy*, 40:5120-5124.

Mali, N., Darandale, S. & Vavia, P. 2013. Niosomes as a vesicular carrier for topical administration of minoxidil: formulation and in vitro assessment. *Drug delivery and Translational Research*, 3(6):587-592.

Martin, G.P. & Lansley, A.B. 2013. Nasal drug delivery. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 657-674).

Martini, F.H., Nath, J.L. & Bartholomew, E.F. 2012. 9th ed. Anatomy and Physiology. Sansome St, SF: Pearson Education Inc.

Mbah, C.J., Uzor, P.F. & Omeje, E.O. 2011. Perspectives on transdermal drug delivery. *Journal of Chemical and Pharmaceutical Research*, 3(3):680-700.

Menon, G.K., Cleary, G.W. & Lane, M.E. 2012. The structure and function of the stratum corneum. *International Journal of Pharmaceutics*, 435(1):3-9.

Mikesh, L.M., Aramadhaka, L.R., Moskaluk, C., Zigrino, P., Mauch, C. & Fox, J.W. 2013. Proteomic anatomy of human skin. *Journal of Proteomics*, 84:190-200.

Moghassemi, S. & Hadjizadeh, A. 2014. Nano-niosomes as nanoscale drug delivery systems: an illustrated view. *Journal of Controlled Release*, 185:22-36.

Murdan, S. 2013. Solutions. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 395-405).

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical Science & Technology today*, 3(9):319.

N'Da, D.D. 2014. Prodrug strategies for enhancing the percutaneous absorption of drugs. *Molecules*, 19(12):20780-20807.

Pawar, J.N., Shete, R.T., Gangurde, A.B., Moravkar, K.K., Javeer, S.D., Jaiswar, D.R. & Amin, P.D.2015?. Development of amorphous dispersions of artemether with hydrophilic polymers via spray drying: physicochemical and in silico studies. *Asian Journal of Pharmaceutical Sciences* (In Press).

Perrin, P. 2015. Human and tuberculosis co-evolution: an integrative view. *Tuberculosis*, 95(1):S112-S116.

Prasanthi, D. & Lakshmi, P.K. 2012. Vesicles-mechanism of transdermal permeation: a review. *Asian Journal of Pharmaceutical and Clinical Research*, 5(1):18-25.

Prausnitz, M.R., Elias, P.M., Franz, T.J., Schmuth, M., Tsai, J.C., Menon, G.K., Holleran, W.M.H. & Feingold, K.R. 2012. Skin barrier and transdermal drug delivery. http://drugdelivery.chbe.gatech.edu/Papers/2012/Prausnitz%20Derm%20Book%20Chapter%202012.pdf Date of access: 4 Apr. 2016.

Rahimpour, Y. & Hamishehkar, H. 2012. Niosomes as carrier in dermal drug delivery. (*In* Sezer, A.D., *ed*. Recent advances in novel drug carrier systems. Rijeka: InTech. p. 141-164).

Rivero-Lezcano, O.M. 2013. *In vitro* infection of human cells with *Mycobacterium tuberculosis*. *Tuberculosis*, 93(2):123-129.

Sachan, R. & Bajpai, M. 2013. Transdermal drug delivery system: a review. *International Journal of Research and Development in Pharmacy and Life Sciences*, 3(1):748-765.

Schito, M. & Dolinger, D.L. 2015. A collaborative approach for "ReSeq-ing" *Mycobacterium tuberculosis* drug resistance: Converge for drug and diagnostic developers. *EBioMedicine Journal*, 2(10):1262-1265.

Sigma-Aldrich. 2013. Safety data sheet: Artemether. http://www.sigmaaldrich.com/MSDS/MSDS/DisplayMSDSPage.do?country=ZA&language=en&productNumber=A9361&brand=SIGMA&PageToGoToURL=http%3A%2F%2Fwww.sigmaaldrich.com%2Fcatalog%2Fproduct%2Fsigma%2Fa9361%3Flang%3Den. pdf Date of access: 5 Nov. 2015.

Silamut, K., Newton, P.N., Teja-Isavadharm, P., Suputtamongkol, Y., Siriyanonda, D., Rasameesoraj, M., Pukrittayakamee, S. & White, N.J. 2003. Artemether bioavailability after oral or intramuscular administration in uncomplicated falciparum malaria. *Antimicrobial agents and chemotherapy*, 47(12):3795-3798.

Tavano, L., Aiello, R., Ioele, G., Picci, N. & Muzzalupo, R. 2014. Niosomes from glucuronic acid-based surfactant as new carriers for cancer therapy: Preparation, characterization and biological. *Colloids and Surfaces B: Biointerfaces*, 118:7-13.

Van Zyl, L., du Plessis, J. & Viljoen, J. 2015. Cutaneous tuberculosis overview and current treatment regimens. *Tuberculosis*, 95(6):629-638.

Vandana, Y, Sipai, A.B.M., Mamatha, Y. & Prasanth, V.V. 2012. Transdermal drug delivery: a technical write up. *Journal of Pharmaceutical and Scientific innovation*, 1(1):5-12.

Venus, M., Waterman, J. & McNab, I. 2010. Basic physiology of the skin. *Surgery*, 29(10):471-474.

Wang, Z., Chen, J., Li, L., Zhou, Z., Geng, Y. & Sun, T. 2015. Detailed structural study of β-artemether: density functional theory (DFT) calculations of Infrared, Raman spectroscopy, and vibrational circular dichroism. *Journal of Molecular Structure*, 1097:61-68.

Wickett, R.R. &Visscher, M.O. 2006. Structure and function of the epidermal barrier. *American Journal of Infection Control*, 34(10):S98-S110.

WHO, see World Health Organization

World Health Organization. 1995. WHO model prescribing information: drugs used in parasitic diseases. http://apps.who.int/medicinedocs/pdf/h2922e/h2922e.pdf Date of access: 11 Jul. 2015.

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 675-697).

Yeom, S., Shin, B.S. & Han, S. 2014. An electron spin-resonance study of non-ionic surfactant vesicles (niosomes). *Chemistry and Physics of Lipids*, 181:83-89.

York, P. 2013. Design of dosage forms. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 7-19).

CHAPTER 3

ARTICLE FOR PUBLICATION IN INTERNATIONAL JOURNAL OF PHARMACEUTICS

Chapter 3 is written in article format for publication in International Journal of Pharmaceutics. The complete author's guide is given in Appendix E. This chapter has been justified to improve reading.

Artemether loaded niosome and proniosome vesicles for topical delivery: Design, characterisation and skin diffusion

Chantell van der Merwe, Minja Gerber, Jan du Preez and Jeanetta du Plessis*

Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001, Potchefstroom, 2520, South Africa

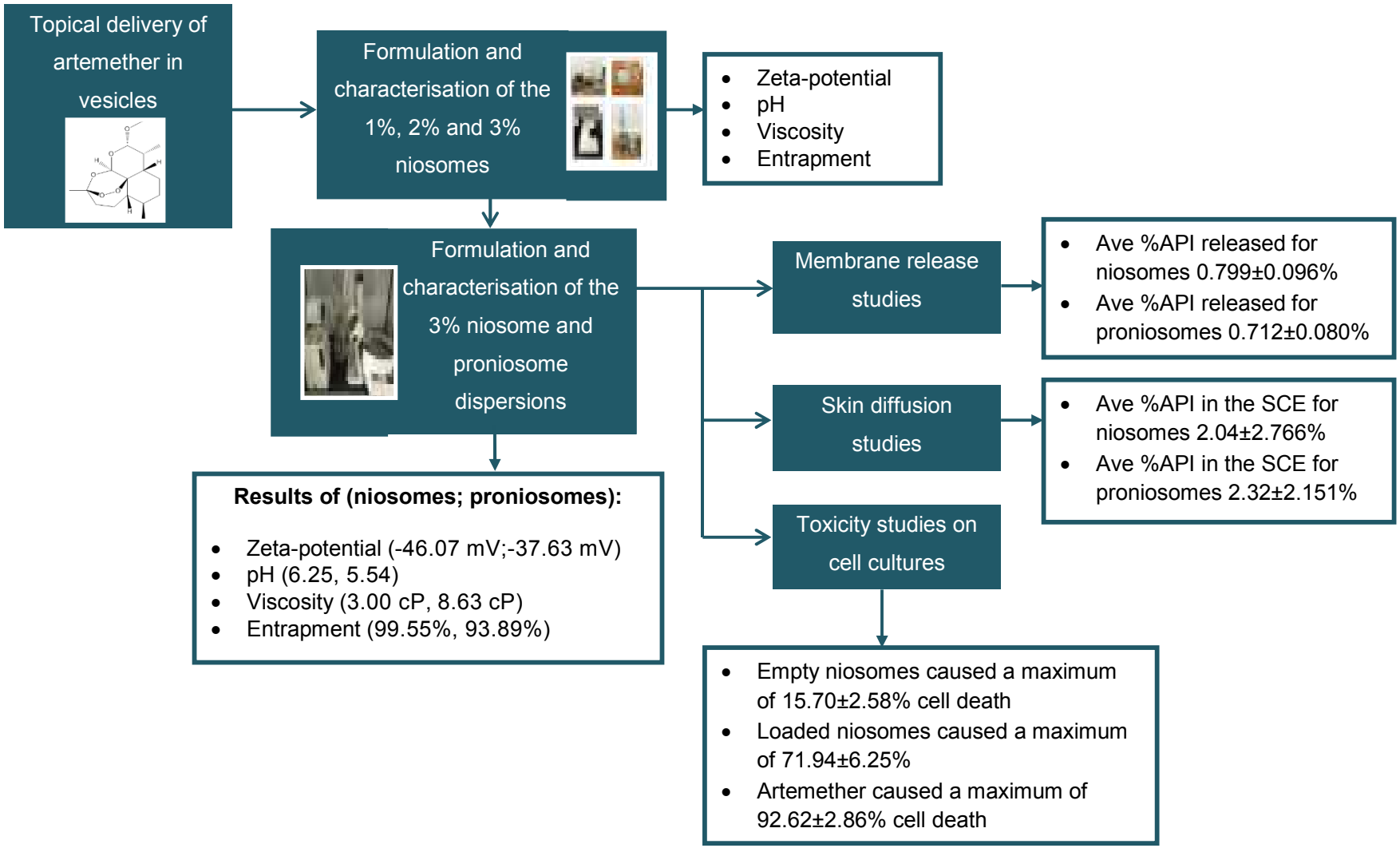
*Corresponding author. Tel: +2718 299 2274; Fax: +2787 231 5432; Email address: Jeneatta.DuPlessis@nwu.ac.za (J Du Plessis)

Abstract

A study for the topical drug delivery of artemether, which would serve as treatment of cutaneous tuberculosis (CTB), was performed as part of the South African Medical Research Council Flagship Project, MALTB-Redox. Topical delivery of therapeutic drugs is a common delivery route, but the skin's barrier creates a challenge for this delivery route. The aim of this study was to formulate artemether entrapped in niosome and proniosome vesicle systems, respectively, and to determine the membrane and skin diffusion of these dispersions. The characterisation of the 3% niosome and proniosome dispersions indicated good physicochemical properties regarding topical drug delivery and therefore, they were chosen as the final dispersions for further analysis. Membrane release studies were executed, which indicated high release of artemether. No artemether could be quantified in the stratum corneum-epidermis after skin diffusion studies. Despite the fact the artemether concentrations acquired in the epidermis-dermis were not quantifiable, targeted delivery did occur. The toxicity of the active artemether and the vesicles systems were determined on HaCaT (immortalised human keratinocytes) cell cultures using an LDH (lactate dehydrogenase) assay; the results indicated only artemether was cytotoxic to cell cultures from a concentration of 150 µg/ml and higher.

Keywords: Artemether, Diffusion, Topical drug delivery, Niosome, Proniosome, Cytotoxicity

Graphical abstract



1 INTRODUCTION

The human skin functions as a delivery route for pharmaceutical drugs to acquire a local effect via topical application. The main obstacle during topical drug delivery is the barrier properties that reside in the different skin layers, mainly the stratum corneum (Bolzinger et al., 2012; Dwivedi et al., 2016; Gillet et al., 2011). The skin mainly consists of three layers, from the outer layer inwards-the epidermis, the dermis and the hypodermis. The stratum corneum, outer layer of the epidermis, is lipophilic and only contains 13% of water, whereas the dermis skin layer is hydrophilic in nature (Bolzinger et al., 2012). Dermal absorption is the movement of compounds across the different skin layers. These movements are divided into two steps, i.e. 1) the penetration of a compound into a specific skin layer and 2) the permeation of the compounds from one layer into the next (Bolzinger et al., 2012).

The aforementioned characteristics of each skin layer cause difficulty regarding the penetration of drugs through the skin. A hydrophilic drug cannot enter the stratum corneum, due to its lipophilic qualities; a lipophilic drug can penetrate into the stratum corneum where it will reside because it cannot permeate through to the hydrophilic dermis (Bolzinger et al., 2012; Williams, 2013).

In this study, artemether was selected as the active ingredient to deliver topically due to the advantages over oral treatment. Artemether is the active ingredient in the herb *Artemisia annua,* which was discovered in 1971 by Chinese scientists; it is also a derivative of the artemisinin family. Artemether has an aqueous solubility of 0.457 mg/ml, which does not fit the criteria for optimum transdermal drug delivery (>1 mg/ml), and would most likely result in being able to penetrate into the stratum corneum, but not diffuse through into the epidermis-dermis (Ansari et al., 2010; 2015; Bolzinger et al., 2012; Human Metabolome Database, 2013:2; Naik et al., 2000; White et al., 2008).

The skin barrier restrictions can be overcome by formulating low aqueous soluble drugs in lipid-based formulations for topical drug delivery. These lipid-based formulations (nano-vesicles) act as drug carriers by encapsulating the drugs and carrying it through the skin

layers. The nano-vesicles possess certain physical and chemical properties empowering them to be optimal for topical drug delivery (Dwivedi et al., 2016; Gautschi et al., 2016).

The success of skin diffusion, especially due to the skin barrier properties, depends mainly on the physicochemical properties of the pharmaceutical drug (Foldvari, 2000). By encapsulating artemether in niosome and proniosome vesicles, the penetration of artemether through the skin should be enhanced regarding skin diffusion studies. Niosome and proniosome vesicles can encapsulate both hydrophilic and lipophilic drugs (Moghassemi & Hadjizadeh, 2014).

Tuberculosis (TB) is an infectious disease with an increased contribution to the yearly total of deaths, with approximately 20 to 30% of the world's population already being infected. Cutaneous tuberculosis (CTB) is a disease resulting in skin manifestations, mainly caused by the bacterium *Mycobacterium tuberculosis,* whose occurrence increases with the increase in TB treatment resistance. In order for a human to have CTB, they must already be infected with TB (Perrin, 2015; Van Zyl et al., 2015:629).

Due to the increasing resistance of *M. tuberculosis* against TB treatments, the amount of treatment options has been reduced. The current TB treatments cause a great deal of discomfort to the patients using them, therefore recently, combination therapy has been researched as means of TB treatment (Haynes, 2013:1-2; Haynes, 2015:3). The artemisinin family has the characteristics required for TB treatment and would serve as one component (oxidant drug) during combination therapy. The topical delivery of artemether against CTB, for an overall decrease in CTB treatment period, is being evaluated.

In the present study, the skin diffusion of artemether encapsulated in niosome and proniosome carrier vesicles was investigated. The aim of this study was to formulate artemether encapsulated in niosome and proniosome vesicles and to characterise them. The membrane release and skin diffusion studies of these dispersions would then be determined, as well as the cytotoxicity of artemether and the niosome vesicles on cultured human keratinocyte (HaCaT) cell cultures using an LDH (lactate dehydrogenase) assay.

2 MATERIALS AND METHODS

2.1 Materials

Artemether was purchased from DB Fine Chemicals (Woodmead, RSA). Chloroform, D-sorbitol and LiChrosolv® acetonitrile were purchased from Merck Millipore (Darmstadt, Germany). Cholesterol was purchased from Sigma-Aldrich (Steinheim, Japan) and Span® 60 from Fluka (Steinheim, Switzerland). Phosphate buffer solution (PBS) reagents, namely uniLAB® sodium hydroxide and univAR® potassium dihydrogen orthophosphate, were purchased from Merck Chemicals (Wadeville, RSA). When Milli-Q® water was required, a Milli-Q® Academic water purification system, which is HPLC grade, was used (Merck-Millipore, Midrand, RSA) throughout the whole study. The HaCaT cell line was a gift from the University of the Witwatersrand (WITS). The cell culture growth medium, which included non-Essential Amino Acids, Dulbecco's Modified Eagle Medium (DMEM), L-Glutamine, phosphate buffered saline (1x) and Foetal Bovine Serum, was purchased from HyClone™ (GE Healthcare Life Sciences, South Logan, Utah). The CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit was purchased from Promega™ (Madison, Wisconsin). The consumables used during the cytotoxicity analysis were purchased from Lonza™ (Basel, Switzerland), which included Penicillin/Streptomycin (Pen/Strep) and Trypsin-Versene® (EDTA), and Trypan Blue Solution from Sigma-Aldrich® (St. Louis, Missouri).

2.2 Methods

2.2.1 HPLC validation

The HPLC method for the determination of artemether was validated. The analytical instrument used during this analysis was the Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA). The column used was Venusil XBP C_{18} (2), reverse phase column, 150 mm in length and 4.6 mm in diameter, with a particle size of 5 µm (Agela Technologies, Newark, DE). The injection volume for artemether was determined as 50 µl, the running time set at 15 min per sample and the retention time was determined between

8.7 and 8.8 min. The solvent and mobile phases were prepared in the same manner, namely acetonitrile/water (ACN/H_2O) in a 70:30 ratio, which were filtered or sonicated.

2.2.2 Standard preparation

The stock solution for a standard curve was used during analysis with the HPLC. Artemether was weighed according to specifications needed in the ACN/water solvent, while making it up to volume in a 25 ml volumetric flask. The preparation of the standard solution alternated between two different methods. The first method was by means of different injection volumes, where the stock solution was injected on the HPLC with the injection volumes of 5.0, 7.5, 10.0, 25.0 and 50.0 µl. The second method was by diluting 10 ml of the stock solution with the ACN/water solvent to 20 ml; this was repeated five times and 50 µl was injected on the HPLC.

2.2.3 Niosome vesicle preparations

The niosome vesicles were prepared using the thin-film hydration (THF) method, according to the method of Moghassemi and Hajizadeh (2014). The surfactant, cholesterol and API (active pharmaceutical ingredient) were dissolved in chloroform (Table 1) then evaporated using a rotary evaporator. A thin-film formed on the side of the round-bottomed flask. The film was hydrated using preheated Milli-Q® water, then sonicated on ice for 2.5 min. The formulation was left to stand at room temperature for at least 2 h to let the niosome vesicles swell and ensure equilibrium.

2.2.4 Proniosome vesicle preparations

The proniosome vesicles were prepared using the slow spray coating method (Udasi et al., 2012). The Span® 60, API and cholesterol were mixed together and then dissolved in chloroform (Table 1). The sorbitol carrier placed inside the round bottomed flask was wetted with the organic mixture using a dropping method. Once all the organic solvent was sprayed onto the sorbitol bed and evaporated, the sample was placed inside a desiccator. When required, the dried powder was hydrated with preheated (±40°C) Milli-Q® water, following sonication on ice for 2.5 min and then left to stand for at least 2 h at room temperature to let the proniosomes swell.

Table 1: Quantities of materials used during the formulation of niosome and proniosome vesicles encapsulating artemether.

2.2.5 Characterisation of the dispersions

The characterisation of three different formulations (1%, 2% and 3%), done to determine which one of the three dispersions would give the best results during further analysis, was performed by measuring the zeta-potential, polydispersity index (PdI), particle size, pH and viscosity of the dispersions; the entrapment efficiency (EE%) of these three dispersions was also determined. The 3% proniosome dispersion was also characterised according to the named properties. The morphology of both the 3% empty niosomes and proniosomes were determined.

2.2.5.1 Zeta-potential, polydispersity index and particle size

The zeta-potential (mV), PdI and droplet size (d.nm) of the dispersions were determined using the Malvern® Zetasizer Nano ZS 2000 (Malvern® Instruments Ltd, Worcester, UK) (Begum, 2014; Shirsand, 2012). For each analysis, the dispersion was diluted by adding one drop of the dispersion to 20 ml of Milli-Q® water and mixing well.

2.2.5.2 Viscosity

The viscosity of all the dispersions was determined using a Brookfield Viscometer DV III Ultra (Middleboro, Massachusetts, USA). Due to the aqueous nature of the dispersions the T-bar spindle 18 (code: SC4-18) was used, taking a reading every minute for 6 min at a temperature of approximately 25°C.

2.2.5.3 pH

The pH of the human skin ranges between 4.5 and 6.0 (Lingan et al., 2011) therefore, in order to deliver a drug topically it must have a pH with close proximity to that of the skin to ensure it would not cause damage (Nair et al., 2013). The pH of the dispersions was measured using a Mettler Toledo® Seven Multi pH meter, equipped with an InLab® 410 electrode (Mettler Toledo, Greifensee, Switzerland).

2.2.5.4 Entrapment efficiency (EE%)

The entrapment efficiency determines the concentration of API trapped inside the vesicle dispersion along with the concentration of free flowing API. The EE% for the niosomes was determined by transferring the dispersion to a 20 ml tube and then centrifuging the dispersion using an Optima L-100 xP ultracentrifuge (Beckman Coulter, USA). The centrifuge was set at a rotation speed of 25000 rpm and a temperature of 4°C for 30 min using the 50.2Ti Rotor.

The supernatant was analysed using a HPLC system, which revealed the unentrapped (free flowing) artemether concentrations. The theoretical entrapped artemether concentration was calculated. The supernatant values, along with the theoretical entrapped artemether concentration, were substituted in Equation 1 to determine the EE%.

$$EE\% = [(Ct-Co)/Ct] \times 100 \qquad \text{Equation. 1}$$

The EE% for the proniosomes was determined in the exact same manner as for the niosomes, except the proniosome dispersion was first diluted by transferring 1 ml to a measuring cylinder and filling up to 25 ml using Milli-Q® water. This dilution was taken into account during the calculation.

2.2.5.5 Transmission electron microscopy (TEM)

The morphology of the 3% niosomes and proniosomes were determined using a FEI Tecnai F2 200 kV high-resolution transmission electron microscope (TEM; Czech Republic, Europe). No active was present in the dispersion during these analyses, as the active could damage the microscope, therefore only the morphology of the unloaded 3% niosomes and proniosomes (placebo) were determined.

2.3 Diffusion studies

2.3.1 Solubility

The aqueous solubility of artemether in water and PBS (pH 7.4) were determined. The aqueous solubility of artemether was of great importance during the diffusion studies, as the PBS (pH 7.4) was used as the receptor phase during the skin diffusion studies and the water

solubility of an API is one of the most important factors, which determines whether the active would be able to penetrate the stratum corneum.

The aqueous solubility of artemether was determined by preparing six test tubes, where 3 ml of Milli-Q® water, as well as 3 ml of PBS (pH 7.4) was transferred to three of the test tubes, respectively. A surplus amount of artemether (to obtain a saturated solution) was added to all six test tubes, the solutions were then vigorously mixed by placing the test tubes inside a shaking bath set at 32°C for 24 hrs. After this period, six HPLC vials were prepared by transferring 2 ml of solvent from each test tube to a separate HPLC vial, these solutions were analysed using the HPLC.

2.3.2 Log D and log P determination

The log D (octanol-buffer distribution coefficient) and log P (octanol-water partition coefficient) values or artemether were determined using the shake-flask method adapted from the methods discussed in OECD council (1995) and Leo et al. (1971). Two separate funnels were prepared in order to determine: 1) the log D and 2) the log P values. Funnel 1 contained the two solvents *n*-octanol and PBS (pH 7.4), while Funnel 2 contained the two solvents *n*-octanol and Milli-Q® water. Both the organic and aqueous phases were individually visible inside the funnels, the top layer being *n*-octanol and the bottom layer the aqueous phase. From both funnels, 20 ml of the PBS (pH 7.4) or Milli-Q® water phase and 20 ml of the organic *n*-octanol phase were transferred into a beaker.

Artemether (1.0 mg) was transferred to both of the 20 ml *n*-octanol phases and even distribution was ensured by placing the solutions on the sonication bath. The sample for the determination of the log D was prepared as follows: from Funnel 1, 3 ml of the *n*-octanol phase containing artemether and 3 ml of the PBS phase were transferred to three test tubes. The sample for the determination of the log P was prepared as follows: from funnel 2, 3 ml of the of *n*-octanol phase containing artemether and 3 ml of the Milli-Q® water was transferred to three test tubes. All six test tubes, each containing 3 ml of aqueous and 3 ml of lipophilic phase, were placed in a shaking water bath preheated to 32°C for 24 h to ensure equilibrium distribution.

The octanol phase (top phase) from all six test tubes were diluted by transferring 1 ml of the top phase to a volumetric flask (10 ml) and making up to volume using methanol. All six of these mixtures were then sonicated. For the HPLC analysis, 1 ml from each of the diluted octanol (6 volumetric flasks) phases were transferred to six separate HPLC vials, 1 ml from the aqueous phases (buffer or water) were also transferred to six separate HPLC vials, making a total of 12 vials analysed.

2.3.3 Receptor phase preparation

Originally, PBS (pH 7.4) was used as the receptor phase during four repetitions of the membrane release studies, but the low artemether concentrations obtained indicated an inadequate solubility of artemether in PBS (pH 7.4), therefore different receptor phases were used for the membrane release and skin diffusion studies.

Membrane release studies are executed to determine whether the vesicle systems release the encapsulated artemether into the receptor phase, hence, artemether must be soluble in the receptor phase. Firstly, the solubility of artemether in a mixture of PBS (pH 7.4) and ethanol (1:9) was determined in the hope of finding a receptor phase equipped for both the skin diffusion and membrane release studies. Artemether was not sufficiently soluble in this mixture, resulting in the search for an organic solvent as the receptor phase. Three organic solvents were analysed, i.e. THF, ethanol (99.9%) and isopropanol. Artemether was only soluble in the THF and ethanol (99.9%), and THF are known to react with the cellotape used during the tape stripping. Since artemether is soluble in ethanol (99.9%) (Nasir et al., 2012) it was used during the membrane release studies as the receptor phase.

Ethanol could not serve as the receptor phase during the skin diffusion studies as it would damage the skin, as well as serve as a penetration enhancer (Lachenmeier, 2008), which would not represent the true *in vivo* environment found during topical drug delivery. During the topical delivery of artemether, it was only expected to find artemether concentrations in the stratum corneum-epidermis and the epidermis-dermis and not in the receptor phase (Uchehi et al., 2014). Consequently, while PBS (pH 7.4) was used as the receptor phase; ethanol (99.9%) was used as the solvent, in which the tape strips (stratum corneum-

epidermis) and the skin pieces (epidermis-dermis) were left overnight to analyse for the presence of artemether concentrations.

2.3.4 Donor phase preparation

During both diffusion studies (skin and membrane), an artemether loaded vesicle dispersion and an empty vesicle dispersion (placebo) was analysed using an HPLC. The preparation of these dispersions was discussed in Sections 2.2.3 and 2.2.4. To ensure complete vesicle swelling and dispersion equilibrium, the final dispersions for both vesicles were left to stand at room temperature for 24 h before executing the diffusion studies. An hour before commencing the study, the dispersions were placed in a water bath preheated to 32°C.

2.3.5 Membrane diffusion studies

Membrane release studies were executed using 12 Franz cell sets, each consisting of a receptor and donor phase. The diffusion areas of these Franz cells were approximately 1.075 cm^2. A magnetic stirrer was placed inside each of the receptor phase compartments. A polyvinylidene difluoride (PVDF), hydrophilic membrane filter with a diameter of 25 mm and a pore size of 0.45 µm (Bonna-Agela Technologies Inc., Wilmington, USA) was placed in between the receptor and donor phase of each Franz cell set (12 per study), which were then tightly greased together using Dow Corning® high vacuum grease and mounted using a clamp.

A water bath containing the receptor phase, ethanol (99.9%), was preheated to 37°C, while the niosome and proniosome dispersions were preheated to 32°C. After the Franz cell sets were greased and clamped, 1 ml of the artemether loaded niosomes/proniosomes were placed into 10 of the donor phase compartments and 1 ml of the unloaded niosome/proniosome in the remaining two donor phase compartments. To prevent evaporation, the donor phases were covered using two pieces of Parafilm® and a cap. All 12 of the receptor phase tubes were filled with 2 ml of ethanol (99.9%) and then placed inside the water bath set at 37°C. After 1 h the ethanol (99.9%) in each receptor compartment was extracted and refilled with fresh ethanol (99.9%); this step was repeated each hour for 6 h.

The extracted receptor phase samples were analysed by HPLC for the artemether concentrations.

2.3.6 Skin preparation

Full-thickness abdominal skin obtained from Caucasian female abdominoplasty was collected from plastic surgeons and kept frozen at -20°C until needed. Patients filled out an informed consent form for the use of their skin for research purposes and all information was kept confidential. The diffusion studies using human skin were conducted in a Type 2 laboratory approved for research using human skin, with the research being approved by the Research Ethics Committee of the North-West University (reference number NWU-00114-11-A5).

Approximately a week before being required, the full-thickness skin was dermatomed (400 µm tick) using the Zimmer™ electric dermatome Model 8821 (Zimmer, Ohio, USA). These skin pieces were stretched out onto filter paper and frozen until the morning of the diffusion study, when they were examined for any stretch marks, defects or excess hair. With the skin still attached to the filtration paper, 12 round skin pieces with the approximate size of the Franz cell diffusion areas were cut out.

2.3.7 *In vitro* diffusion studies

The exact same preparation method was performed as discussed in Section 2.4.2, with three exceptions. The first exception was the receptor phase used was PBS (pH 7.4), the second was the prepared dermatomed skin (Section 2.4.3) was placed in between the donor and receptor compartments instead of the PVDF membrane filters and the last exception was the extractions were every 2 h followed by the refilling of the receptor compartment with fresh PBS (pH 7.4) for a total of 12 h, concluding to the analysis of six extractions per Franz cell using the HPLC.

2.3.8 Tape stripping

After the final PBS (pH 7.4) extraction at 12 h, the skin between each Franz cell set was removed, separated from the filtration paper and lightly wiped clean. The stratum corneum-epidermis of each skin piece was removed by placing a piece of Scotch® Magic Tape™ onto

the upper part of the skin, lightly rubbing the tape and then removing it. For each skin piece, 16 strips of Scotch® Magic Tape™ were cut and used; the first piece of tape was discarded as it contained the remaining dispersion, while the rest of the tape pieces were placed into a polytop containing 5 ml of ethanol. The remaining skin (diffused) was cut up into small pieces and placed into a separate polytop also containing 5 ml of ethanol. Ultimately, there were 12 polytops containing tape strips and 12 containing cut up pieces of skin. All 24 polytops were placed in the fridge (±4°C) for at least 8 h, after which each sample was transferred to separate HPLC vials by means of filtration and then analysed for the presence of artemether concentrations. This method was adapted from the method described in Pellet et al. (1997).

2.3.9 Data analysis

For the membrane release studies, the individual artemether concentration released from the vesicles per Franz cell, as well as the average cumulative amount per area (µg/cm^2) of artemether released from the vesicle carriers for all 10 Franz cells were determined over a period of 6 h. The flux (µg/cm².h) was determined from the slope of the graphs and the average flux (µg/cm².h) was calculated.

For the skin diffusion studies, the average concentration artemether that diffused through the skin into the receptor phase was determined. Regarding the tape stripping analysis, the average concentration artemether (µg/ml) that diffused into the stratum corneum-epidermis and epidermis-dermis for each individual Franz cell was calculated.

2.4 *In vitro* cytotoxicity determination

The toxicity of artemether and the niosome vesicle dispersions (artemether encapsulated and placebo) were determined on HaCaT cell cultures. The LDH assay was used for the cytotoxicity analysis. HaCaT cells were grown in the growth media (DMEM) at a temperature of 37°C in a laboratory with a humidified atmosphere of 5% CO_2.

For the LDH-assay, the cytotoxicity of artemether was determined by preparing a stock solution, which was done by weighing 50 mg of artemether and dissolving it in 10 ml methanol. Three different concentrations of the artemether stock solution were analysed,

i.e. 75 µg/ml, 150 µg/ml and 300 µg/ml, which were further diluted with DMEM, up to a final volume of 100 µl per well. The empty and the loaded niosomes were prepared as explained in Section 2.2.3 and 2.2.4. The concentration of artemether present in the samples was consistent for all those containing artemether in order to compare the results.

2.4.1 LDH assay

The cytotoxicity of artemether and the niosome dispersions were determined by means of the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit. A 96-well was prepared by transferring $2x10^4$ of the cells grown in DMEM to each well. Five different samples were analysed, i.e. 1) a positive control, 2) a negative control, 3) an artemether stock solution, 4) the empty niosome dispersion and 5) the loaded niosome dispersion. For the analysis of the positive control only, the cells were present in the wells, while the wells for the negative control contained only 100 µl of DMEM and no cells. Samples 3 to 5 were added to the required cell containing wells and then the plate was incubated for 12 h at 37°C. Eleven hours and 15 min (45 min before 12 h) into the incubation time, 10 µl of the 10X Lysis Solution was added to the wells containing the positive control. After incubation, 50 µl from each of the occupied wells was transferred to a fresh 96-well. The plate was incubated for 30 min in total darkness at room temperature. After incubation, the Stop solution was added to all the occupied wells; the absorbance of LDH enzyme present at a wavelength between 490-492 nm using a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) was measured between 0 and 1 h after adding the Stop solution.

3 RESULTS AND DISCUSSION

3.1 Formulation and characterisation of the niosome and proniosome dispersions

Three different niosome dispersions were formulated, 1%, 2% and 3% of the API, respectively. Artemether and the lipophilic compounds (cholesterol and Span ®60) were dissolved in cholesterol and then roto-evaporated. The only difference between these three formulations was the different amount of chemicals included during the preparation of the dispersions. After rehydration of the film that formed on the side of the round bottomed flask

and after evaporation and sonication, the dispersions displayed a liquid solution, which was milky in colour. All three dispersions were characterised according to the characteristics measured.

Table 2: Physical characteristics of the three niosome dispersions

The particle size distribution (d.nm) of a dispersion should be as small as possible for optimal results during topical drug delivery (Sezgin-Bayinder & Yuksel, 2012). The results of the particle size distribution between the three dispersions varied quite a lot, but the 3% dispersions had the smallest value, as seen in Table 2.

The zeta-potential value indicates whether the electrical charge on a particle is negative or positive, and thereby specifies the electrokinetic potential of a colloidal system. With regards to topical drug delivery, 1) the zeta-potential value of a particle reveals the stability and release rate of the compound from the vesicle systems and 2) nano-particles with values, higher than +30 and -30, especially highly negative values, are considered stable and should deliver ideal results during diffusion studies, in respect of the zeta-potential (Bayinder & Yuksel, 2010; Honary & Zahir, 2013a, 2013b; Nnamani et al., 2014). The zeta-potential values for all three dispersions fell within the criteria range (Table 2).

The EE% for all three dispersions was very high (Table 2), therefore it should have high bioavailability, as almost all of the added artemether was encapsulated inside the vesicle systems (Raslan, 2013).

The 3% niosome dispersion was the only one where a viscosity reading was registered by the instrument, consequently this dispersion gave the better results.

The pH values of the 2% and 3% dispersions fell between the pH range for normal human skin, being between 4.5 and 6.0 (Lingan et al., 2011); the 1% dispersion had a pH value below the criteria and could therefore harm the skin (Lingan et al., 2011; Nair et al., 2013).

The PdI measurement of a dispersion indicates whether the dispersion is uniform or not. The PdI results of the 3% niosome dispersion were the closest to the value 1, indicating a

polydispersed dispersion. The 1% and 2% dispersions were between 0 and 1 and are therefore not monodisperse or polydispersed (Nobbman, 2004).

Taking all six characteristics of the three dispersions displayed in Table 2 into consideration, the formulation chosen (3% niosome dispersion) was considered to deliver the best results during further analysis.

A 3% proniosome dispersion was formulated to correlate with the chosen 3% niosome dispersion. The morphology of both of the empty niosome and proniosome dispersion was determined using a FEI Tecnai G2 200 kV high resolution TEM. The vesicles sizes of the niosomes ranged from 106.17 nm to 269.05 nm, whereas the proniosome vesicle sizes ranged from 52.83 nm to 527.70 nm. Most of these vesicle sizes fell within the criteria range of between 50-500 nm for topical drug delivery (Table 3) (Uchechi et al., 2014).

Table 3: Physical characteristics of the 3% niosome and proniosome dispersions

3.2 Aqueous solubility

The aqueous solubility of artemether in water was determined as 0.11±0.002 µg/ml and its solubility in PBS as 0.09±0.003 µg/ml; the aqueous solubility of a compound used for transdermal drug delivery should be larger than 1 mg/ml (Naik et al., 2000). Even though the solubility of artemether in PBS and water were below the criteria for transdermal drug delivery, it was still argued that the topical delivery of artemether could be possible by means of a vesicle system; also, one cannot use only one parameter and thereby rule out the possibility of transdermal delivery.

3.3 Log D and log P determination

The log D and log P values of artemether were determined as 2.35±0.067 and 2.26±0.117, respectively, which falls within the criteria range of between 1 and 3 for optimal skin permeation (Hadgraft, 2004; Mbah, 2011). These results indicated artemether was more lipophilic, and drugs that are more lipophilic in nature are more favourable for penetration into the stratum corneum (lipophilic). The topical delivery of highly lipophilic drugs can be

problematic, as the drug resides in the stratum corneum and does not diffuse into the epidermis and dermis layers below the stratum corneum (Naik et al., 2000; Williams, 2013).

3.4 Membrane diffusion studies

Fig 1: Average cumulative amount of artemether released per area from the niosomes during the membrane release study after 6 h (n=10)

Fig 2: Average cumulative amount of artemether released per area from the proniosomes during the membrane release study after 6 h (n=10)

The concentrations of artemether acquired from the receptor phase (ethanol (99.9%)) of all 10 Franz cells specified that the release of artemether from both vesicle systems were successful. The average flux of the niosomes was calculated as 73.58±8.64 µg/cm².h, and for the proniosomes as 63.91±7.17 µg/cm².h. The average percentage artemether that diffused from the membrane into the receptor phase was 0.799±0.096% for the niosomes and 0.712±0.080% for the proniosomes. Figures 1 and 2 represent the average cumulative amount of artemether released per area from the niosomes and proniosomes, respectively. When comparing the release of artemether from the niosome and proniosome vesicles, it was clear the two vesicle systems were closely associated, but the niosomes presented the better drug release. Sorbitol (present in the proniosome dispersion) is known to cause slow drug release during *in vitro* permeation studies. The presence of this compound could therefore be the reason why better drug release was observed from the niosome vesicles (Chandra & Sharma, 2008).

3.5 Diffusion studies

During both diffusion studies (skin and membrane), an artemether loaded vesicle dispersion and an empty vesicle dispersion (placebo) were analysed using an HPLC. The transdermal delivery of artemether presented no artemether concentrations in the receptor phase, which was expected. Topical drug delivery is used for local effects and niosome vesicles are known to have an ability to reduce the systemic absorption of a drug (Uchehi et al*.,* 2014).

3.6 Tape stripping

Tape stripping was performed to determine whether any artemether concentrations were present in the stratum corneum-epidermis and the epidermis-dermis.

3.6.1 Stratum corneum-epidermis

The analysis of the tape strips, soaked in ethanol (99.9%) for 8 h would determine the concentration of artemether that resided in the stratum corneum-epidermis; in this instance, no artemether concentrations were identified. The lipophilic nature of artemether indicated that the chances of released artemether residing in the stratum corneum instead of diffusing into the epidermis-dermis were likely (Williams, 2013). The absence of artemether in the stratum corneum-epidermis could be an indication of targeted drug delivery if artemether diffused into the epidermis-dermis (Karim et al., 2010).

3.6.2 Epidermis dermis

The cut up skin pieces placed in ethanol (99.9%) for 8 h were analysed for the presence of artemether concentrations in the epidermis-dermis. The niosomes had an average artemether concentration of 2.04±2.766 µg/ml in the epidermis-dermis of which artemether was only identified in four of the 10 Franz cells. The proniosomes had an average artemether concentration of 2.32±2.151 µg/ml in the epidermis-dermis, where only five out of the eight viable Franz cells presented artemether concentrations.

When comparing the two vesicle systems, the proniosome vesicles gave slightly higher artemether concentrations. The average artemether concentrations identified in both of these vesicles were quantified, but below the limit of quantitation (LOQ) of artemether was determined as 5.08 µg/ml, thus it was not reliable.

The presence of artemether concentrations identified in the epidermis-dermis indicated topical drug delivery did however occur. The topical delivery of artemether can nevertheless be improved by increasing the amount of artemether added to the formulation, which is normally between 2-5 mg/cm^2 for topical drug delivery (Herkenne et al., 2008). Other methods to enhance topical delivery, including chemical, biochemical or physical permeation enhancers, should also be investigated (Prausnitz et al., 2012; Prausnitz & Langer, 2008).

The unsuccessful skin permeation can be due to two reasons, the first being the vesicles physical characteristics and the second being due to the solubility of artemether. The physical characteristics of the vesicles are related to the charge of the dispersion. According to the zeta-potential values of the niosome and proniosome vesicles (Table 3), the dispersions were negatively charged; the surface of the skin is also negatively charged, which could have led to the skin repelling the dispersions. According to Gillet et al. (2011), positively charged vesicles are likely to penetrate the skin more efficiently than the negatively charged vesicles, which would in turn accumulate in the epidermis-dermis. The second reason for unsuccessful skin permeation could be due to the solubility of artemether in ethanol, compared to the solubility of artemether in PBS (pH 7.4). The ethanol (99.9%) used during the membrane release studies acted as a driving force to force the artemether out of the vesicles. Artemether, being lipophilic, has an affinity for organic solvents. The PBS (pH 7.4) used during the skin permeation studies are an aqueous solvent and artemether does not have an affinity for it.

3.7 Toxicity analysis

The cytotoxicity of the active artemether, artemether loaded niosomes and the blank niosomes were determined using an LDH assay.

3.7.1 LDH assay

The cytotoxicity of artemether and the niosome dispersions were analysed using an LDH assay on HaCaT cell cultures.

Fig 3: Percentage cell death caused after 12 h of incubation using the LDH assay. Each of the three bars grouped together represents one sample, while the three different colours represent the different concentrations of artemether that were present, i.e. the blue bar represents the negative control, the purple bars 0.5% of the sample, the red bars 1.0% of the sample and the green bars 2.0% of the sample.

Table 4: Percentage HaCaT cell viability after 12 h incubation of artemether and the vesicle dispersions

López-García et al. (2014) stated the %cell viability caused by a compound is categorised by the following: with a %cell viability lower than 40% it is believed to be strong cytotoxic, a %cell viability from 40-60% it is believed to be moderate cytotoxic, a %cell viability from 60-80% it is considered weak cytotoxic and with a %cell viability higher than 80% it is considered non-cytotoxic.

The %cell death caused by the three samples (Figure 3) was proportional regarding the increase in sample concentration, signifying the assay itself was not problematic and worked. Artemether concentrations of 75 µg/ml, 150 µg/ml and 300 µg/ml were present in 0.5%, 1.0% and 2.0%, respectively, of the artemether stock solution and loaded niosome dispersion that were subjected to the cell cultures.

All the empty niosomes displayed a non-cytotoxicity towards the HaCaT cells, in view of the 0.5%, 1.0% and 2.0% concentrations causing a %cell death of 10.27±1.40%, 12.08±1.94%, 15.70±2.58%, respectively, as presented in Fig 3 (López-García et al., 2014).

The loaded niosomes, from the 0.5%, 1.0% and 2.0% concentrations caused a %cell death of 13.34±2.56%, 24.56±4.10% and 71.94±6.25%, respectively. The 0.5% concentration of the loaded niosomes was regarded as non-cytotoxic with a %cell viability of 86.66%, while the 1.0% concentration was regarded as weakly cytotoxic with a %cell viability of 75.44%. The 2% concentration of the loaded niosome was regarded as strongly cytotoxic towards the HaCaT cell cultures since it had a %cell viability of 28.06% (López-García et al., 2014).

The cytotoxicity analysis of the artemether stock solution revealed a %cell viability of 19.70±3.50%, 42.44±7.74% and 92.62±2.86% from the 0.5%, 1.0% and 2.0% concentrations, respectively. According López-García et al. (2014), the 0.5% concentration stock solution was regarded as non-cytotoxic, having a %cell viability of 80.30%; having a %cell viability of 57.56% and 7.38% the 1.0% and 2% stock solution concentration was considered moderately and strongly cytotoxic, respectively, to the HaCaT cell cultures.

4 CONCLUSION

The 3% vesicle dispersion was chosen as the optimum dispersion for further analysis of membrane and diffusion studies, as well as the cytotoxicity analysis, as it had the required physicochemical properties for topical drug delivery.

The membrane release studies executed on the 3% niosome and proniosome dispersions presented artemether concentrations in the receptor phase (ethanol (99.9%)) indicating both these vesicle systems released the entrapped artemether.

During the tape stripping, no artemether concentrations were identified in the stratum corneum-epidermis, but artemether was present in the epidermis-dermis. Artemether concentrations were acquired in four of the 10 Franz cells for the niosomes and in five of the eight Franz cells for the proniosomes. The artemether concentrations found in the epidermis-dermis were lower than the LOQ value for artemether and therefore these values were not reliable but could be an indication that targeted drug delivery occurred (Karim et al., 2010). The topical delivery of artemether however should be improved by adding permeation enhancers, using a different vesicle system or increasing the concentration of artemether present in the formulation (Herkenne et al., 2008; Prausnitz et al., 2012:2070).

The cytotoxicity determination of artemether and the vesicles using an LDH assay indicated the 2.0% artemether stock solution and loaded niosome dispersion displayed strong cytotoxicity towards the HaCaT cells. However, the 1.0% artemether stock solution and niosome dispersion was regarded as moderately and weak cytotoxic. The 5.0% stock solution and loaded niosome, along with all of the empty niosomes, were regarded as non-cytotoxic to the HaCaT cells.

The probable reason for artemether being toxic to the HaCaT cells, which are a type of T-cell (Wu et al., 2015), could be due to artemether causing a decrease in the proliferation rate of T-cells proportionally to increasing the concentration of artemether present. In view of the significant differences between *in vitro* and *in vivo* environments regarding absorption, metabolism, the distribution process and excretion during the *in vivo* studies, these results cannot be compared to *in vivo* studies (Yoon et al., 2012).

ACKNOWLEDGEMENTS

We would like thank the South African National Research Foundation (NRF) (Grants no. IFRR81178 and CPRR90569), the South African Medical Research Council (MRC) for the Flagship Project MALTB-Redox, and the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom Campus, South Africa, for their financial support during this research study.

Disclaimer

The findings and opinions expressed, as well as the conclusions made, were those of the author and are not necessarily to be attributed to the NRF.

References

Ahn, B-N., Kim, J-A., Kong, C-S., Seo, J. & Kim, S-K. 2012. Protective effect of (2'S)-columbianetin from *Corydalis heterocarpa* on UVB-induced keratinocyte damage. J Photochem Photobiol. 109, 20-27.

Ansari, M.T., Karim, S., Ranjha, N.M., Shah, N.H. & Muhammad, S. 2010. Physicochemical characterization of artemether solid dispersions with hydrophilic carriers by freeze-dried and melt methods. Arch Pharm Res. 3(6), 901-910.

Ansari, M.T., Hussain, A., Nadeem, S., Majeed, H., Saeed-Ul-Hassan, S., Tariq, I., Mahmood, Q., Khan, A.K. & Murtaza, G. 2015. Preparation and characterization of solid dispersions of artemether by freeze-dried method. BioMed Res. Int. 1-11.

Bayinder, Z.S. & Yuksel, N. 2009. Characterization of niosomes prepared with various nonionic surfactants for paclitaxel oral delivery. J Pharm Sci. 99(4), 2049-2060.

Begum, M.Y., Dasari, S., Sudhakar, M., Lakshmi, B.V.S. & Manga, K. Development and evaluation of co-encapsulation stavudine and lamivudine niosomes for the controlled delivery. Der Pharmacia Sinica. 5(1), 1-10.

Bolzinger, M-A., Brainçon, S., Pelletier, J. & Chevalier, Y. 2012. Penetration of drugs through skin, a complex rate-controlling membrane. Curr Opin Colloid Interface Sci. 17(3), 156-165.

Dwivedi, A., Mazumder, A., Fox, L.T., Brümmer, A., Gerber, M., du Preez, J.L., Haynes, R.K. & du Plessis, J. 2016. *In vitro* skin permeation of artemisone and its nano-vesicular formulations. Int J Pharm. 503(1-2), 1-7.

Foldvari, M. 2000. Non-invasive administration of drugs through the skin: challenges in delivery system design. Pharm. Sci. Technol. Today. 3(12), 417-425.

Gautschi, N., Bergström, C.A.S. & Kuentz, M. 2016. Rapid determination of drug solubilization versus supersaturation in natural and digested lipids. Int J Pharm. 513(1-2), 164-174.

Gillet, A., Compère, P., Lecomte, F., Hubert, P., Ducat, E., Evrard, B. & Piel, G. 2011. Liposome surface charge influence on skin penetration behaviour. Int J Pharm. 411(1-2), 223-231.

Han, X., Gelein, R., Corson, N., Wade-Mercer, P., Jiang, J., Biswas, P., Finkelstein, J.N., Elder, A. & Oberdörster, G. 2011. Validation of an LDH assay for assessing nanoparticle toxicity. Toxicol. 287(1-3), 99-104.

Haynes, R. 2013. SA MRC flagship proposal executive summary. Oct. 2013. [Correspondence]. 29 Jul. 2015, Potchefstroom.

Haynes, R.K. 2015. Development of oxidant and redox drug combinations for treatment of malaria, TB and related diseases. [PowerPoint presentation].

Honary, S. & Zahir, F. 2013a. Effect of zeta potential on the properties of nano-drug delivery systems – a review (Part 1). Trop J Pharm Res. 12(2), 255-264.

Honary, S. & Zahir, F. 2013b. Effect of zeta potential on the properties of nano-drug delivery systems – a review (Part 2). Trop J Pharm Res. 12(2), 265-273.

ISO (International Organization of Standardization) 10993-5. 2009. Biological evaluation of medical devices – part 5: Test for *in vitr*o cytotoxicity, third ed. ISO, Geneva.

Lingan, M.A., Sathali, A.A.H., Kumar, M.R.V. & Gokila, A. 2011. Formulation and evaluation of topical drug delivery system containing clobetasol propionate niosomes. Sci Revs Commun. 1(1), 7-17.

López-García, J., Lehocký, M., Humpolíček, P. & Sáha, P. 2014. HaCaT keratinocytes response on antimicrobial atelocollagen substrates: extent of cytotoxicity, cell viability and proliferation. J Funct Biomater. 5(2), 43-57.

Moghasemi, S & Hajizadeh, A. 2014. Nano-niosomes as nanoscale drug delivery systems: An illustrated review. J Control Release. 185, 22-36.

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. Pharm. Sci. Technol. Today. 3(9), 318-326.

Nair, A., Jacob, S., Al-Dhubaib, B., Attimarad, M. & Harsha, S. 2013. Basic considerations in the dermatokinetics of topical formulations. Braz J Pharm Sci. 49(3), 423-434.

Nnamani, P.O., Hansen, S., Windbergs, M. & Lehr, C-L. 2014. Development of artemether-loaded nanostructured lipid carrier (NLC) formulation for topical application. Int J Pharm. 477(1-2), 208-217.

Nobbman, U. 2014. Polydispersity – what does it mean for DLS and chromatography? http://www.materials-talks.com/blog/2014/10/23/polydispersity-what-does-it-mean-for-dls-and-chromatography/ (accessed 31.08.16).

Perrin, P. 2015. Human and Tuberculosis co-evolution: An integrative view. Tuberculosis. 95(1), S112-S116.

Raslan, M.A-E. 2013. Effect of some formulation variables on the entrapment efficiency and in vitro release of ketoprofen from ketoprofen niosomes. J Coast Life Med. 1(2),15-22.

Riss, T.L., Moravec, R.A., Niles, A.L., Benink, H.E., Worzella, T.J. & Minor, L. 2013. Assay guidance manual: cell viability assays. http://www.ncbi.nlm.nih.gov/books/NBK144065/pdf/mttassays.pdf (accessed 21.03.15).

Sezgin-Bayindir, Z. & Yuksel, N. 2012. Investigation of formulation variables and excipient interaction on the production of niosomes. AAPS Pharm Sci Tech. 13(3), 826-835.

Shirsand, S.B., Para, M.S., Nagendrakumar, D., Kanani, K.M. & Keerthy, D. 2015. Formulation and evaluation of Ketoconazole niosomal gel drug delivery system. Int J Pharm. Investigation. 2(4), 201-207.

Stockert, J.C., Blázques-Castro, A., Cañete, M. & Horobin, R.W. 2012. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histo. 114(8), 785-796.

Udasi, T.A., Wankhade, V.P., Ingle, L.M., Atram, S. & Tapar, K.K. 2012. Proniosome: A novel approach to vesicular drug delivery system. Int J Pharmacy Pharm Sci. 3(1), 1-6.

Uchechi, O., Ogbonna, J.D.N & Attama, A.A. 2014. Nanoparticles for dermal and transdermal drug, in: Sezer, A.D., (Eds.), Application nanotechnology in Drug Delivery, Nsukka: InTech. pp. 193-235.

Van Zyl, L., du Plessis, J. & Viljoen, J. 2015. Cutaneous tuberculosis overview and current regimens. Tuberculosis. 95(6), 629-638.

Wang, G., Zhang, J., Dewilde, A.H., Pal, A.K., Bello, D., Therrien, J.M., Braunhut, S.J. & Marx, K.A. 2012. Understanding and correcting for carbon nanotube interferences with a commercial LDH cytotoxicity assay. Toxicol. 299(2-3), 99-111.

Wu, J., Li, H. & Li, M. 2015. Effects of artemether on the proliferation, apoptosis and differentiation of keratinocytes: potential application for psoriasis treatment. Int J Clin Exp Med. 8(5), 7069-7078.

Yoon, M., Campbell, J.L., Andersen, M.E. & Clewell, H.J. 2012. Quantitative *in vitro* to *in viv*o extrapolation of cell-based toxicity assay results. Crit Rev Toxicol. 42(8), 633-652.

Table 1: Quantities of materials used during the formulation of niosome and proniosome vesicles encapsulating artemether

Materials	Quantity	
	Niosome (3%)	**Proniosome (3%)**
Artemether	±300 mg	±300 mg
Span® 60	±300 mg	±300 mg
Cholesterol	±600 mg	±600 mg
Chloroform	10 ml	10 ml
Milli-Q® water	10 ml	10 ml
Sorbitol	-	±3 g

Table 2: Physical characteristics of all three niosome dispersions

Characteristics	1%	2%	3%
Particle size (d.nm)	479.10	963.40	279.00
Zeta-potential (mV)	-48.20	-49.90	-46.10
EE% (%)	98.60	99.32	99.55
Viscosity (cP)	0.00	0.00	9.96
pH	3.63	4.92	4.84
PdI	0.53	0.55	0.39

Table 3: Physical characteristics of the 3% niosome and proniosome dispersions

Vesicle system	Characteristics					
	Zeta-potential (mV)	PdI	Size distribution (d.nm)	Viscosity (cP)	pH	EE% (%)
Niosomes with API	-46.07	0.37	286.20	3.00	6.25	99.55
Niosome placebo	-42.17	0.22	180.57	3.39	6.29	None
Proniosomes with API	-37.63	0.86	798.13	8.63	5.54	93.89
Proniosome placebo	-39.60	0.45	185.07	5.04	6.36	None

Table 4: Percentage HaCaT cell viability after 12 h incubation of artemether and the vesicle dispersions

Sample	75 µg/ml artemether	150 µg/ml artemether	300 µg/ml artemether
Niosome placebo	70.64%	57.01%	38.68%
Niosome with API	72.08%	59.05%	31.13%
Stock solution	70.72%	32.01%	24.25%

FIGURE LEGENDS

Fig 1: Average cumulative amount of artemether released per area from the niosomes during the membrane release study after 6 h (n=10)

Fig 2: Average cumulative amount of artemether released per area from the proniosomes during the membrane release study after 6 h (n=10)

Fig 3: Percentage cell death caused after 12 h of incubation using the LDH assay. Each of the three bars grouped together represents one sample, while the three different colours represent the different concentrations of artemether that were present, i.e. the blue bar represents the negative control, the purple bars 0.5% of the sample, the red bars 1.0% of the sample and the green bars 2.0% of the sample.

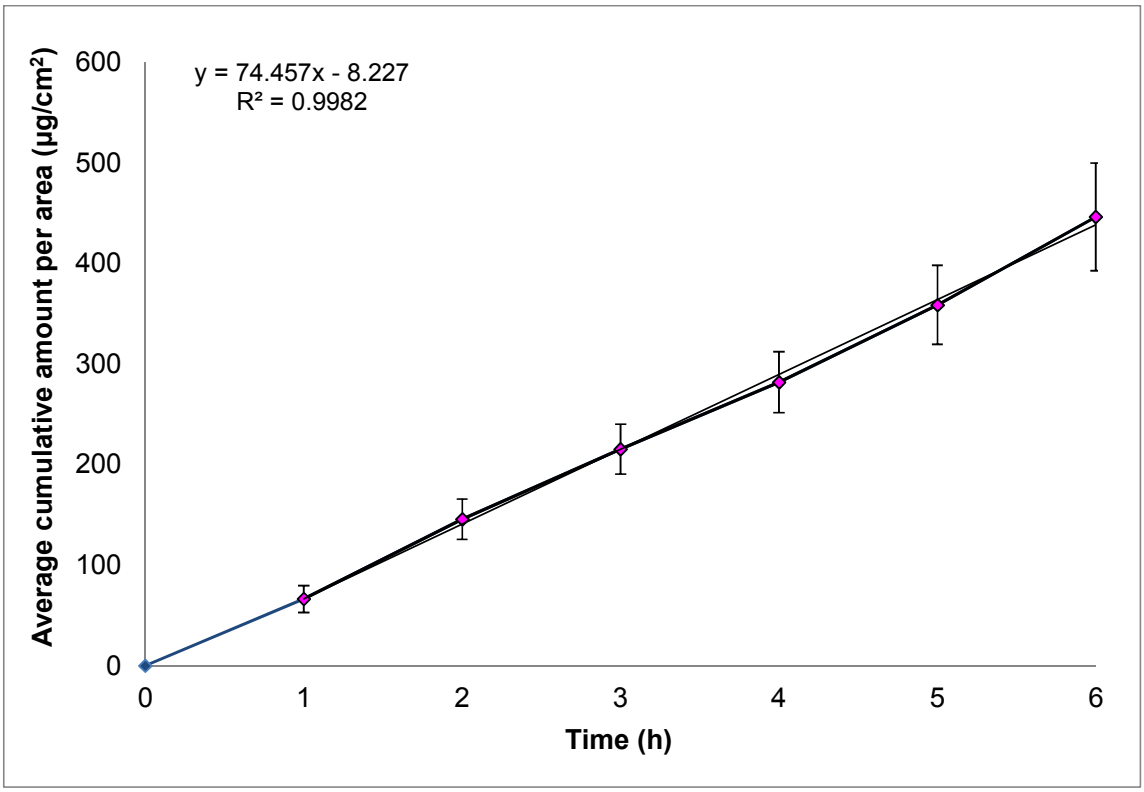


Fig 1: Average cumulative amount of artemether released per area from the niosomes during the membrane release study after 6 h (n=10)

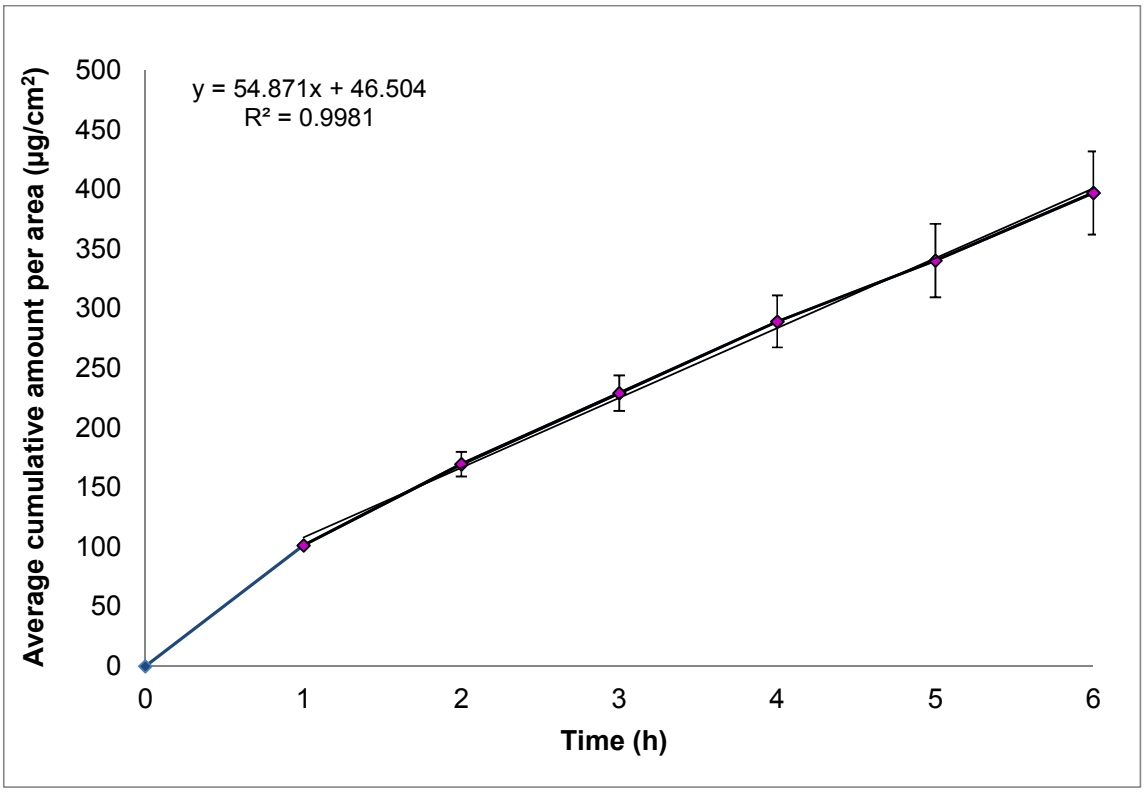


Fig 2: Average cumulative amount of artemether released per area from the proniosomes during the membrane release study after 6 h (n=10)

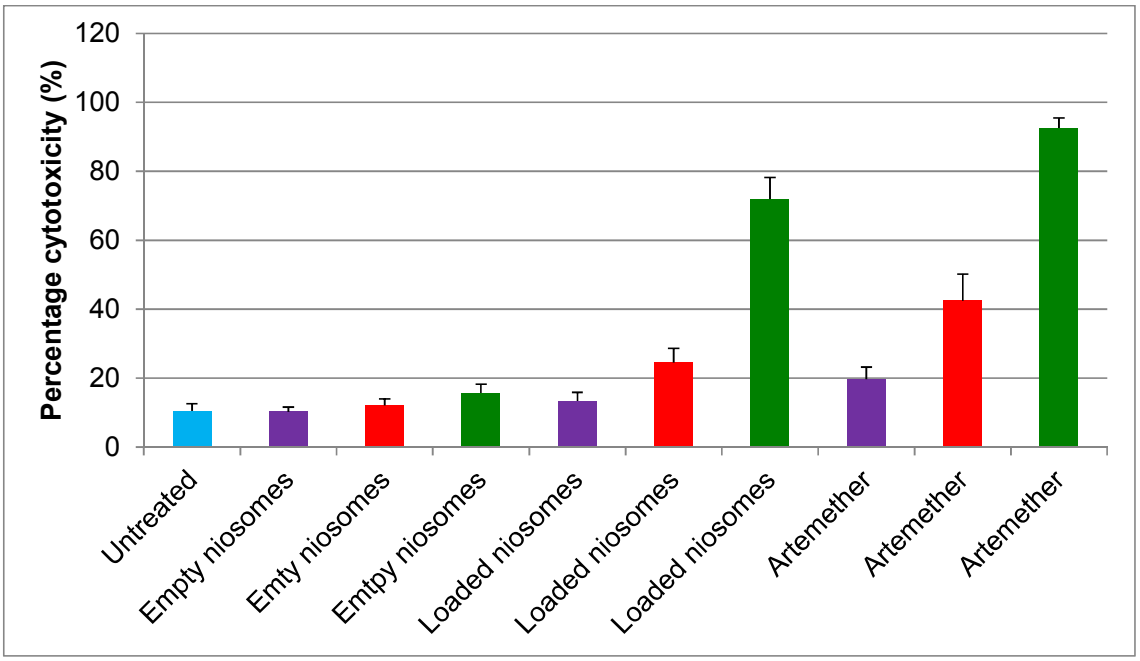


Fig 3: Percentage cell death caused after 12 h of incubation using the LDH assay. Each of the three bars grouped together represents one sample, while the three different colours represent the different concentrations of artemether that were present, i.e. the blue bar represents the negative control, the purple bars 0.5% of the sample, the red bars 1.0% of the sample and the green bars 2.0% of the sample.

CHAPTER 4

CONCLUSION AND FUTURE RECOMMENDATIONS

The topical delivery of a drug entails the delivery of a therapeutically active drug onto the skin for a local effect. Topical drug delivery is the most relevant delivery route for skin disease treatments (Shilakari *et al*., 2013:77; More *et al*., 2016:196). TB is a global epidemic disease, mainly caused by the bacterium *M. tuberculosis*, which causes many human deaths each year (Hershkovitz *et al*., 2008:1). CTB is a secondary skin illness due to the main infection of TB. This infection results in skin manifestations, which is not only painful, but has a psychological effect on the patient. The increasing resistance of TB against the multi-drug treatments are directly proportional to the increase in the occurrence of CTB (Dos Santos *et al*., 2014:219; Van Zyl *et al*., 2015:629). The recent aim for treatment against TB is the use of combination therapy, where an oxidant and redox drug is combined into one. Artemether and its artemisinin family have the necessary characteristics to act as treatment of TB, and will therefore represent the oxidant drug during the combination therapy (Haynes, 2013:1-2; Haynes, 2015:3-8; Miller *et al*., 2011:2076). The topical delivery of conventional formulations have not yet proved to be successful, because the permeation characteristics of the skin layers, mostly the stratum corneum, results in difficulty during drug transport. New techniques that increase the permeation of drugs during topical delivery have been researched and include drug carrier vesicles, i.e. niosomes and proniosomes (Ahmed, 2016:245; Gillet *et al*., 2011:223; Shilakari *et al*., 2013:77).

The aim of this study was to determine whether it was possible to deliver the low aqueous soluble artemether topically, while encapsulated in a carrier vesicle. The second aim was to establish the cytotoxicity of artemether on cell cultures.

The objectives of this study were to:

- Validate a HPLC method that would obtain adequate concentrations of artemether during analysis.
- Determine the aqueous solubility along with the log D and log P of artemether.
- Formulate optimum artemether (as API) entrapped in niosomes.
- Characterise three different niosome dispersions to select the optimum dispersion based upon the overall best characteristics regarding topical drug delivery.
- Formulate and characterise the chosen percentage proniosome dispersion.

- Perform membrane release studies for the purpose of determining whether the entrapped artemether is released from the vesicle systems.
- Determine whether topically applied artemether, entrapped inside the vesicles, can permeate through the different skin layers to the target-site (dermis), by making use of tape stripping and diffusion studies.
- Determine whether the application of artemether or any vesicle systems results in cytotoxicity towards cell cultures.

An HPLC method was developed and validated for the assessment of artemether concentrations, with the expert help of Prof Jan du Preez from the Analytical Technology Laboratory (ATL). This method was used to determine the artemether concentrations during the analysis of the standard artemether solutions and the niosome and proniosome dispersions.

Three artemether encapsulated niosome dispersions containing different percentages of API (1%, 2% and 3%), were formulated and characterised. The optimum dispersion was chosen based upon the overall best physical characteristics. Six different parameters were determined, i.e. zeta-potential, vesicle size, size distribution, viscosity, pH and the entrapment efficiency. Overall, the 3% niosome dispersion encapsulating artemether had the best characteristics for topical drug delivery; the proniosome formulation was therefore prepared to have an API concentration of 3%. The entrapment efficiency for the niosome and proniosome dispersions were calculated as 99.55% and 93.91%, respectively.

The aqueous solubility of artemether in water and PBS (pH 7.4) were determined as 0.11 ± 0.002 mg/ml and 0.09 ± 0.003 mg/ml, respectively. According to Naik *et al*. (2000:319), the aqueous solubility of a drug must be larger than 1 mg/ml for optimum results during transdermal drug delivery. The log D value (at pH 7.4) of artemether was calculated as 2.35 ± 0.067 and the log P value as 2.26 ± 0.117, which correlates with a log P drug requirement of between 1 and 3 for adequate skin permeation.

After completing the membrane release studies, using PBS (pH 7.4) as the receptor phase, it was detected that artemether does not dissolve in the PBS (pH 7.4) and therefore gave concentrations lower than the LOQ of artemether. Solubility studies on artemether in the lipophilic solvents ethanol (99.9%), THF and isopropanol, were determined; the solubility of artemether in ethanol (99.9%) was the best and therefore ethanol (99.9%) was used as the receptor phase during the membrane release studies.

The second set of membrane release studies using ethanol as receptor phase were executed and quantifiable concentrations of artemether were obtained. The membrane release diffusion results for the niosome dispersion encapsulating the API gave an average flux of

73.58 ± 8.64 μg/cm^2.h, while the average percentage artemether diffused was 0.799%. For the proniosome dispersion encapsulating the API, an average flux of 63.91 ± 7.17 μg/cm^2.h was obtained along with an average percentage artemether diffused of 0.712%. These results indicated the vesicles did release the entrapped artemether.

The skin diffusion studies were conducted using PBS (pH 7.4) as the receptor phase, as ethanol would damage the skin and during topical drug delivery, the API should not permeate as far as the circulatory system and PBS (pH 7.4) resembles the circulatory system better than ethanol (Lachenmeier, 2008:6; Rahimpour & Hamishehkar, 2012:141). After the final extraction at 12 h, the skin was stripped 15 times. The tape strips (15) and skin were dissolved in ethanol (99.9%) to determine the concentration of artemether that resided in the different skin layers. No artemether concentration was acquired in the stratum corneum-epidermis (tape strips) for either the niosome or the proniosome dispersions. An average artemether concentration of 2.04 ± 2.766 μg/ml was obtained in the epidermis-dermis for the niosome dispersion and 2.32 ±2.151 μg/ml for the proniosome dispersion. Only a few of the 10 Franz cell units, four of the 10 for the niosomes and five of eight for the proniosomes, contained artemether in their epidermis-dermis skin layers. The average artemether concentration for both these vesicle systems was below the limit of quantification (LOQ) and even though the results were quantified, they were not dependable; nevertheless, artemether was found in the epidermis-dermis. The permeation of artemether into the epidermis-dermis can however be improved by adding penetration enhancers, changing the vesicle carrier system or increasing the concentration of artemether in the dispersion (Herkenne *et al*., 2008; Prausnitz *et al*., 2012:2070).

The main reasons the artemether concentrations for the topical delivery were lower than expected may possibly be that the physical characteristics of the vesicle system played a role. The negative charge of both the vesicle and the skin could have caused these two compounds to repel each other; this also could have led to the accumulation of artemether in the epidermis-dermis (Gillet *et al*., 2011:223-224). Another reason could be due to the lipid solubility of artemether. The aqueous solubility of artemether determined as 0.11 mg/ml in water and 0.9 mg/ml in PBS (pH 7.4), indicated that artemether is not soluble in aqueous solvents. The membrane release studies indicated that artemether was released from the vesicles, but the receptor phase used was ethanol (99.9%) and as artemether is lipophilic, it would have an affinity for the ethanol (99.9%). The receptor phase used during the skin diffusion studies, PBS (pH 7.4) is aqueous and therefore it could have resulted in artemether residing in the vesicles due to not having an affinity for PBS (pH 7.4).

The toxicity levels of artemether and the niosome vesicles were determined using the cell line Cultured Human Keratinocyte (HaCaT) by means of the lactase dehydrogenase (LDH) assay.

The LDH assay indicated that artemether was cytotoxic to the HaCaT cells with a %cell death larger than 30% (ISO, 2009:9), from a concentration of 150 µg/ml. The unloaded niosomes were non-cytotoxic, but the loaded niosomes indicated cytotoxicity from a concentration of 300 µg/ml and also, artemether was only partially released from the vesicles. However, these results could not be directly compared to *in vivo* environments, as there are other contributing factors in the *in vivo* environments, i.e. metabolism, excretion, distribution and absorption (Yoon *et al*., 2012:634).

The topical delivery of artemether encapsulated in niosomes and proniosomes, however unreliable, was still found in the epidermis-dermis and therefore can be seen as successful, as the vesicle systems fulfilled their function in delivering artemether to the epidermis-dermis (target area) (Karim *et al*., 2010:374). The low concentrations of artemether obtained in the epidermis-dermis during the skin diffusion studies can however be improved by increasing the permeation of artemether. This can be done via several methods, one of them is to increase the concentration of artemether in the formulation, i.e. for topical drug delivery one dose usually contains 2 – 5 mg/cm^2 API (Herkenne *et al*., 2008:87); other methods are to add chemical, physical or biochemical enhancers (Herkenne *et al*., 2008:8; Morrow *et al*., 2007:39) to the formulation or to research the use of a different nano-vesicle system.

Future recommendations:

- Extract receptor phase once after 12 h during the skin diffusion studies.
- Exploring other nano-vesicle systems to carry artemether, improving the solubility of artemether enough for adequate permeation into the skin.
- Use a different API formulated in a vesicle system, which also works well as treatment against CTB.

References

Ahmed, T.A., El-Say, K.M., Aljaeid, B.M. & Fahmy, U.A. 2016. Transdermal glimepiride delivery system based on optimized ethosomal nano-vesicles: Preparation, characterization, *in vitro*, *ex vivo* and clinical evaluation. *International Journal of Pharmaceutics*, 500(1-2):245-254.

Dos Santos, J.B., Figueiredo, A.R., Ferraz, C.E., De Oliveira, M.H., Da Silva, P.G. & De Medeiros, V.L.S. 2014. Cutaneous tuberculosis: epidemiologic, etiopathogenic and clinical aspects – Part 1. *Anais Brasileiros de Dermatologia*. 89(2):219-228.

Gillet, A., Compère, P., Lecomte, F., Hubert, P., Ducat, E., Evrard, B. & Piel, G. 2011. Liposome surface charge influence on skin penetration behaviour. *International Journal of Pharmaceutics*, 411(1-2):223-231.

Haynes, R. 2013. SA MRC flagship proposal executive summary. October 2013. [Correspondence]. 29 Jul. 2015, Potchefstroom.

Haynes, R.K. 2015. Development of oxidant and redox drug combinations for treatment of malaria, TB and related diseases. [PowerPoint presentation].

Hershkovitz, I., Donoghue, H.D., Minikin, D.E., Besra, G.S., Lee, O.Y-C., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Bar-Gal, G.K. & Spigelman, M. 2008. Detection and molecular characterization of 9000-year-old Mycobacterium tuberculosis from a neolithic settlement in the eastern Mediterranean. *PLoS ONE*, 3(10):1-6.

ISO (International Organization of Standardization) 10993-5. 2009. Biological evaluation of medical devices – part 5: Test for *in vitr*o cytotoxicity. 3rd ed. Geneva: ISO.

Shilakari, G., Singh, D. & Asthana, A. 2013. Novel vesicular carriers for topical drug delivery and their application's. *International Journal of Pharmaceutical Science Review and Research*, 21(1)77-86.

Miller, M.J., Walz, A.J., Zhu, H., Wu, C., Moraski, G., Möllman, U., Tristani, E.M., Crumbliss, A.L., Ferdig, M.T., Checkley, L., Edwards, R.L. & Boshoff, H.I. 2011. Design, synthesis and study of a mycobactin-artemisinin conjugate that has selective and potent activity against tuberculosis and malaria. *Journal of the American Chemical Society*,133(7):2076-2079.

More, S.B., Nandgude, T.D. & Poddar, S.S. 2016. Vesicles as a tool for enhanced topical drug delivery. *Asian Journal of Pharmaceutics*, 10(3):196-209.

Naik, A., Kalia, Y.N and Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical Science & Technology Today*, 3(9):318-326.

Uchechi, O., Ogbonna, J.D.N. & Attama, A.A. 2014. Nanoparticles for dermal and transdermal drug delivery. (*In* Sezer, A.D., *ed*. Application of nanotechnology in drug deliver. Nsukka: InTech. p. 193-235).

Van Zyl, L., Du Plessis, J. & Viljoen, J. 2015. Cutaneous tuberculosis overview and current treatment regimens. *Tuberculosis*, 95(6):629-638.

Yoon, M., Campbell, J.L., Andersen, M.E. & Clewell, H.J. 2012. Quantitative *in vitro* to *in vivo* extrapolation of cell-based toxicity assay results. *Critical Reviews in Toxicology*, 42(8):633-652.

York, P. 2013. Design of dosage forms. (*In* Aulton, M.E. & Taylor, K.M.G. *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 7-19).

APPENDIX A

METHOD VALIDATION FOR THE HPLC ASSAY OF ARTEMETHER

A.1 Introduction

In order to obtain an optimum method for the analysis of artemether alone, as well as in formulated vesicles, the validation of the HPLC method for artemether was performed under the supervision of Prof Jan du Preez. This validation was done to ensure the HPLC method for artemether was sensitive, responsive and consistent in the determination of the presence, as well as the concentration of the active (artemether) in the formulated vesicles. The method validation of artemether was executed jointly with Ms Esmari van Jaarsveld, as both of us investigated artemether as an API during our research. A single set of data was thus recorded.

A.2 Chromatographic conditions

The different chromatographic conditions regarding the HPLC analysis of artemether was determined during the method validation and discussed in Table A.1.

Table A.1: Chromatographic conditions used for the analysis of artemether

HPLC conditions for artemether	
Analytical instrument	An Agilent 1100 Series HPLC system equipped with a gradient pump, autosampler, UV detector and ChemStation Rev. A.10.02. data acquisition and analysis software was used (Agilent Technologies, Palo Alto, CA)
Column	A Venusil XBP, C_{18} (2), reverse phase column with dimensions of 150 x 4.6 mm and a particle size of 5 µm was used (Angela Technologies, Newark, DE)
Mobile phase	Analysis for the rest of the validation was done using a mixture of acetonitrile (ACN) and Milli-Q® water in a ratio of 70:30 (filtered/sonicated)
Solvent	Mixture of ACN and Milli-Q® water in a ratio of (70:30) (filtered/sonicated)
Injection volume	50 µl
Run time	15 min
Retention time	Approximately 9.469 min
Flow rate	1.0 ml/min
Detection	Using UV at 216 nm

A.3 Standard preparation

Standard solutions were prepared by weighing artemether according to the concentration needed, dissolving it in the solvent and making it up to volume in a 25 ml volumetric flask. An alternation of two different methods was used to prepare standard curves, i.e. 1) by means of dilutions and 2) by means of different injection volumes. When conducting the first method, five dilutions were prepared by taking 10 ml of the standard solution and using a pipette, making up to volume (20 ml) with the solvent. From each of these dilutions, 2 ml was placed in separate vials and from that, 50.0 µl was injected in duplicate. When executing the second method, a standard solution was prepared in a 25 ml volumetric flask, of which 2 ml was transferred to a vial. From this one vial, different injection volumes were injected on the HPLC in duplicate, i.e. 5.0, 7.5, 10.0, 25.0 and 50.0 µl.

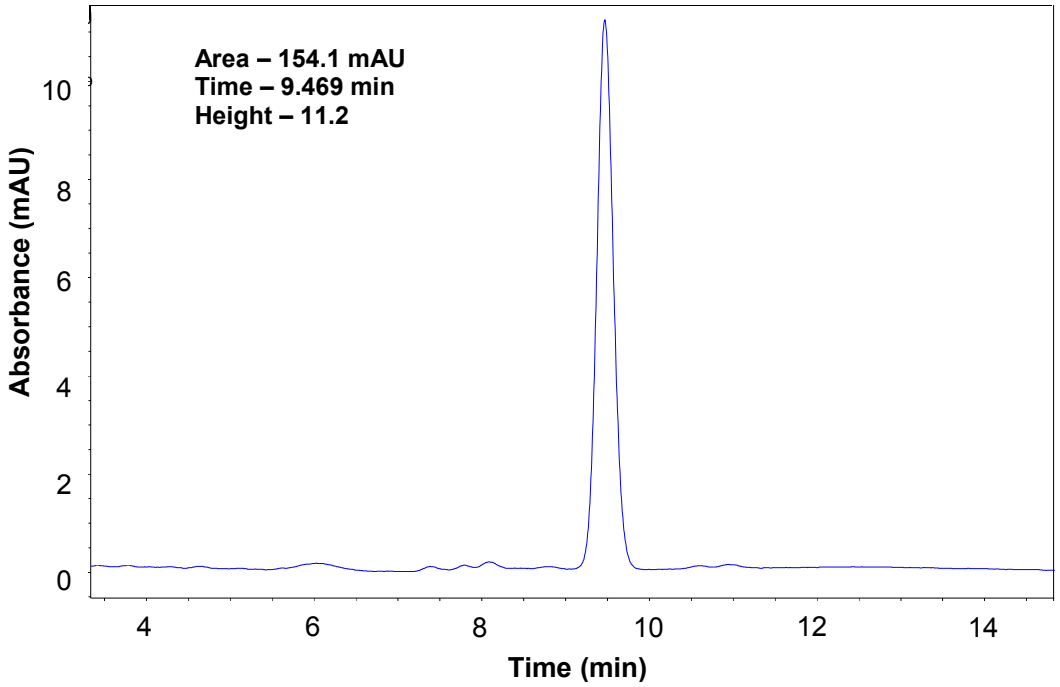


Figure A.1: HPLC chromatogram of a standard solution of artemether

A.4 Validation parameters

A.4.1 Linearity

During this analysis, the linearity between different concentrations (ranges) of one sample were determined. The concentrations of the samples must therefore be directly proportional to the area ranges (results) of the samples. These different ranges can be obtained by either preparing different dilutions of a standard sample or by preparing different samples, each having a different sample mass. During the analysis of linearity a minimum of five different

samples must be used (ICH, 2005:5&8). The acceptance criteria for the linearity of a method is a regression coefficient yield of $r^2 \geq 0.997$ (Shabir, 2006:7).

A standard solution was prepared by weighing 20.4 mg of artemether and transferring it into a 25 ml volumetric flask. The volumetric flask was made up to volume with ACN/water (70:30) mixture, then 10 ml of the standard solution was transferred into another 20 ml volumetric flask using a pipette and made up to volume using the ACN/water (70:30) mixture; this was the first dilution. Six more dilutions were prepared each time, obtaining 10 ml from the previous prepared dilution; this yielded seven dilutions in total, excluding the stock solution.

Table A.2: Concentrations and peak areas of artemether standards

Sample	Concentration (µg/ml)	Peak area		
		1	2	Average
Dilution 7	6.13	7.00	6.60	6.80
Dilution 6	12.25	15.60	15.50	15.55
Dilution 5	25.50	35.40	36.70	36.05
Dilution 4	51.00	78.10	78.10	78.10
Dilution 3	102.00	149.40	146.50	147.95
Dilution 2	204.00	298.60	300.90	299.75
Dilution 1	408.00	613.20	612.80	613.00
Stock solution	816.00	1252.90	1250.80	1251.85

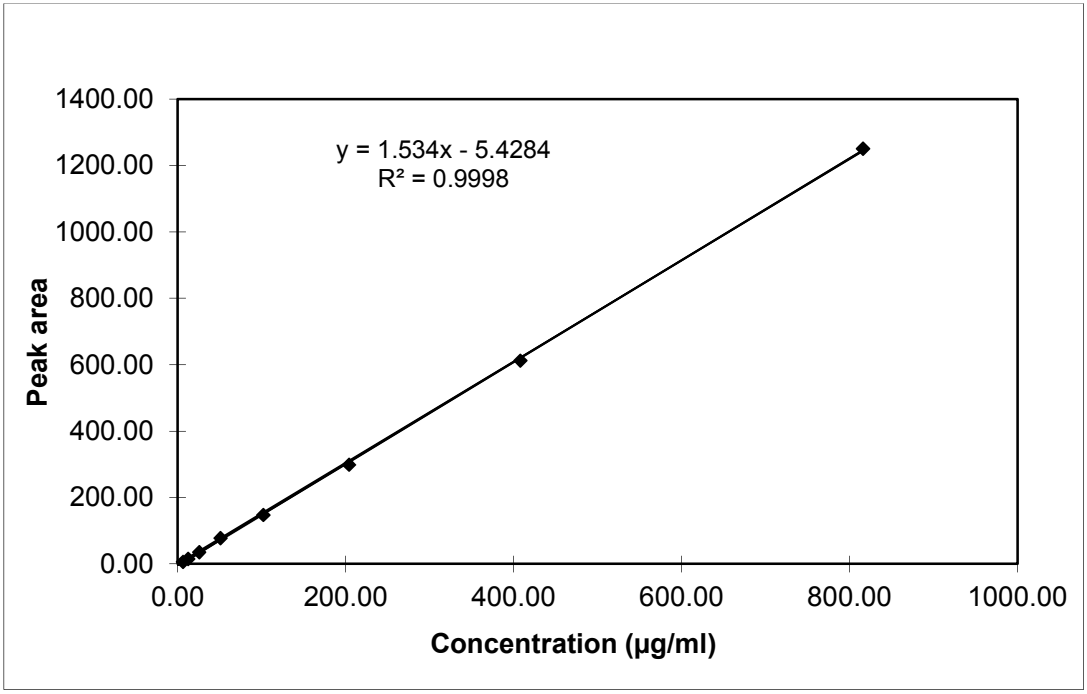


Figure A.2: Linear regression curve of artemether standards

From Figure A.2, it is observed that the method for artemether was linear over the concentration of 6.0 – 816.0 µg/ml. The regression value of 0.9998 falls within the accepted criteria range (Shabir, 2006:7), which indicates a good linearity of the analysis system.

A.4.2 Accuracy

The accuracy of an analytical procedure determines how closely related the true value obtained and the accepted reference value are to one another (ICH, 2005:4). The acceptance criterion for accuracy is a percentage recovery of 98 – 102% (Shabir, 2006:9).

The first step in the accuracy procedure is to choose one point on the regression line from the linearity results obtained. The concentration, 408 µg/ml, obtained from the regression line was chosen as the parameter for further analysis. The amount (mass) of artemether present in this dilution was 10.2 mg. Three samples with this concentration were prepared and two dilutions from each sample were prepared.

The masses of artemether weighed:

Sample 1:	10.22 mg
Sample 2:	10.16 mg
Sample 3:	10.25 mg
Standard:	11.56 mg

For Sample 1, 10.22 mg was transferred into a 25 ml volumetric flask, made up to volume using the ACN/water (70:30) mixture and then placed in the ultra-sonication bath to ensure a thoroughly dissolved solution. This stock solution was diluted twice: for the first dilution, 10 ml of the stock solution was transferred to a 20 ml volumetric flask and made up to volume using the ACN/water (70:30) mixture; the second dilution, was prepared by taking 10 ml of the first dilution and transferring it to another 20 ml volumetric flask and made up to volume using the ACN/water (70:30) mixture. This procedure was followed for all three samples.

A standard solution was prepared to obtain a standard curve. Artemether was weighed as 11.56 mg, transferred into a 25 ml volumetric flask and made up to volume using the ACN/water (70:30) mixture. Five dilutions were made from the stock solution: the first dilution was prepared by taking 5 ml from the stock solution and making up to volume in a 10 ml volumetric flask using the ACN/water (70:30) mixture; the rest of the dilutions were prepared exactly the same way, each time obtaining 5 ml from the previously prepared dilution.

Table A.3: Standard peak areas

Sample (Standard)	Concentration spiked (µg/ml)	Peak area		
		1	2	Average
Dilution 5	14.45	21.4	13.8	17.6
Dilution 4	28.90	41.1	40.9	41.0
Dilution 3	57.80	86.6	86.8	86.7
Dilution 2	115.60	162.3	160.9	161.6
Dilution 1	231.20	360.9	359.1	360.0
Stock solution	462.40	713.7	713.2	713.5

Table A.4: Accuracy parameters of artemether

Artemether sample (mg)	Concentration spiked (µg/ml)	Peak area			Recovery	
		1	2	Average	mg/100 ml	%
10.250	410.00	629.10	628.70	628.90	407.75	99.45
10.220	408.80	638.60	639.00	638.80	414.11	101.30
10.160	406.40	617.00	617.00	617.00	400.11	98.45
5.125	205.00	311.20	311.00	311.10	203.75	99.39
5.110	204.00	317.60	317.00	317.30	207.73	101.83
5.080	203.20	304.50	304.10	304.30	199.38	98.12
2.563	102.50	153.70	153.70	153.70	102.71	100.20
2.555	102.20	158.80	158.80	158.80	105.98	103.70
2.540	101.60	149.20	149.50	149.35	99.92	98.34
					Mean	**100.98**
					SD	1.51
					%RSD	1.50

The accuracy method for artemether yielded a mean recovery of 100.98% over a concentration range of 101.60 – 410.00 µg/ml. This percentage recovery falls within the acceptable criteria range (Shabir, 2006:9), therefore it is acceptable. The standard deviation (SD) indicates how closely related the different values of the %recovery results are in relation to the mean value, therefore a low SD value is required. The %relative standard deviation (%RSD) is just the SD value expressed as a percentage.

A.4.3 Precision

Precision is an analytical procedure where one standard (homogenous) solution is prepared and multiple samples of this standard solution are analysed under the exact same conditions (method). The closeness in agreement between the data obtained from all of these samples is

called the precision. This analytical procedure also involves the quantitative determination for the presence of any impurities in the sample (ICH, 2005:8-10; USP, 2012:879).

Precision is divided into three sections, i.e. 1) repeatability, 2) intermediate precision and 3) reproducibility. Only the repeatability and reproducibility were determined during this method experimentation.

A.4.3.1 Repeatability (intraday precision)

This analysis shows the closeness between the results obtained from the repetition of an analytical procedure over a short time range, conducted by one analyst using the same equipment (USP, 2012:879). The repeatability of artemether was determined using the same sample preparation and results obtained from the analysis of the accuracy of artemether (Section A.3.2). According to Shabir (2006:9), the %RSD of the intraday precision for a method validation must be ± 2%.

Table A.5: Repeatability (intraday precision) of artemether

Artemether sample (mg)	Concentration spiked (µg/ml)	Peak area			Recovery	
		1	2	Average	mg/100 ml	%
10.250	410.00	629.10	628.70	628.90	413.53	100.86
10.220	408.80	638.60	639.00	638.80	419.98	102.73
10.160	406.40	617.00	617.00	617.00	405.77	99.84
5.125	205.00	311.20	311.00	311.10	206.35	100.66
5.110	204.00	317.60	317.00	317.30	210.39	103.13
5.080	203.20	304.50	304.10	304.30	201.92	99.37
2.563	102.50	153.70	153.70	153.70	103.74	101.21
2.555	102.20	158.80	158.80	158.80	107.06	104.76
2.540	101.60	149.20	149.50	149.35	100.90	99.31
					Mean	101.32
					SD	1.76
					%RSD	**1.73**

The repeatability of the artemether method gave a %RSD of 1.73%, which falls within the acceptable criteria range (Shabir, 2006:9).

A.4.3.2 Reproducibility (interday precision)

This analysis shows the variance present between the results obtained from the same analytical procedure conducted in different laboratories (USP, 2012:879). The acceptance criteria for interday precision is a %RSD smaller than ≤ 2% (Shabir, 2006:9).

The interday precision for Day 1 was determined using the repeatability data (Section A.3.3.1). For the reproducibility of Days 2 and 3 the same method as for Day 1 was followed, the only difference was the amount of artemether used. The amounts were as follow:

Day 2:

Sample 1: 10.18 mg

Sample 2: 10.15 mg

Sample 3: 10.18 mg

Standard: 5.13 mg

Day 3:

Sample 1: 10.17 mg

Sample 2: 10.15 mg

Sample 3 10.19 mg

Standard: 5.10 mg

Table A.6: Reproducibility (interday precision) of artemether

Mass (mg)	Day 1	Day 2	Day 3	Between days
5.11	100.66	99.08	100.42	
5.08	103.13	97.59	96.07	
5.13	99.37	99.36	97.71	
Mean	101.05	98.68	98.06	99.26
SD	1.56	0.78	1.79	1.94
%RSD	1.54	0.79	1.83	**1.95**

The interday precision for artemether had a %RSD of 1.95%, which falls within the acceptable criteria range (Shabir, 2006:9).

A.4.4 Ruggedness

The same sample preparation was conducted for the system stability and the system repeatability. A stock solution was prepared by weighing artemether (5.08 mg), transferring it into a 100 ml volumetric flask and making it up to volume using the ACN/water (62:38) mixture. The stock solution was transferred to two HPLC vials.

A.4.4.1 System stability

One of the two vials mentioned in Section A.3.5 was used for the 24 h stability analysis. The HPLC system injected 50 µl of the sample, every hour for 25 h starting at 0 h. The degradation percentage over this period of 25 h was determined. The time (hours) it takes for the sample to degrade to as much as 2% is the maximum time that the sample is viable.

Table A.7: System stability analysis of artemether

Time(h)	Peak area	%Remaining
0	74.90	100.00
1	75.50	100.80
2	74.50	99.47
3	74.70	99.73
4	75.00	100.13
5	74.60	99.60
6	74.50	99.47
7	73.90	98.66
8	73.70	98.40
9	73.80	98.53
10	73.90	98.66
11	74.30	99.20
12	74.70	99.73
13	74.70	99.73
14	74.70	99.73
15	74.90	100.00
16	74.90	100.00
17	75.30	100.53
18	74.90	100.00
19	75.00	100.13
20	75.20	100.40
21	75.30	100.53
22	75.80	101.20
23	76.60	102.27
24	77.00	102.80
25	76.60	102.27
Mean	74.96	**100.08**
SD	0.82	1.09
%RSD	1.09	1.09

The stability of the artemether analysis method used showed that no degradation took place after 25 h; therefore, artemether is stable and viable for at least 25 h.

A.4.4.2 System repeatability

The other vial prepared (Section A.3.5) was used for the analysis of the system repeatability. From this sample 50 µl was injected seven times. According to the acceptable criteria the system repeatability must have a %RSD of 1% (Shabir, 2006:11).

Table A.8: System repeatability of artemether

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

The %RSD for the system repeatability of the artemether method is 0.54%, which falls within the acceptable criteria range, therefore, the system repeatability is acceptable (Shabir, 2006:11).

A.4.5 Robustness

During this analytical procedure, deliberate, but small, variations were made to the specific sample's method parameter, in order to establish whether the sample is affected by the variations. The degree to which the sample is affected by the variations determines the reliability of the sample used under normal conditions (ICH, 2005:5).

A standard sample was prepared: 5.00 mg of artemether was transferred to a 100 ml volumetric flask and made up to volume using the ACN/water (70/30) mixture. The standard sample was injected on the HPLC under normal conditions, which served as the template (control) to determine whether variation took place. The conditions of the artemether method were changed twice, in order to observe whether there were differences in the results. The different conditions changed were the injection volume (µl), flow rate (ml/min) and wavelength (nm) as seen in Table A.9.

Table A.9: Robustness of artemether method

Sample	Injection volume (µl)	Flow rate (ml/min)	Wavelength (nm)	Area	Retention time
Standard	50.00	1.00	216.00	83.55	9.36
1	45.00	1.20	210.00	86.47	7.79
2	55.00	0.80	220.00	96.57	11.67

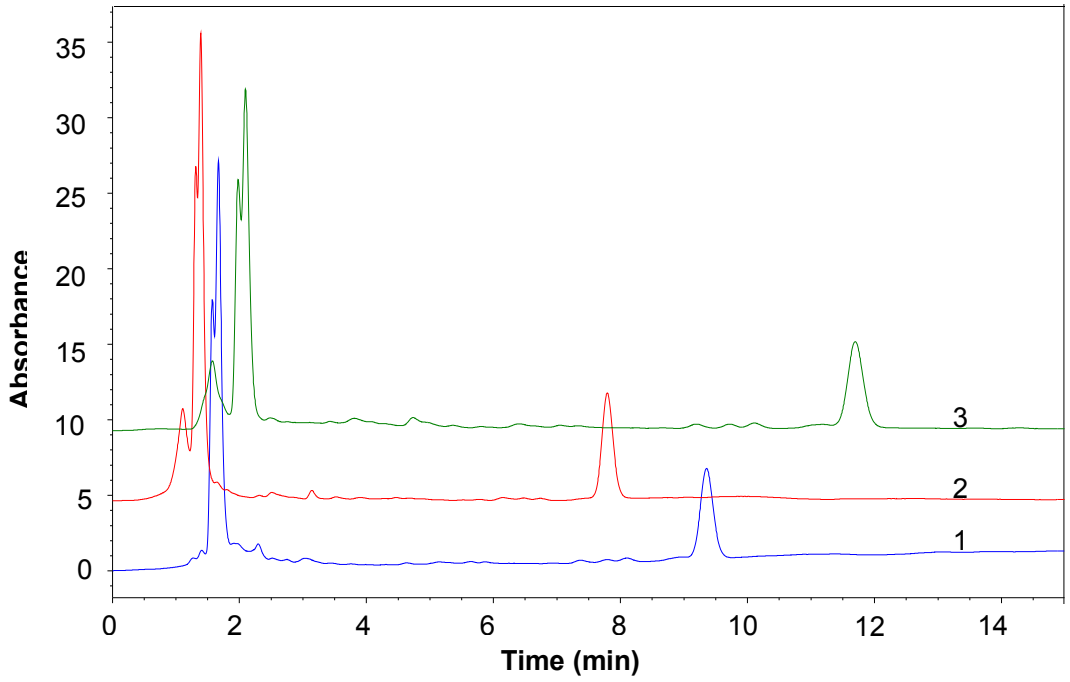


Figure A.3: Chromatograph from the HPLC, showing the graphs of artemether due to changing the HPLC conditions, i.e. 1) the standard chromatograph with a peak area of 83.55 mAU and retention time of 9.36 min, 2) Sample 1 chromatograph with a peak area of 86.47 mAU and a retention time of 7.79 min and 3) Sample 2 chromatograph with a peak area of 96.57 mAU and a retention time of 11.67 min.

Figure A.3 illustrates that the system condition changes did not cause great variation for the peaks obtained for artemether, in comparison to the standard peak (blue in colour).

In Table A.9, the retention times of Samples 1 and 2 changed respectively to the conditions altered and the peak areas obtained do not show significant variation, therefore the conclusion can be drawn that the reliability of the sample is good and acceptable.

A.4.6 Specificity

This analysis shows the ability to discriminate between the analyte and any other components that could be present during analysis and which could possibly interfere. These other

components include impurities, degradation products and matrix components (USP, 2014:1159). When there are no visible peaks interfering with the retention time of the analyte, the method is selective.

A blank sample containing only a mixture of ACN/water (70/30) was injected onto the HPLC system to illustrate and ensure the solvent does not contain any interfering peaks. A standard solution was prepared by transferring 5.00 mg of artemether to a 100 ml volumetric flask and making it up to volume using the solvent. Using a pipette, 1 ml of the standard solution was transferred to three different test tubes. Three different compounds were added to the three tubes, i.e. 200 µl of ammonium hydroxide (NH_4OH), 200 µl of hydrochloric acid (HCl) and 200 µl of hydrogen peroxide (H_2O_2), respectively. These three vials, along with one vial containing only the solvent and another containing the standard solution, were injected on the HPLC.

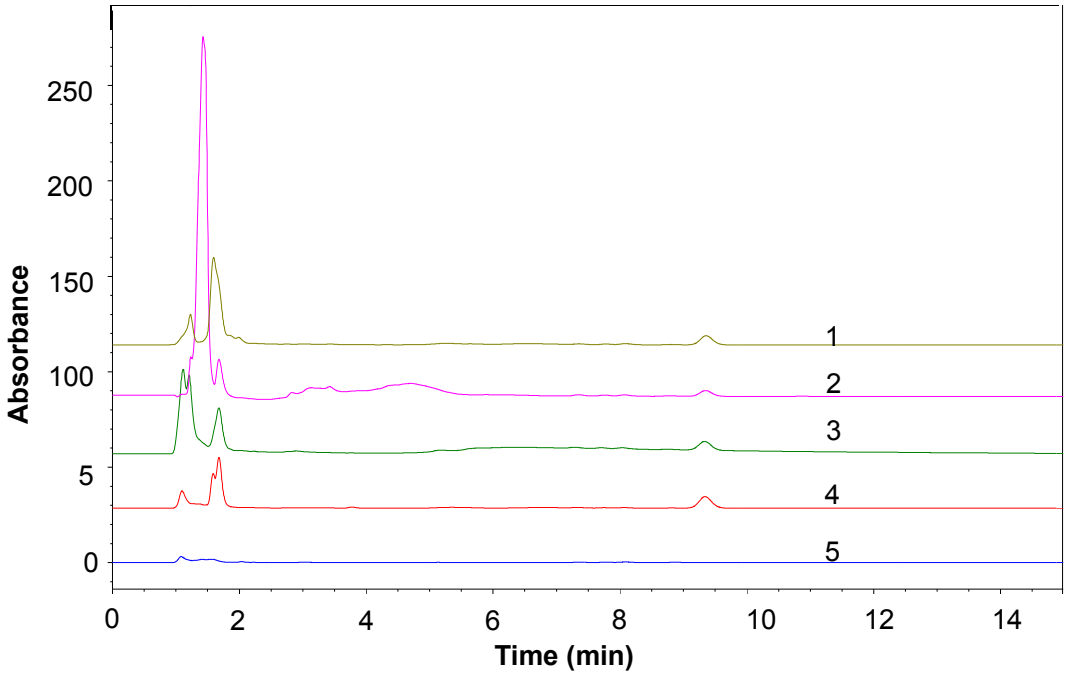


Figure A.4: Chromatograph showing the specificity of artemether in relation to 1) H_2O_2, 2) HCl, 3) NH_4OH 4) a standard and 5) a blank solvent.

Table A.10: Specificity of artemether

Sample	Peak area 1	Peak area 2	Average peak area
Blank	0.00	0.00	0.00
Standard	83.70	84.20	83.95
NH_4OH	63.20	63.40	63.30
HCl	41.20	41.10	41.15
H_2O_2	65.60	65.60	65.60

According to Figure A.4 and Table A.10, the three tested samples did not interfere at all with the retention times and chromatograph of artemether.

A.4.7 Limit of detection and limit of quantitation

During the analytical procedure limit of detection (LOD), the lowest amount (concentration) of analyte that can be detected was determined. Limit of quantitation (LOQ) is the determination of the lowest amount of analyte that can be determined with acceptable accuracy and precision (ICH, 2005:5).

Table A.11: LOD and LOQ of artemether

Weighed (5.08 mg)	LOD	LOQ	LOQ	LOQ	LOQ
Injection volume	10.00	20.00	30.00	40.00	50.00
Concentration (µg/ml)	**10.16**	**20.32**	30.48	40.64	50.8
Peak area	1.20	2.40	3.70	4.70	5.70
	1.00	2.40	3.80	4.70	5.70
	1.50	2.30	3.50	4.60	5.70
	1.40	2.50	3.70	4.70	5.70
	1.10	2.30	3.60	4.70	5.90
	1.40	2.40	3.50	4.50	5.60
	1.20	2.20	3.40	4.90	5.60
Mean	1.26	2.36	3.60	4.69	5.70
SD	0.17	0.09	0.13	0.11	0.09
%RSD	**13.35**	**3.83**	3.64	2.40	1.62

The LOD and LOQ values of artemether were determined by preparing a diluted solution from the stock solution prepared for the ruggedness analysis (Section A.3.5.). The dilution was prepared by taking 5 ml of the stock solution and transferring it to a 50 ml volumetric flask and making it up to volume using the ACN/water (70:30) mixture. This solution was thoroughly mixed, by means of the ultrasonic bath and then transferred to one vial. The sample was

injected in different volumes (10, 20, 30, 40 and 50 µl), and seven injections were made per injection volume, therefore achieving 35 analyses.

The detection limit of artemether at a concentration of 10.16 µg/ml has a %RSD of 13.35% and a quantitation limit of 20.32 µg/ml, with a %RSD of 3.83%, which falls within the criteria range of ≤ 15% (Rathmann, 2015:54).

A.5 Conclusion

According to all the analyses done and results obtained, the HPLC method validation for the artemether analysis was determined as sensitive, responsive and consistent. Therefore, the presence of artemether concentrations obtained using the HPLC will always be correct; this method is repeatable. This method should also be appropriate and reliable for the analysis of artemether encapsulated in formulated vesicles.

References

ICH Expert Working Group. 2005. ICH harmonized tripartite guideline: validation of analytical procedures: text and methodology Q2(R1). International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use.http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf Date of access: 15 Jul. 2015.

Rathmann, D., Rijntjes, E., Lietzow, J. & Köhrle, J. 2015. Quantitative analysis of thyroid hormone metabolites in cell culture samples using LC-MS/MS. *European Thyroid Journal*, 4(1):51-58.

Shabir, G.A. 2006. Step-by-step analytical methods validation and protocol in the quality system compliance industry. (*In* Institute of validation technology, *ed*. Analytical methods validation. Duluth: Advanstar Communications. p. 4-14).

USP see United States Pharmacopeia

United States Pharmacopeia. 2012. (1225) Validation of compendial procedures USP 37 - NF32. http://www.drugfuture.com/Pharmacopoeia/usp35/PDF/0877-0882%20[1225]%20VALIDATION%20OF%20COMPENDIAL%20PROCEDURES.pdf Date of access: 15 Nov. 2016.

APPENDIX B

FORMULATION OF ARTEMETHER ENCAPSULATED IN NIOSOMES AND PRONIOSOMES FOR TOPICAL DELIVERY

B.1 Introduction

Topical delivery of a drug is the deliverance of the drug onto the skin for a local effect. CTB is caused as a result of having the disease TB. In order to decrease the treatment period of CTB, it can be treated using targeted methods. These skin manifestations can be treated locally by means of topical drug applications (Rahimpour & Hamishehkar, 2012:141; Van Zyl *et al*., 2014:1-2).

The stratum corneum (outer most layer of the skin) acts as a rate-limiting barrier, causing difficulty during transdermal drug delivery across the skin. The diffusion of a drug through the different skin layers is directly proportional to the physicochemical properties of the drug. Colloidal vesicle systems, i.e. niosomes and proniosomes, have unique characteristics that are optimum for topical delivery, for example, increasing the absorption of the drug into the skin by entrapping the drug (Foldvari, 2000:417; Prauznitz & Langer; 2008:2065; Rahimpour & Hamishehkar, 2012:1).

The formula for these vesicles must be improved and then characterised until the optimum vesicle systems are obtained. The characterisation of the formulated vesicle systems will determine whether the vesicles changed the properties of the dispersion to such an extent that it will be suitable for membrane and skin diffusion studies. The characteristics of the niosome and proniosome dispersions should comply with the characteristics of the skin and be suitable for topical drug delivery in order to deliver successful results.

B.2 Materials

Several materials were used during the preparation of the vesicle dispersions with artemether entrapped. Different instruments were used in the formulation process as well as during the characterisation of the vesicle dispersions. These materials and instruments, used during the formulation and characterisation process, will be discussed in Figure B.1 and Tables B.1 and B.2.

Figure B.1: Some of the instruments used during the characterisation and preparation of niosomes and proniosomes: 1) viscosity meter, 2) pH meter, 3) Zetasizer and 4) sonicator

Table B.1: Information about the materials used during the formulation of niosomes

Niosomes			
Materials	**Suppliers**	**Batch numbers**	**Function in the dispersion**
Artemether	DB Fine chemicals	120702	API
Cholesterol	Sigma	421393	Additives
Span® 60	Fluka	85546	Surfactant
Chloroform	Merck	102245	Organic solvent
Milli-Q® water	Rephile	Direct Pure UP	Hydration agent

Table B.2: Information about the materials used during the formulation of proniosomes

Proniosomes			
Materials	**Suppliers**	**Batch numbers**	**Function in the dispersion**
Artemether	DB Fine chemicals	120702	API
Cholesterol	Sigma	421393	Additives
Span® 60	Fluka	85546	Surfactant
Chloroform	Merck	102245	Organic solvent
Milli-Q® water	Rephile	Direct Pure UP	Hydration agent
Sorbitol	Merck	107758	Hydrophilic carrier of the niosomes

B.3 General method for vesicle preparation

B.3.1 General method for the preparation of niosomes

In general there are several different methods available to prepare a niosome vesicle system, i.e. 1) thin-film hydration method (TFH), 2) hand shaking method (HSM), 3) ether injection method (EIM), 4) "bubble" method, 5) reverse phase evaporation method (RPE) and 6) freeze and thaw method (FAT), to mention a few (Moghassemi & Hadjizadeh, 2014:28-29).

It was decided the TFH method would be used during this study as, according to research, it is the most used method and gives good results. During the TFH method a rotary evaporator vacuum is used. Firstly, the surfactants, cholesterol and organic solvent are mixed together; if the drug used is lipophilic, it is also added to this mixture. This mixture is then placed inside a round bottom flask and attached to the rotary vacuum evaporator and placed inside a preheated water bath. The rotary vacuum evaporator removes the organic phase from the mixture, leaving a dry thin film on the side of the flask. The thin film is then hydrated with an aqueous solution. i.e. Phosphate buffered saline (PBS, a water-based salt solution or water, while adding gentle agitation to the mixture. If the drug used is hydrophilic, it will be added to the aqueous solution during hydration. The hydration process must take place at a temperature that is above the transition temperature of the surfactant, causing niosomes to form (Gurjar *et al*., 2014:427; Moghassemi & Hadjizadeh, 2014:28).

B.3.2 General method for the preparation of proniosomes

There are three different methods that can be used for the preparation of proniosomes: 1) the slurry method, 2) the slow spray coating method and 3) co-acervation phase separation method. During this study it was decided that the slow spray coating method would be used for the preparation of the proniosomes (Kaur *et al*., 2014:719-720; Udasi *et al*., 2012:3).

A water soluble carrier is used, i.e. sorbitol or maltodextrin. The carrier coats the niosomes and in turn, is covered with a thin film of surfactant. During the preparation of proniosomes, the carrier (sorbitol) is placed into a round bottomed flask. The surfactant, organic solvent and drug (if it is lipophilic) are mixed together and then lightly sprayed onto the sorbitol without over wetting the sorbitol bed. The round bottomed flask is then lowered into the preheated water bath connected to the rotary vacuum evaporator and left to dry. When the sorbitol is dry, the next aliquot of surfactant mixture is added onto the sorbitol bed and again left to dry. This step is repeated until all the surfactant is applied onto the sorbitol bed and the sorbitol is dry. The dry powder is transferred into a beaker and placed inside a desiccator overnight, or until it is needed. This dried powder is called the proniosomes and can be stored; when these vesicles are needed, it is hydrated. Upon hydration of the dried powder it transforms into niosomes (Sambhakar, 2012:236-237).

B.4 The development process of a product

During the development process of a product there are three steps, i.e. pre-formulation, early formulation and final formulation. These three steps are discussed below.

B.4.1 Pre-formulation of a vesicle

During pre-formulation, a research study is done on the drug that will be used in the preparation of the dispersion, prior to the physical formulation of a vesicle-system. The pre-formulation is done to ensure that a safe, stable and effective dosage form is created. During the pre-formulation procedure the physical and chemical properties of the drug is researched and determined. These properties include the drug solubility, partition coefficient, degradation process and formulation of other related drugs (Sahitya *et al.,* 2012:2311; Vilegave *et al*., 2013:1). This procedure was discussed and conducted by the means of a literature study.

B.4.2 Early formulation of niosomes and proniosomes

During the early formulation a trail-and-error approach was used where previously tested vesicle dispersions were prepared, tested and evaluated.

B.4.2.1 Problems encountered during the formulation process of a vesicle

B.4.2.1.1. Problems during the formulation of a niosome

During the first attempts, the tested niosome dispersion, developed by Miss Candice Csongradi (Csongradi, 2015:148), was prepared. There were many problems with this method regarding the drug, artemether. The hotplate used for the removal of the organic phase resulted in the

formation of a burnt sticky paste and the characteristics of the vesicles regarding the zeta-potential and encapsulation were not optimum.

It was decided the rotary evaporator would be used to dry the organic solution, as according to the articles it is the most common method used when preparing vesicles. The rotary evaporator regulates the set temperature automatically; therefore manual regulation is not needed as in the case of the hotplate.

Due to the difficulties encountered during the formulation process, this method was further researched and it became clear the wrong ratio of artemether, cholesterol and Span® 60 was used, it should be 1:2:1 instead of 1:1:2, therefore it was changed.

The temperature of the water bath used with the rotary evaporator was first set at 90 °C, but that caused the solution to burn and form hard bubbles like a crust, therefore the temperature was decreased to 40 °C. The rotary evaporator has a setting initiating the round bottomed flask to rotate; this rotation has a speed range (rpm) that stretches from 20 to 280. During the formulation of niosomes the rotation speed was set at 280 rpm. The medium round bottomed flask (± 250 ml) was used during the niosome dispersion preparation, because a larger surface area resulted in increased evaporation time. The Büchi R - 100 Rotavapor was used for the evaporation of the organic phases during the preparation of the vesicle systems.

Problems concerning the zeta-potential, particle size and encapsulation efficiency (EE%, amount of artemether entrapped inside the vesicle system) were still encountered. It was determined, concerning the zeta-potential, particle size and polydispersity index (PdI), the niosome dispersion must first be diluted 25 times (1 ml dispersion dissolved 24 ml of Milli-Q® water) in order to obtain acceptable results. The original rotation speed of 14 000 rpm used during the ultracentrifugation for the EE% determination, was changed to 25 000 rpm.

B.4.2.1.2 Problems during proniosome formulation

The proniosome dispersions were prepared using the rotary evaporator. The temperature of the water bath was reduced from 90 °C to 40 °C. First, a small round bottomed flask (250 ml) was used but it caused the dried particles to splatter into the nozzle of the flask which led to a loss of product, therefore a larger flask (500 ml) was used, which also accelerated the evaporation process. During the EE% determination, problems were encountered with regard to the ultracentrifugation. After the ultracentrifugation of the hydrated proniosomes, the dispersion was frozen, independent of whether the temperature was 4 °C or 25 °C, or whether the running time was 30 min or 5 min. A different method was tried by adding 1 ml of the dispersion to 24 ml of Milli-Q® water and then ultracentrifuging the sample for 30 min at 4 °C at a rotation speed of 25 000 rpm; this worked.

B.4.3 Final vesicle formula

The final vesicle formula was acquired on a trial-and-error approach and concerned the determination and selection of the optimal percentage vesicle dispersion. This selection took place by formulating three different percentage niosome dispersions (1%, 2% and 3%), following their characterisation. The best formula was chosen based upon the overall best dispersion characteristics. Six different characteristics were measured, namely:

- Zeta-potential
- Particle size (PdI)
- Viscosity
- pH
- EE%
- Morphology

B.4.3.1 Zeta-potential, particle size and PdI

In relation to the measurement of the zeta-potential, particle size (Z–average measured in d.nm) and the PdI of the vesicles, the Malvern® Zetasizer Nano ZS 2000 (Malvern® Instruments Ltd, Worcestershire, UK) was used. The niosome dispersions were prepared, following their dilution, by placing 20 ml of Milli-Q® water into a polytop and adding one drop of the dispersion solvent to the water using a syringe. This mixture was mixed thoroughly and placed inside a cuvette, each sample was analysed in triplicate.

Table B.3: Zeta-potential results of the different percentage niosomal dispersions

Niosome dispersion	Measurement			Average zeta-potential	Result quality
	1	2	3		
1%	-44.70	-45.00	-48.20	-46.60 ± 2.263	Good
2%	-49.70	-47.50	-49.90	-49.03 ± 1.332	Good
3%	-45.70	-46.40	-46.10	-46.067 ± 0.351	Good

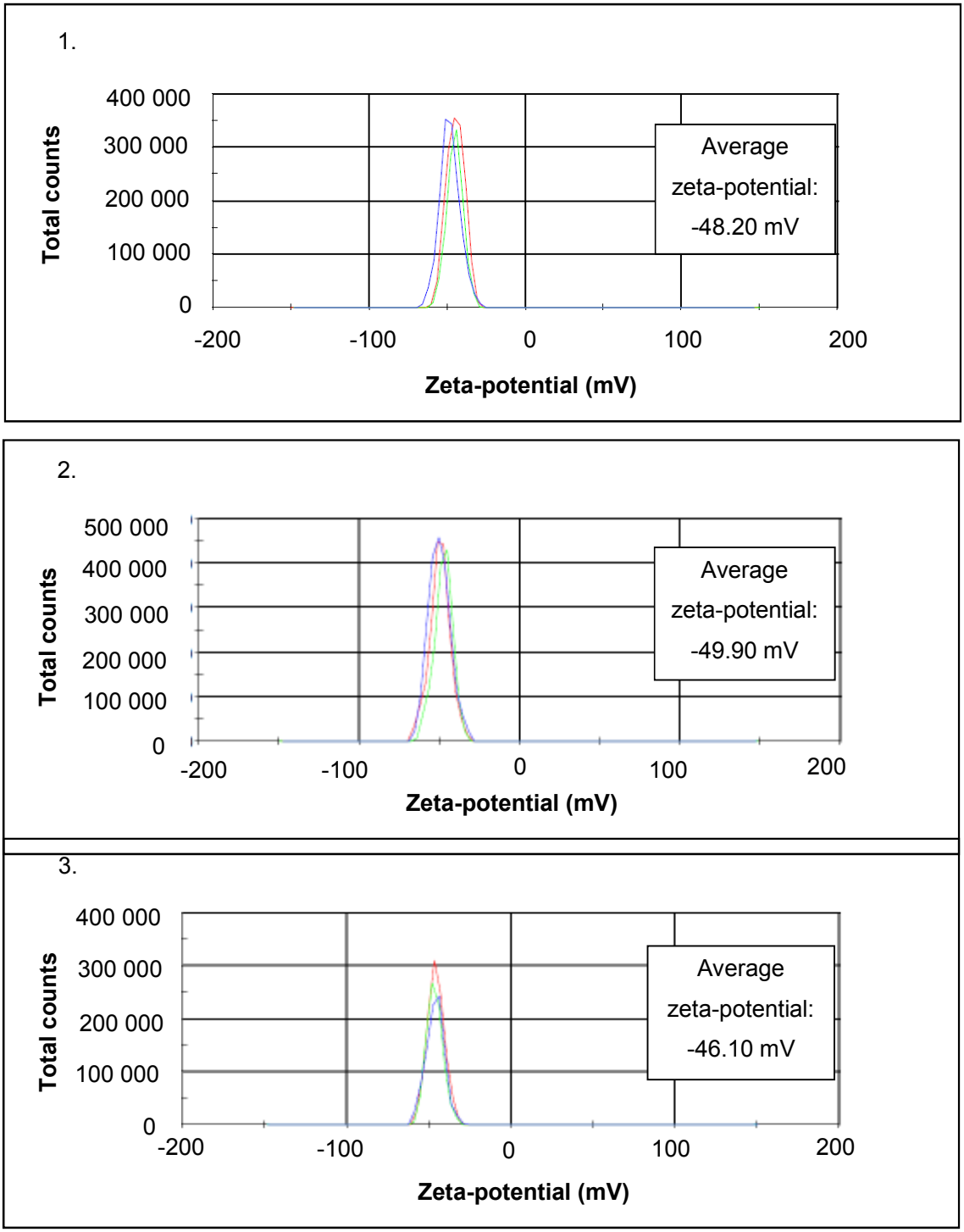


Figure B.2: Zeta-potential distribution of the niosome dispersions representing the comparison between three measurements obtained for each formula: 1) 1% artemether encapsulated in niosomes, 2) 2% artemether encapsulated in niosomes and 3) 3% artemether encapsulated in niosomes

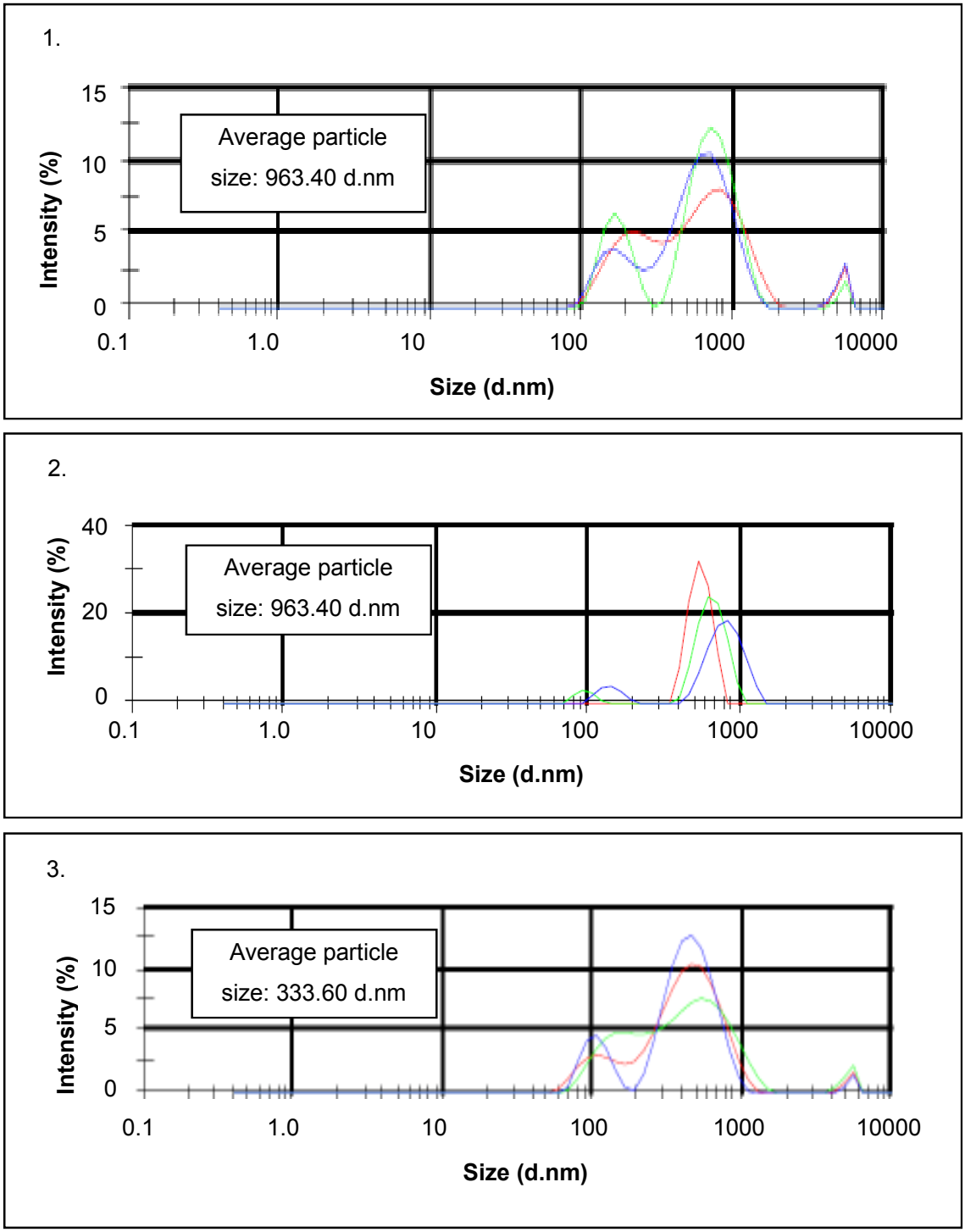


Figure B.3: Size distribution of niosome dispersions representing the comparison between three measurements obtained for each formula: 1) 1% artemether encapsulated in niosomes, 2) 2% artemether encapsulated in niosomes and 3) 3% artemether encapsulated in niosomes.

Table B.4: Polydispersity index (PdI) of the different percentage niosomal dispersions

Niosome dispersion	Measurement			Average PdI	Result quality
	1	2	3		
1%	0.584	0.530	0.532	0.55 ± 0.031	Good
2%	0.664	0.656	0.545	0.62 ± 0.067	Not good
3%	0.362	0.362	0.390	0.37 ± 0.016	Good

Table B.5: Size of niosomes (d.nm) in the different percentage niosomal dispersions

Niosome dispersion	Measurement			Average size	Result quality
	1	2	3		
1%	468.900	458.400	479.100	468.80 ± 10.350	Good
2%	1118.000	1172.000	963.400	1084.47 ± 108.268	Not good
3%	289.400	290.200	279.000	286.20 ± 6.248	Good

B.4.3.2 Viscosity

The viscosities of the vesicle systems were determined using the Brookfield Viscometer DV III Ultra (Middleboro, Massachusetts, USA; Begum *et al*., 2014:3; Shirsand *et al*., 2012:203). The three different niosome dispersions (1%, 2% and 3%) were formulated. An hour before the analysis took place, the water bath, which was connected to the Brookfield, was preheated and set to 25 °C then the vesicle dispersions were placed inside it. The T-bar spindle 18 (code: SC4-18) was used for the analyses of the viscosity of the different vesicle dispersions. Approximately 10 ml of the dispersion was placed inside the big sample adapter container and the analyses were done at a temperature of 25 °C for a total of 6 min. A measurement was taken every minute; therefore seven measurements were obtained per sample.

Table B.6: Results of the viscosity measurements of the different percentage niosomal dispersions

Niosome dispersion	Measurement (cP)							Average viscosity
	1	2	3	4	5	6	7	
1%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3%	9.960	9.960	9.960	9.960	9.960	9.96	9.96	9.96 ± 0.00

The 3% niosome dispersion was the only sample that gave a viscosity reading; the 1% and 2% niosome dispersions were too aqueous in texture, therefore no viscosity was registered.

B.4.3.3 pH

The pH of the vesicle systems was determined using a Mettler Toledo® Seven Multi pH meter equipped with an InLab® 410 electrode (Mettler Toledo, Greifensee, Switzerland). The electrodes of the pH meter was placed inside the dispersion and the calculate button pressed. After a couple of minutes the measurement stabilised and could be recorded. The pH of normal human skin is between 4.5 and 6, therefore, for optimum results regarding topical delivery the pH value of the vesicle dispersions should be the same as this, as a higher or lower pH value would harm the skin (Lingan *et al*., 2011:9; Nair *et al*., 2013:425).

Table B.7: pH measurements of the different percentage dispersions

Niosome dispersion	Measurement			Average pH
	1	2	3	
1%	3.620	3.640	3.620	3.63 ± 0.012
2%	4.900	4.920	4.930	4.92 ± 0.015
3%	4.840	4.840	4.840	4.84 ± 0.000

The pH of the 1% noisome dispersion fell below the criteria range. The 2% and 3% noisome dispersions gave pH readings above 4.5; therefore both these dispersions would not harm the skin and could be used for topical delivery (Nair *et al*., 2013:425).

B.4.3.4 Entrapment efficiency (EE%)

The EE% determines the amount of active (artemether) encapsulated inside the vesicles. The vesicle dispersions were placed inside 20 ml plastic test tubes respectively, and all the tubes had the same volume of solution within them. The vesicle dispersions were centrifuged using an Optima L-100 xP ultracentrifuge (Beckman Coulter, USA), at a rotation speed of 25 000 rpm, and at a temperature of 4 °C for 30 min using the 50.2Ti Rotor.

The proniosome dispersion was first diluted by taking 1 ml of the dispersion and filling up to 25 ml using Milli-Q® water, which was then centrifuged at a rotation speed of 25 000 rpm at a temperature of 4 °C for 30 min. The centrifugation process produced two separate phases, the supernatant phase contained the free un-entrapped API, while the pellet contained the artemether entrapped vesicles. Only the supernatant was analysed. The EE% was analysed using Equation B.1.

B.4.3.5.1 Calculation of the encapsulation efficiency of the niosomes

A 200 µg/ml concentration API standard solution was prepared as discussed in Section A.3. The default injection volume (50 µl) contained an artemether concentration of 10 µg/50 µl.

Table B.8: Calculation of the standard curve values

Sample	Injection volume	Concentration (µg/ml)	(µg/50 µl)	Area average
Dilution 4	5	20	1.0	29.85
Dilution 3	7.5	30	1.5	45.20
Dilution 2	10	40	2.0	60.60
Dilution 1	25	100	5.0	153.05
Standard	50	200	10.0	309.85

The encapsulation efficiency of artemether was determined by calculating the concentration of the artemether entrapped inside the niosome vesicles. The concentrations of the encapsulated artemether were determined for all three different niosome dispersions (1%, 2% and 3%). The EE% was determined using Equation B.1 (Nii & Ishii, 2005:200) in conjunction with the standard solution results.

$$EE\% = [(Ct-Co)/Ct] \times 100 \qquad \textbf{Equation B.1}$$

- Ct is the total concentration of artemether suspended in the dispersion
- Co is the concentration artemether that was free and un-entrapped (in the supernatant)

Three different amounts of artemether were weighed, 1) 1% dispersion – 100.4 mg, 2) 2% dispersion – 200.8 mg and 3) 3% dispersion – 300.5 mg for the preparation of the niosome dispersions.

The chromatograph results obtained from the standard solution were used to calculate the total concentration of artemether suspended in the dispersion (Ct) using Equation B.2.

$$C_1V_1 = C_2V_2 \qquad \textbf{Equation B.2}$$

- C_1 = Concentration of standard
- V_1 = Area of standard
- C_2 = Unknown value
- V_2 = Area of the supernatant

The percentage API encapsulated in all three percentage niosome dispersions was determined using this equation.

The EE% of the 1, 2 and 3% niosome dispersions were calculated as 99.30%, 99.66% and 99.77%, respectively. A high EE% signifies a high concentration of the API added to the dispersion were entrapped inside the vesicle, which will ensure higher bioavailability as well as a reduction in the amount of dosages needed (Raslan, 2013:17). All three niosome dispersions had high entrapment values, therefore all gave optimal results.

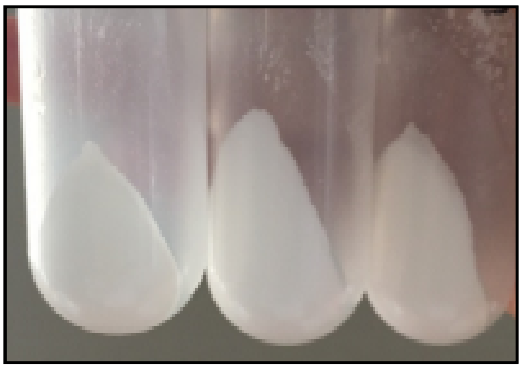


Figure B.4: The niosome dispersion pellets (containing the entrapped artemether) which formed after the ultracentrifugation

All the above characteristics were taken into account for the determination of the optimum formula.

Table B.9: Summary of the different percentage niosome dispersions regarding their characteristics

Characteristics	1%	2%	3%
Particle size (d.nm)	479.10	963.40	279.00
Zeta-potential (mV)	-48.20	-49.90	-46.10
EE% (%)	99.30	99.66	99.77
Viscosity	0.00	0.00	9.96
pH	3.63	4.92	6.25
PDI	0.53	0.55	0.39

Table B.9 presents a summary of all the characteristics obtained for the three different percentage niosome dispersions and the overall comparison between the different percentage dispersions.

The zeta potential and EE% values obtained for all three niosome dispersions (1%, 2% and 3%) were good and acceptable for topical delivery (Nnamani *et al*., 2014:214; Raslan, 2013:17). The pH values obtained from the 2% and 3% dispersions fell between the ranges of normal human skin (Nair *et al*., 2013:42).

Regarding the viscosity measurements, the 3% niosome was the only dispersion that was viscous enough to give a value, the other two dispersions were too aqueous in texture. The PdI and particle size distribution values of the 3% niosome dispersion were the smallest, indicating this dispersion was the most stable (Nobbman, 2014:1; Sezgin-Bayinder & Yuksel, 2012:833).

Overall, the 3% niosome dispersion had the best characteristic needed for topical drug delivery and was chosen as the best formula to give optimal results regarding the diffusion studies and further analysis during this study.

B.4.3.5 Transmission electron microscopy (TEM)

The morphology of the 3% niosomes and proniosomes was determined by means of a FEI Tecnai G2 200 kV high resolution TEM (Czech Republic, Europe). During the morphology analyses of the niosomes and proniosomes, no active can be present in the dispersion due to the sensitivity of the TEM. The 3% niosomes and 3% proniosomes were therefore formulated without any active (artemether), hence the use of the empty vesicles (placebos).

Preparation method for TEM:

- The prepared dispersion was diluted by adding six drops of the vesicle dispersion into ± 10 ml of Milli-Q® water then mixed thoroughly.
- One drop of the solution was placed on a copper grid and the excess solution was removed using filter paper.
- The solution was left to penetrate for ± 1 min.
- After 1 min, osmium was dropped onto the solution (the osmium evaporated and then oxidised the lipids in the dispersion).
- The dispersion was coloured with uranyl acetate, which led to binding sites forming once the dispersion bonded with the protein.
- This was followed by the staining of the dispersion with lead citrate, which attached to the binding sites that formed.
- The dispersion on the copper grid was left to dry for ±10 min following the analysis of the vesicles using the TEM (Bayindir & Yuksel, 2012:826).

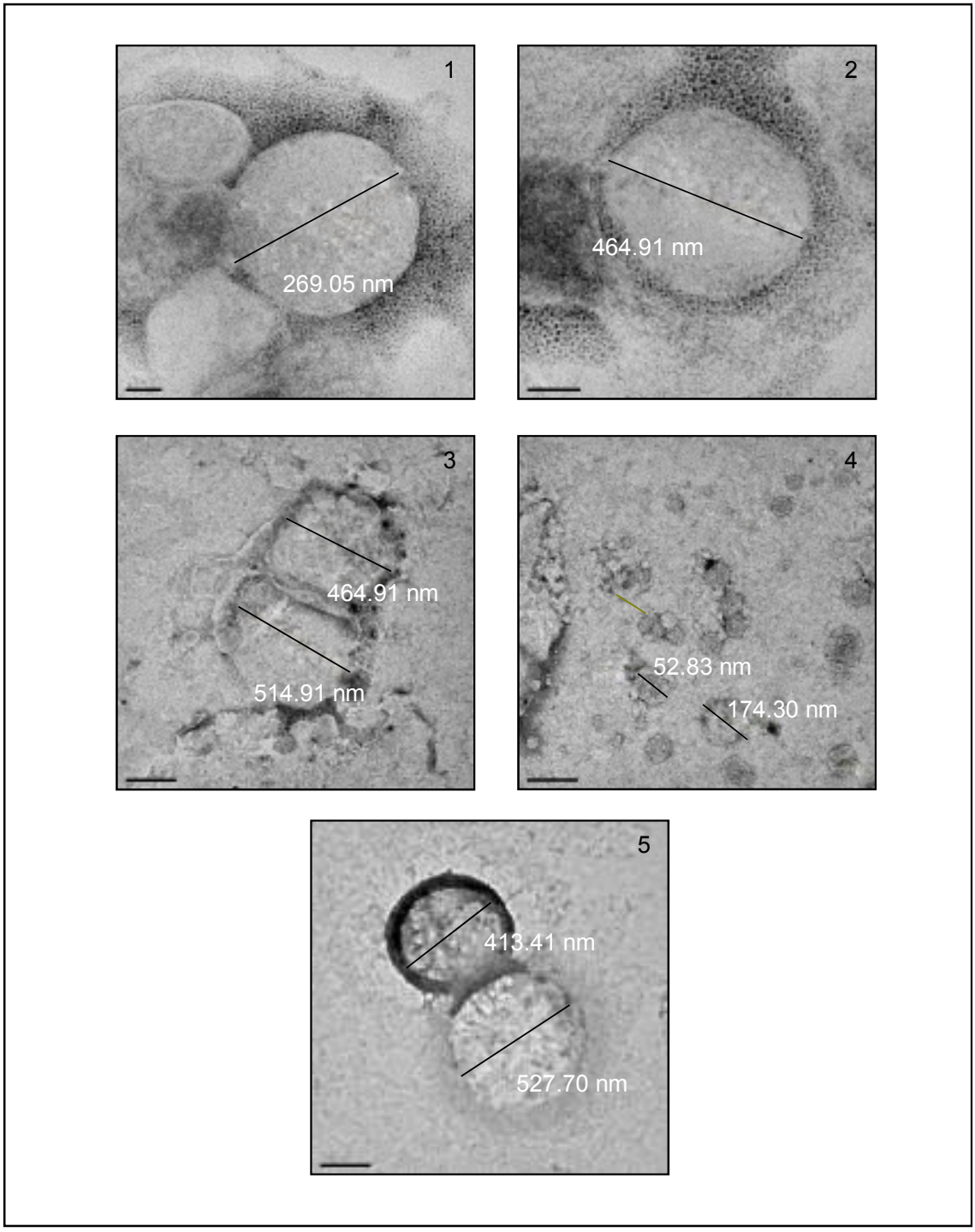


Figure B.5: Vesicle TEM analysis pictures: 1) niosome with a size of 269.05 nm, 2) niosome with a size of 199.70 nm, 3) two proniosomes with sizes 464.91 and 514.91 nm, 4) two proniosomes with sizes 52.83 and 174.30 nm 5) two proniosomes with the sizes 413.41 and 527.70 nm.

The acceptable particle size for drug delivery is between 50 and 500 nm. The sizes of the niosomes and proniosomes were between the criteria parameter, therefore the formulation, with regard to vesicle size, gave good results (Uchechi *et al*., 2014:193).

B.4.3.6 Final niosome formulation

Table B.10: Materials and quantities used during the final formulation of niosomes

Niosome (3%)		
Materials	**Quantity**	**Ratio**
Artemether	± 300 mg	1
Span® 60	± 300 mg	1
Cholesterol	± 600 mg	2
Chloroform	10 ml	
Milli-Q® water	10 ml	

The final niosome with active (artemether) formula:

- Artemether, Span® 60 and cholesterol was weighed according to Table B.13 and mixed in a beaker.
- The powder mixture was dissolved in ± 10 ml of chloroform.
- The solution was placed inside a small round bottom flask (± 250 ml).
- The flask was attached to the rotary evaporator and placed inside a preheated water bath set at a temperature of ± 40 °C.
- The solution was kept on the rotary evaporator until a thin layer (between see through and white in colour) formed on the side of the flask without any fluid. The drying of the solution on the rotary evaporator took between ± 0.5 – 1.0 h, under a pressure set at 200 mbar and a speed of 280 rpm.
- During the film hydration, a magnetic stirrer was placed inside the flask (to ensure gentle agitation) and 10 ml of lukewarm Milli-Q® water was added. The hydration took place on a magnetic heat-plate and was finished when a smooth milky colour formed.
- The solution was transferred to a beaker and left to cool to room temperature (25 °C).
- When the solution reached room temperature it was sonicated for 2.50 min while on ice.
- After sonication the dispersion was left to stand (at room temperature) for at least 2 h for the vesicles to swell.

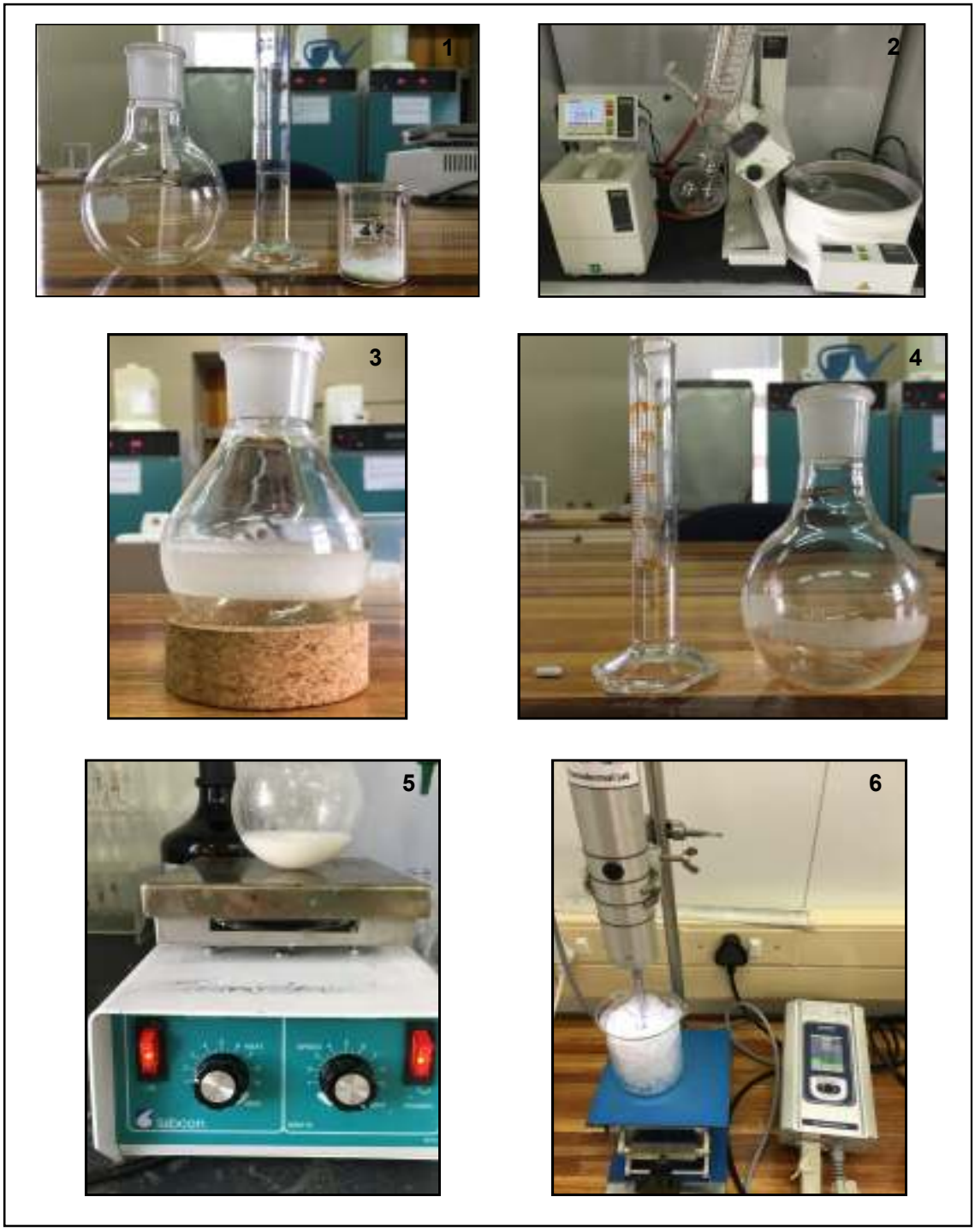


Figure B.6: Formulation process of the 3% niosomes: 1) the mixed powder chemicals, metered chloroform (10 ml) and the round bottomed flask that was used; 2) the rotary vacuum evaporator used; 3) the thin film that formed after all the chloroform evaporated; 4) the dried film in the round bottomed flask, magnetic stirrer and Milli-Q® water (10 ml) used for the hydration step; 5) the hydrated solution; 6) the sonication of the solution, which took place on ice.

During the formulation of niosomes without any active (placebo), the aforementioned method was followed, except no artemether was included in the solution. The drying time on the rotary evaporator vacuum for the empty niosomes (placebo) took ± 30 min.

Considering the optimum encapsulation results and the very good physicochemical characteristics results obtained from the 3% niosome dispersion (with and without API), the interaction between the formula chosen and the active ingredient worked well together. The high entrapment values indicate that high concentrations of artemether should diffuse through the stratum corneum barrier during the diffusion studies.

B.4.3.7 Final proniosome formulation

Since the 3% niosome dispersion was selected as the optimum formula, only 3% proniosome (with and without the API) dispersions were prepared. The standard curve discussed in Table B.8 was used for the EE% calculation of the 3% proniosomes.

B.4.3.7.1 Calculation of the encapsulation efficiency of the 3% proniosomes

The encapsulation efficiency of artemether was determined by calculating the concentration of the artemether entrapped inside the proniosome vesicles. The supernatant concentration was analysed on the HPLC in triplicate, therefore three concentrations were calculated.

The EE% was determined using Equation B.1 (Nii & Ishii, 2005:200) in conjunction with the standard solution results.

$$EE\% = [(Ct-Co)/Ct] \times 100 \qquad \textbf{Equation B.1}$$

- Ct is the total concentration of artemether suspended in the dispersion
- Co is the concentration artemether that was free and un-entrapped (in the supernatant)

The amount of artemether used during the preparation of the proniosome dispersion was 600.40 mg.

The chromatograph results obtained from the standard solution were used to calculate the total concentration of artemether suspended in the dispersion (Ct) using Equation B.2.

$$C_1V_1 = C_2V_2 \qquad \textbf{Equation B.2}$$

- C_1 = Concentration of standard
- V_1 = Area of standard
- C_2 = Unknown value
- V_2 = Area of the supernatant

The percentage API encapsulated in all three samples of the 3% proniosome dispersion was determined using this equation.

The EE% of the 3% proniosome dispersion was calculated as 93.83%, 94.00% and 93.91%, respectively. Eventually, the average EE% for the proniosome dispersion was 93.91 ± 0.085%, which is very good.

Figure B.7: The proniosome dispersion pellet containing the artemether entrapped vesicles, which formed after the ultracentrifugation

B.4.3.7.2 Final method for 3% proniosome dispersion

Table B.11: Materials and quantities used during the final formulation of proniosomes

Proniosome (3%)		
Materials	**Quantity**	**Ratio**
Artemether	± 300 mg	1
Span® 60	± 300 mg	1
Cholesterol	± 600 mg	2
Sorbitol	± 3 g	10
Chloroform	10 ml	3
Milli-Q® water	10 ml	3

The final proniosome dispersion with active (artemether) formula:

- Artemether, Span® 60 and cholesterol were weighed according to Table B.18 and mixed in a beaker.
- The powder mixture was dissolved in ± 10 ml of chloroform.
- The sorbitol was transferred to a large round bottomed flask (± 500 ml).
- The solution was dripped onto the sorbitol bed without over wetting the bed.

- The flask was attached to the rotary evaporator and placed inside a preheated water bath set at a temperature of ± 40 °C.
- The solution was kept on the rotary evaporator until all the sorbitol was dry and laid at the bottom of the flask.
- The procedure of dripping the solution onto the sorbitol, followed by the drying thereof, was repeated until all the solution had dripped onto the sorbitol bed and was dry, forming little swollen white grains. This drying process took between 30 and 60 min.
- The dry proniosomes were placed inside a beaker, which was stored in a desiccator overnight.
- The hydration process took place the day after the formulation process, or when it was needed.
- During the proniosomal hydration, a magnetic stirrer was placed inside the beaker (for gentile agitation) and 10 ml of lukewarm Milli-Q® water was added by dripping it onto the dry grains. The hydration took place on a magnetic heat-plate and was complete when a smooth milky-coloured fluid was obtained. This step caused the proniosome to transform into niosomes.
- The solution was left to cool to room temperature (25 °C).
- When the solution reached room temperature it was sonicated for 2.5 min whilst on ice.
- After sonication, the hydrated proniosome dispersion was left to stand (at room temperature) for at least 2 h so the vesicles could swell.

During the formulation process of proniosome dispersions without any active (placebo), the exact method from above was followed, without adding the artemether. The drying process of the sorbitol on the rotary evaporator vacuum of the empty proniosomes (placebo) took between 30 and 60 min.

Figure B.8: Formulation process of the 3% proniosomes: 1) the sorbitol (3 g) in the round bottomed flask, measured chloroform and mixed chemicals used; 2) the proniosomes after all the solution was dried onto the sorbitol; 3) the dried proniosomes; 4) the ingredients used during the hydration; 5) the hydration took place on a magnetic heat-plate; 6) the final proniosomal dispersions after hydration and sonication.

B.5 Characteristics of the final 3% vesicle systems

Once the final noisome and proniosome methods were determined and prepared, the physicochemical characteristics of these final vesicle dispersions were measured for a final time. These results, regarding the niosomes and proniosomes with API and the placebo's, will be discussed in Table 12 and Figure B.9.

Table 12: Final results obtained from the physicochemical characteristics of the 3% niosome and proniosome dispersions, with and without active.

Vesicle system	Characteristics					
	Zeta-potential (mV)	**PDI**	**Size distribution (d.nm)**	**Viscosity (cP)**	**pH**	**EE%**
Niosomes with API	-46.07	0.37	286.20	3.00	6.25	99.50
Niosome placebo	-42.17	0.22	180.57	3.39	6.29	None
Proniosomes with API	-37.63	0.86	798.13	8.63	5.54	93.91
Proniosome placebo	-39.60	0.45	185.07	5.04	6.36	None

The measurements of the physicochemical properties (including zeta-potential, PDI, size, viscosity and pH) of the niosome and proniosome dispersions were done 2 h after their preparation and again 24 h later, in order to determine whether the dispersions were stable for 24 h. According to the results obtained, the vesicle systems are stable for at least 24 h

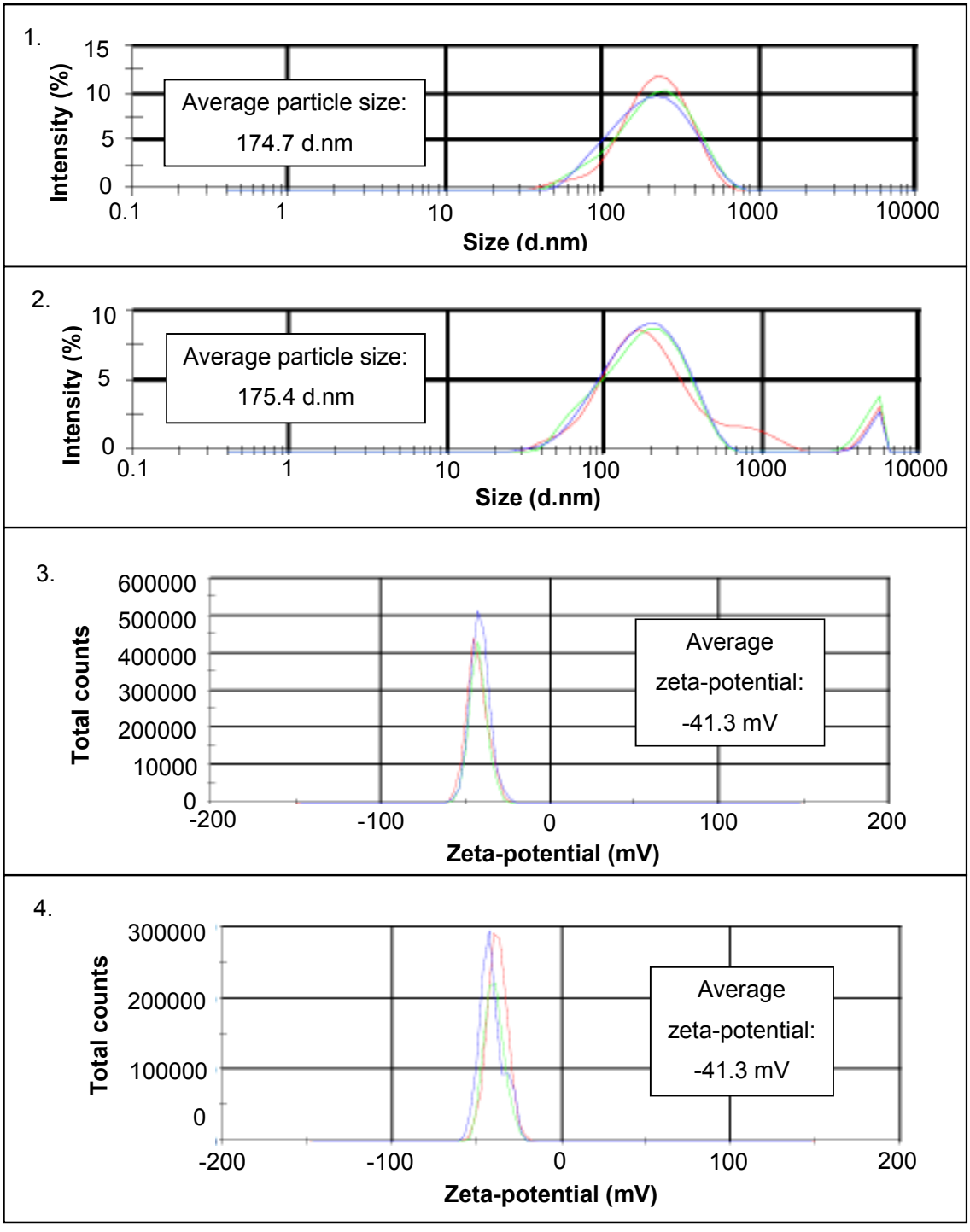


Figure B.9: The results (in triplicate) obtained for the placebo samples from the Zetasizer: 1) particle sizes of the niosome placebo; 2) particle size of the proniosome placebo; 3) zeta-potential of the niosome placebo; 4) zeta-potential of the proniosome placebo.

B.6 Discussion

During the formulation process of the niosomes and the proniosomes (with and without active), numerous changes were made to the early formula used as well as the standard methods, which took some time, but ultimately the best formulas were chosen. The 3% niosome and proniosome dispersions were chosen and shall be used for further experimental studies.

According to the physicochemical properties, the zeta-potential, particle sizes, PdI, viscosity and pH of the 3% vesicle dispersions, the vesicle systems should deliver good results during further analysis.

References

Bayindir, Z.S. & Yuksel, N. 2010. Characterization of niosomes prepared with various non-ionic surfactants for paclitaxel oral delivery. *Journal of Pharmaceutical Sciences*, 99(4):2049-2060.

Bayindir, Z.S. & Yuksel, N. 2012. Investigation of formulation variables and excipient interaction on the production of niosomes. *American Association of Pharmaceutical Scientists*, 13(3):826-835.

Begum, Y.M, Dasari, S., Sudhakar, M., Lakshmi, B.V.S. & Manga, K. 2014. Development and evaluation of co-encapsulated stavudine and lamivudine niosomes for the controlled delivery. *Der Pharmacia Sinica*, 5(1):1-10.

Csongradi, C. 2015. The effect of solid-state forms on the topical delivery of roxithromycin. Potchefstroom: NWU. (Dissertation - MSc).

Foldvari, M. 200. Non-invasive administration of drugs through the skin: challenges in delivery system design. *Pharmaceutical science & technology today*, 3(12):417-425.

Gurjar, P., Naik, N. & Chouksey, S. 2014. Niosome: a promising pharmaceutical drug delivery. *International Journal of Pharmaceutics and Drug Analysis*, 2(5):425-431.

Honary, S & Zahir, F. 2013. Effect of zeta potential on the properties of nano-drug delivery systems-a review (part 1). *Tropical Journal of Pharmaceutical Research*, 12(2):255-264.

Honary, S & Zahir, F. 2013. Effect of zeta potential on the properties of nano-drug delivery systems-a review (part 2). *Tropical Journal of Pharmaceutical Research*, 12(2):265-273

Kaur, R., Nagpal, M., Sidhu, R., Singh, S. & Jain, U.K. 2014. A review on proniosome, a promising carrier for delivery of drug across membranes. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(9):714-727.

Lingan, M.A., Sathali, A.A.H., Kumar, M.R.V. & Gokila, A. 2011. Formulation and evaluation of topical drug delivery system containing clobetasol propionate niosomes. *Scientific Reviews and Chemical Communications*, 1(1):7-17.

Moghassemi, S. & Hajizadeh, A. 2014. Nano-niosomes as nanoscale drug delivery systems: An illustrated review. *Journal of Controlled Release*, 185:22-36.

Nair, A., Jacob, S., Al-Dhubaib, B., Attimarad, M. & Harsha, S. 2013. Basic considerations in the dermatokinetics of topical formulations. *Brazilian Journal of Pharmaceutical Sciences*, 49(3):423-434.

Nii, T. & Ishii, F. 2005. Encapsulation Efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method. *International Journal of Pharmaceutics*, 298(1):198-205.

Nnamani, P.O., Hansen, S., Windbergs, M. & Lehr, C.L. 2014. Development of artemether-loaded nanostructured lipid carrier (NLC) formulation for topical application. *International Journal of Pharmaceutics*, 477(1-2):208-217.

Nobbman, U. 2014. Polydispersity - what does it mean for DLS and chromatography? http://www.materials-talks.com/blog/2014/10/23/polydispersity-what-does-it-mean-for-dls-and-chromatography/ Date of access: 31 Aug. 2016.

Particle Sciences –Technical Brief. 2012. An overview of the zeta potential. http:// http://www.particlesciences.com/news/technical-briefs/2012/overview-of-zeta-potential.html Date of access: 28 Jan. 2016.

Prausnitz, M.R. & Langer, R. 2008. Transdermal drug delivery. *Nature Biotechnology*, 26(11):1261-1268.

Rahimpour, Y. & Hamishehkar, H. 2012. Niosomes as carrier in dermal drug delivery. http://dx.doi.org/10.5772/51729 Date of access: 9 Mar. 2015.

Raslan, M.A. 2013. Effect of some formulation variables on the entrapment efficiency and in vitro release of ketoprofen from ketoprofen niosomes. *Journal of Life Me*dicine, 1(2):15-22.

Sahitya, G., Krishnamoorthy, B. & Muthukumaran, M. 2012. Importance of pre-formulation studies in designing formulations for sustained release dosage forms. *International Journal of Pharmacy & Technology*, 4(4):2311-2331.

Sambhakar, S., Singh, B., Paliwal, S. & Mishra, P.R. 2012. Sorbitol based proniosomes to improve the permeability and stability of an oral cephalosporin. *International Journal of Drug Delivery*, 4(2):236-245.

Sezgin-Bayindir, Z. & Yuksel, N. 2012. Investigation of formulation variables and excipient interaction on the production of niosomes. *American Association of Pharmaceutical Scientists*, 13(3):826-835.

Shirsand, S.B., Para, M.S, Nagendrakumar, D., Kanani, K.M. & Keerthy, D. 2012. *International Journal of Pharmaceutical Investigation*, 2(4):201-207.

Udasi, T.A., Wankhade, V.P., Ingle, L.M., Atram, S. & Tapar, K.K. 2012. Proniosome: a novel approach to vesicular drug delivery system. *International Journal of Pharmacy and Pharmaceutical Science Research*, 3(1):1-6.

Uchechi, O., Ogbonna, J.D.N & Attama, A.A. 2014. Nanoparticles for dermal and transdermal drug (*In* Sezer, A.D., *ed*. Application nanotechnology in Drug Delivery. Nsukka: InTech. p. 193-235).

Van Zyl, L., du Plessis, J. & Viljoen, J. 2015. Cutaneous tuberculosis overview and current treatment regimens. *Tuberculosis*, 95(6):629-638.

Vilegave, K., Vidyasagar, G. & Chandankar, P. 2013. Preformulation studies of pharmaceutical new drug molecule & products: an overview. *American Journal of Pharmacy and Health Research*, 1(3):1-20.

APPENDIX C

TOPICAL DELIVERY OF ARTEMETHER ENCAPSULATED IN NIOSOME AND PRONIOSOME DISPERSIONS

C.1 Introduction

Topical drug delivery is the delivery of drugs directly onto the skin for either local effects or systemic purposes. In this study, the topical delivery of artemether for local effects was determined. Topical drug delivery has several advantages when compared to other drug delivery routes, i.e. the oral or intravenous delivery routes. These advantages include: 1) deliverance of a drug to a specific site, 2) constant drug release, 3) no first-pass metabolism and 4) patients are more compliant, to mention a few. The good barrier properties of the skin, mostly residing in the stratum corneum, would make the topical delivery of a drug (especially a lipophilic drug) very difficult, therefore, vesicles were used as API carriers (Leite-Silvia *et al*., 2012:384-386; Ueda, 2009:750). Artemether has an aqueous solubility of 0.457 mg/ml (Human Metabolome database, 2013:2) and according to Naik *et al*. (2000:319); a drug should have an aqueous solubility larger than 1 mg/ml for transdermal drug delivery. The purpose of the niosome and proniosome vesicle systems were to serve as colloidal carriers of artemether. Lipid based vesicle carriers, such as niosome vesicles, have certain characteristics, such as being able to entrap both hydrophilic and hydrophobic compounds, giving it the capacity to diffuse across the skin. When an unfavourable API is entrapped inside a vesicle, it will be carried across the outer membrane (More *et al*., 2016:198; Shatalebi, 2010: 113; Uchechi *et al*., 2014:211).

Membrane release studies were done using polyvinylidene difluoride (PVDF) membranes to determine whether the encapsulated artemether was being released from the niosome and proniosome dispersions. Skin diffusion studies were performed using niosomes and proniosomes as vesicle carriers. These vesicle systems were used to determine whether the encapsulated artemether could cross the stratum corneum barrier and reside in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED) skin layers, which are the traits of topical delivery.

Prior to the execution of the membrane release and skin diffusion studies, the solubility of artemether in PBS (pH 7.4) and water, together with its log D and log P values, were determined. The samples analysed during the solubility, log D, log P and diffusion studies were analysed using HPLC.

C.2 Materials

Table C.1: Materials used during the membrane release and skin diffusion studies

Materials	Quantity during membrane study	Quantity during skin study
Caps	12	12
Clamps	12	12
Franz cells (donor and receptor phases)	12	12
HPLC vials	72	96
Magnetic stirrers (small)	12	12
Membranes	12	-
Needles	14	14
Parafilm® pieces	24	24
Plastic tubes	14	14
Polytops with caps	-	24
Syringes	16	16
Skin (pieces)	-	12
Tape strips	-	192
Vacuum grease	1 Tube	1 Tube
Water baths	2	2

C.3 Methods

C.3.1 Determination of the artemether concentration with HPLC

The determination of the concentration of artemether after the membrane release and skin diffusion studies was performed by means of HPLC (see Sections A.2 and A.3). Sampling was performed at predetermined times during the membrane release study and the receptor phase was analysed for the presence of artemether. During the skin diffusion studies, the receptor phase, tape strips (SCE) and the cut up skin pieces (ED) were analysed for the presence of artemether.

The HPLC analysis was done using a Venusil XBP, C_{18} (2), reverse phase column with a length of 150 mm, a diameter of 4.6 mm and a particle size of 5 µm (Agela Technologies, Newark, DE). The mobile phase used was the same as with the drug method validation (Section A.2); i.e. ACN and Milli-Q® water in a ratio of 70:30. The running time per sample was set at 15 min, with an injection volume of 50 µl. The solvent used for the preparation of the standards was ACN/Milli-Q® water in a ratio of 70:30.

C.3.2 Preparation of PBS (pH 7.4)

PBS (pH 7.4) was prepared by weighing NaOH (3.074 g) and $K_2H_2PO_4$ (13.61 g) and then dissolving it in 800 ml and 500 ml of Milli-Q® water, respectively. These two solutions were then mixed together and filled up to 2 l using Milli-Q® water. The mixture was thoroughly mixed by placing a magnetic stirrer inside the mixture and then positioning this onto a magnetic stirring plate. The pH of the solution was measured and ammonium drops were added until the pH was acquired as 7.4.

C.3.3 Aqueous solubility of artemether

The solubility of artemether was determined in both water and PBS (pH 7.4). According to literature, artemether is lipophilic, therefore a low water solubility was expected (Human Metabolome database, 2013:2; Sunil *et al*., 2010:93). Diffusion studies are mostly performed with PBS (pH 7.4) as the receptor phase, therefore the solubility of artemether was determined in PBS (pH 7.4).

Six test tubes were prepared; 3 ml of PBS (pH 7.4) was transferred to three tubes and 3 ml of Milli-Q® water to the remaining three tubes. An excess amount (to obtain a saturated solution) of artemether was added to each of the six tubes, followed by vigorous mixing of all the test tubes, by placing them inside a shaking water bath, set at 32 °C, for 24 h. After this period, 2 ml of each test tube solution was obtained and transferred to six separate HPLC vials and analysed using the HPLC.

C.3.4 Log D and log P determination of artemether

The shake-flask method was used to determine the log D and log P values, adapted from the methods described in the OECD council (1995:2-3) and Leo *et al*. (1971:537). In order to determine, a) the distribution of artemether in *n*-octanol and buffer and b) the partition of artemether in *n*-octanol and water, two separating funnels were prepared. One funnel contained the two immiscible solvents *n*-octanol and PBS at pH 7.4 (Funnel 1), whilst the other funnel contained the two immiscible solvents, *n*-octanol and Milli-Q® water (Funnel 2). In both funnels, the aqueous phase and the organic phase were distinctively visible with the aqueous phase as the bottom layer and the *n*-octanol the top layer. From both funnels, 20 ml was acquired from the *n*-octanol phases and from the PBS (pH 7.4) or Milli-Q® water phases and transferred to a beaker.

Artemether (1.0 mg) was added to both of the 20 ml *n*-octanol phases and both solutions were placed on the sonication bath for even distribution of artemether in the *n*-octanol. Six test tubes were prepared, three for the octanol-buffer (log D) and three for the octanol-water (log P). The

log D was prepared as follows: 3 ml of the *n*-octanol and artemether mixture was transferred to three test tubes, followed by 3 ml of the PBS obtained from Funnel 1. The log P was prepared as follows: 3 ml of the *n*-octanol and artemether mixture was transferred to three test tubes along with 3 ml of the Milli-Q® water obtained from Funnel 2. Eventually, all six tubes contained 6 ml of solution with the API, consisting of a 3 ml lipophilic- and 3 ml aqueous phase; all six tubes were placed inside a shaking water bath set at 32 °C for 24 h to obtain an equilibrium distribution.

After 24 h, six volumetric flasks (10 ml) were prepared. From all six of the test tubes, 1 ml was taken from the top phase (octanol) and transferred to separate volumetric flasks. All the volumetric flasks were filled up to 10 ml using methanol and placed onto the sonication bath. The diluted octanol (1 ml) was acquired and placed in six separate HPLC vials, thereafter 1 ml of the bottom phase (buffer or water) was transferred to six separate HPLC vials. These 12 vials were analysed on the HPLC system.

C.3.5 Preparation of donor phase for the diffusion studies

During each diffusion study (membrane and skin), an unloaded vesicle dispersion (placebo) and a vesicle dispersion, encapsulating artemether was analysed with HPLC. Both dispersions were prepared as described in Sections B.4.3.6 and B.4.3.7. The niosomes were freshly prepared along with the hydration of the proniosomes 24 h before the execution of the diffusion studies to ensure the vesicles would swell sufficiently and that the dispersion reached equilibrium. After preparation, the dispersions were left at room temperature, covered with Parafilm®. One hour before the diffusion study would commence, the dispersions were placed in a water bath set at 32 °C.

C.3.6 Preparation of the receptor phase for the diffusion studies

The receptor phase used during the membrane release studies and the skin diffusion studies differed due to the low aqueous solubility of artemether (lipophilic API) in PBS (pH 7.4). PBS (pH 7.4) was originally used as the receptor phase during the membrane release studies, but after four repetitions, it was determined that the artemether concentrations found in the receptor phase were too low. Consequently, it was concluded that artemether was not soluble enough in PBS (pH 7.4).

The purpose of the membrane release studies was to determine whether the vesicles would release the entrapped artemether, therefore, artemether must be soluble in the receptor phase. During the search for a receptor phase, which could be used during both the membrane release and the skin diffusion studies, the solubility of artemether was determined in a mixture of ethanol and PBS (pH 7.4) in the ratio of 1:9. However, it was found that artemether was not

soluble enough in ethanol:PBS (1:9). Subsequently, the solubility of artemether was determined in three different organic solvents, i.e. ethanol (99.9%), THF and isopropanol. Artemether was not at all soluble in isopropanol, but was soluble in THF and ethanol (99.9%). THF could not be used as receptor phase as it has shown to react with the cellotape used during tape stripping. Consequently, ethanol (99.9%) was selected as the receptor phase during the membrane release studies, since artemether was soluble in ethanol (99.9%) (Nasir *et al*., 2012:1165).

Ethanol was not used as the receptor phase during the skin diffusion studies, because it would not suffice. During skin diffusion studies, ethanol would serve as a penetration enhancer, because it would extract the lipids from the stratum corneum due to its affinity for lipids (Lachenmeier, 2008). Ethanol would also damage the skin and therefore the true topical delivery of artemether in an *in vivo* environment would not be represented (Lachenmeier, 2008). The use of two different receptor phases for the membrane release studies and skin diffusion studies was not considered a problem, as it was expected that artemether would only be delivered topically (SCE and ED) and not in the receptor phase during the skin diffusion studies (Uchehi *et a*l., 2014:212). Therefore PBS (pH 7.4) was used as the receptor phase during the skin diffusion studies, while ethanol (99.9%) was used as the solvent in which the tape strips (SCE) and pieces of skin (ED) were placed overnight after the skin diffusion studies.

C.3.7 Membrane release studies

The apparatus used in the membrane and skin diffusion studies are shown in Figure C.1. The vesicle systems (niosomes and proniosomes), with and without (placebo) the API, were prepared and left to stand for 24 h before the commencement of the membrane release study in order for the vesicles to swell out. One hour before the diffusion would start, the water baths were switched on, one at 37 °C and the other at 32 °C. The receptor phase (ethanol (± 250 ml)) was placed inside the 37 °C water bath and the donor phase inside the 32 °C water bath. The flat sides of the receptor and donor compartments of all 12 Franz cell units were greased using Dow Corning® high vacuum grease. A magnetic stirring rod was placed inside the receptor compartment of each Franz cell. The membrane was placed on top of the receptor compartment (rough side facing upwards) and thereafter the two compartments were placed on top of each other and sealed at the sides using the Dow Corning® high vacuum grease. The receptor and donor phases of all 12 Franz cell units were clamped together.

Figure C.1: Diffusion study workstation: 1) vacuum grease used; 2) Franz cells with membrane/skin in between; 3) clamped Franz cells; 4) syringes with needles and tubes used for the extraction of the receptor phases; 5) Franz cells placed inside the water bath; 6) extracted receptor phases in HPLC vials.

While holding the cell downwards, the receptor compartment tube was filled with 2 ml of the receptor phase using a syringe, making sure no bubbles formed inside the receptor phase. The

donor phase containing the active (1 ml) was placed inside the donor compartment of 10 Franz cells and 1 ml of the donor phase containing the placebo into the remaining two Franz cells. All the donor compartment openings were sealed with two pieces of Parafilm® and a cap to prevent evaporation of the donor phase. The 12 Franz cells were placed on top of the magnetic stirrer plate inside the water bath set at 37 °C and the water bath was closed with a lid. Once the first cell was placed, the time was noted, and 1 h after the noted time, the first extraction was performed.

After the first hour of the release study, the volume of the ethanol in the receptor phase compartment tube was noted, to determine whether it stayed the same, increased or decreased. The noted ethanol was extracted from each receptor compartment using a syringe with an injection needle and a tube attached. The receptor compartments were then refilled with fresh 37 °C ethanol. The extracted receptor phases were placed into numbered HPLC vials, respectively. A standard was prepared to run in conjunction with the vials containing the extracted receptor phase on the HPLC. Every hour for 6 h an extraction was made, concluding with six extractions per Franz cell in total. After each extraction, the extracted receptor phases were placed onto the HPLC system for analysis. The analysis of the artemether membrane release studies, using the HPLC, ran between 25 – 27 h in total. Sufficient mobile phase was prepared for the analysis of all of the samples, which was approximately 1700 ml in total. This membrane release method was adapted from the methods described in Joshi (2012) and Ternullo *et al*. (2017:336).

C.3.8 Skin diffusion

C.3.8.1 Skin preparation

Full thickness Caucasian female skin was obtained from abdominal plastic surgeries. Once the skin was obtained, it was kept frozen at - 20 °C until required for preparation. Each patient donating skin completed an informed consent form giving us permission to use their skin for research purposes. The skin diffusion studies were executed in a Type 2 laboratory that has been approved for research on human skin by the Research Ethics Committee of the North-West University (reference number: NWU-00114-11-A5).

A few days before it was needed, the skin was defrosted and the full-thickness of skin was inspected. The skin parts without any stretch marks, scars or too much hair were prepared using the Zimmer™ electric dermatome Model 8821 (Zimmer, Ohio, USA), to cut the skin to a 400 µm thickness. The dermatomed skin was placed onto Whatman® filter paper with the stratum corneum facing upwards. The skin was stretched out tightly, avoiding any folds. The Whatman® filter paper containing one donor was wrapped inside one piece of aluminium foil,

with the preparation date and the name of person who performed the preparation written upon it. This aluminium foil was placed inside a plastic bag and kept frozen at - 20 °C until required.

The morning of the skin diffusion study, the frozen skin was cut out into circles with a diameter approximately the same as that of the diffusion area of the donor and receptor compartments. The cut out skin was placed on top of the diffusion area of the receptor compartment, with the stratum corneum facing upwards and the filter paper facing downwards.

C.3.8.2 Diffusion study preparation

The skin diffusion of artemether encapsulated in the niosomes and proniosomes was executed in the same manner as discussed in Section C.3.7, with three exceptions. The first exception was that instead of membranes, the prepared dermatomed skin (Section C.3.8.1) was placed between the receptor and donor phases. Secondly, the receptor phase was PBS (pH 7.4) instead of ethanol (99.9%) and lastly, the receptor compartments were extracted and replaced with fresh PBS (pH 7.4) every 2 h for up to 12 h; therefore there were six extractions in total.

C.3.8.3 Tape stripping

The following tape stripping method executed was adapted from the method described in Pellet *et al*. (1997:94). After the final sample extraction (at 12 h), the skin was removed from the Franz cell units without damaging it. The excess donor phase was removed from the skin by lightly patting it with paper towel. The skin was then stretched and pinned onto a filter paper. Sixteen strips of Scotch® Magic Tape™ were cut for each skin piece (192 strips in total) and were used to remove the top layer of the skin, the SCE. The first strip was discarded because it contained excess donor phase. The other 15 strips containing parts of the SCE were all placed inside one polytop containing 5 ml of ethanol, ensuring the ethanol covered all of the strips. This was done for all 12 pieces of skin. After tape stripping, the diffused skin (ED) was cut into small pieces and placed into a separate polytop containing 5 ml ethanol; this resulted in 24 polytops, all of which were placed in the fridge for 8 h and then transferred to HPLC vials (24 in total) by means of filtration. These 24 samples were run on the HPLC for analysis, which took between 7 and 8 h.

C.4 Results and discussion

C.4.1 Aqueous solubility of artemether

The solubility of artemether in water and PBS (pH 7.4) was found to be 0.11 ± 0.002 mg/ml and 0.09 ± 0.003 mg/ml, respectively (Table C.2). According to Naik *et al*. (2000:319), the aqueous solubility of a drug should be above 1 mg/ml to give optimum results during topical and transdermal drug delivery. The solubility of artemether in both water and PBS (pH 7.4) was below the criteria set for optimal drug delivery. Due to the fact the aim of this study was to

deliver artemether topically it was argued that it could still be viable to investigate vesicle delivery of the drug.

Table C.2: Solubility of artemether in water and PBS (pH 7.4)

Sample	Injection volume (µl)	Peak area			Concentration	
		1	2	Mean	(µg/ml)	mg/ml
PBS (pH 7.4)	50	133.6	141.6	137.60	87.03	0.09
	50	148.5	145.1	146.80	92.82	0.09
	50	140.3	143.8	142.05	89.83	0.09
Average of each sample						**0.09 ± 0.003**
%RSD						**2.63**
Water	50	160.4	165.4	162.90	102.96	0.10
	50	167.6	171.9	169.75	107.28	0.11
	50	168.5	165.5	167.00	105.55	0.11
Average of each sample						**0.11 ± 0.002**
%RSD						**1.68**

C.4.2 Log D and log P of artemether

Table C.3: Determination of the log P and log D values of artemether

Sample	Mean	Concentration (µg/ml)	Ko/w	Log P or log D
Octanol-water	6964.00	4387.2	208.21	2.32
	6983.85	4399.7	200.41	2.30
	7137.30	4496.4	199.1	2.30
	6873.70	4330.3	193.37	2.29
	5077.00	3198.5	138.35	2.14
	6655.35	4192.8	170.66	2.23
Average log P				**2.26 ± 0.117**
%RSD				**1055.94**
Octanol-PBS	6835.79	4306.4	217.37	2.34
	7073.40	4456.1	235.8	2.37
	6788.35	4276.5	216.55	2.34
	6771.95	4266.2	352.74	2.55
	7003.15	4411.8	152.25	2.18
	6515.60	4104.7	215.06	2.33
Average log D				**2.35 ± 0.067**
%RSD				**2557.07**

During this study, the log D and log P of artemether was determined and found to be 2.35 ± 0.067 and 2.26 ± 0.117, respectively (Table C.3). The log D and log P of a drug should be between 1 and 3 to ensure the best results regarding permeation into and through the skin (Hadgraft, 2004:292; Mbah, 2011:680). Artemether falls within the criteria and therefore could qualify as a candidate for topical delivery. However, drugs that are slightly lipophilic are more favourable for topical drug delivery, but highly lipophilic drugs could be problematic due to the stratum corneum also being lipophilic; highly lipophilic drugs would stay in the stratum corneum instead of diffusing through to the next skin layers, i.e. the epidermis and dermis (Naik *et al*., 2000:319; Williams, 2013:680).

C.4.3 Membrane release studies

Membrane release studies were performed to determine whether the encapsulated artemether was being released from the carrier vesicles, niosomes and proniosomes.

Table C.4: Release of artemether from the two different vesicle carriers

Formulation	Ave flux (µg/cm².h)	Ave %released
Niosome encapsulating artemether	73.58 ± 8.64	0.799 ± 0.096
Proniosome encapsulating artemether	63.91 ± 7.17	0.712 ± 0.080

When comparing the two vesicle systems with each other in regards to their membrane release results (Table C.4), it is observed that the niosome carriers had better drug release than that of the proniosome carriers. According to Chandra and Sharma (2008:190), the presence of sorbitol in a formulation could result in slow release during *in vitro* diffusion studies. The proniosome vesicles contain sorbitol, which could be the reason for better release observed from the niosome carriers.

The concentrations of artemether that diffused through the PVDF membranes into the receptor phase (ethanol) in all 10 Franz cells were an indication that the vesicles did release the entrapped artemether. The average cumulative amount per area of artemether released from the niosomes and proniosomes is shown in Figures C.2 and C.4, respectively. The small standard deviation was an indication of repeatable results.

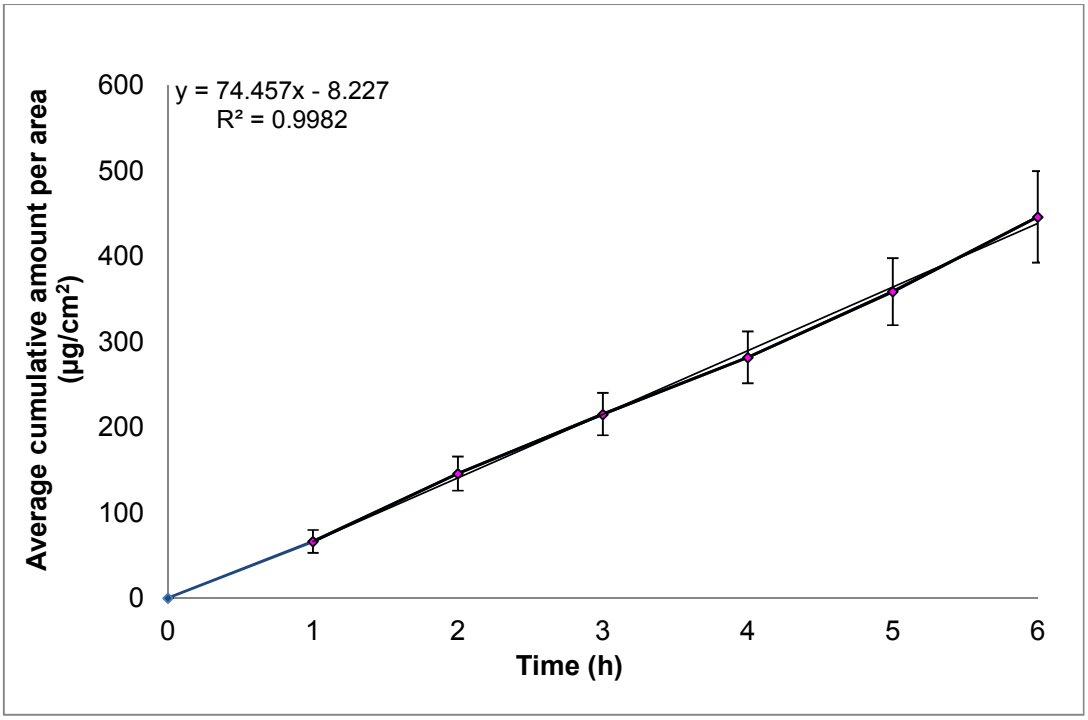


Figure C.2: The average cumulative amount per area (µg/cm^2) of artemether released from the niosome carriers into the receptor phase over a period of 6 h (n = 10)

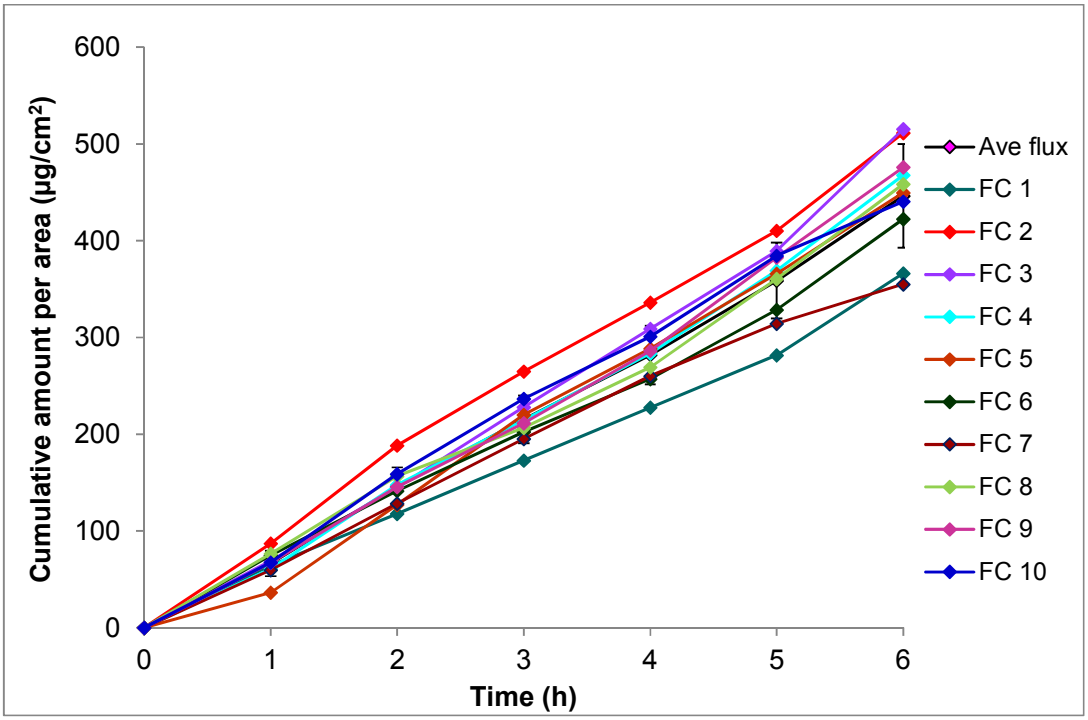


Figure C.3: Cumulative amount per area (µg/cm^2) of artemether released from the niosome carriers over a period of 6 h for each individual Franz cell (n = 10)

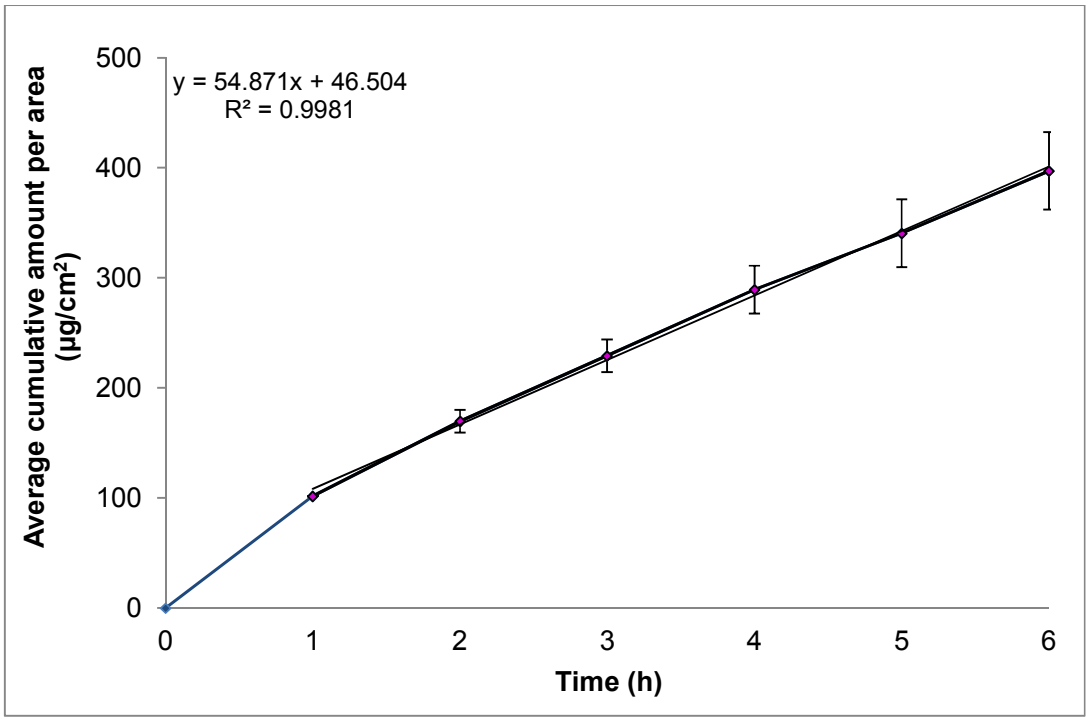


Figure C.4: The average cumulative amount per area (µg/cm^2) of artemether released from the proniosome carriers into the receptor phase over a period of 6 h (n = 10)

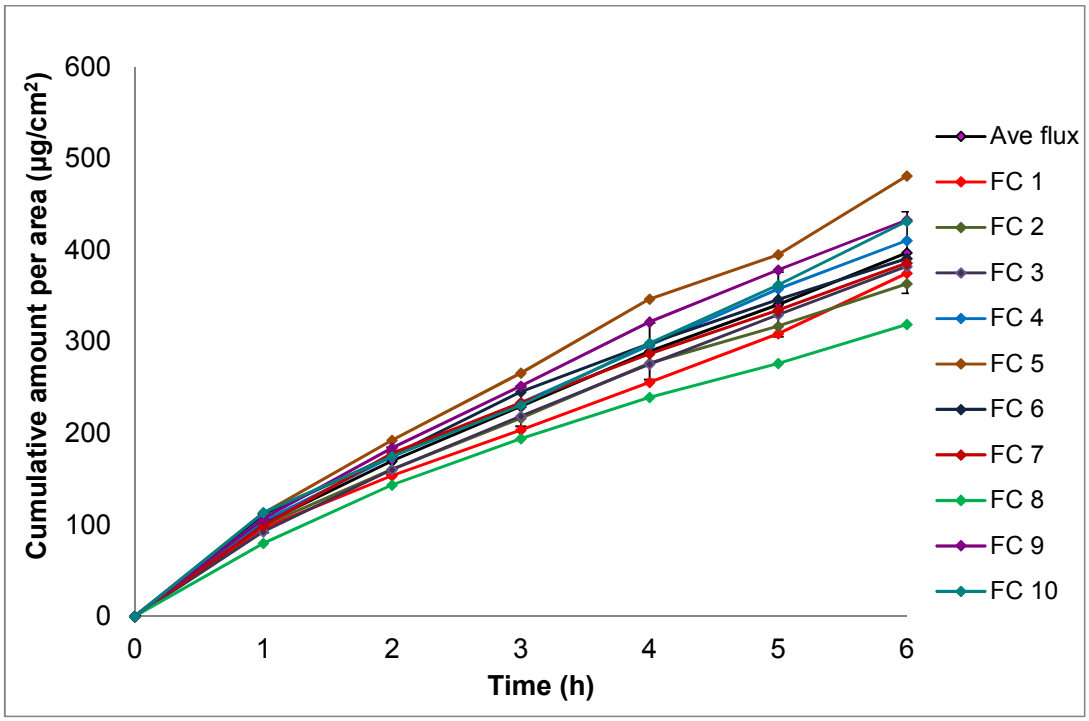


Figure C.5: Cumulative amount per area (µg/cm^2) of artemether released from the proniosome carriers over a period of 6 h for each individual Franz cell (n = 10)

C.4.4 Skin diffusion studies

C.4.4.1 Diffusion study

During the *in vitro* skin permeation studies, it was expected to find low to no artemether concentrations in the receptor phase, seeing that the target for this study was topical and not transdermal drug delivery and the niosome vesicle systems have the ability to reduce systemic absorption (Uchehi *et al.,* 2014:212). Therefore, to determine whether systemic absorption occurred, the concentration of artemether in the receptor phase was analysed.

Two different skin donors were used during the skin permeation studies of the niosome and proniosome carriers due to a shortage of skin donors. For the analysis of the niosomes study, six pieces from both donors were used, while during the analysis of the proniosomes study, five pieces of skin from donor 1 and seven pieces from donor 2 were used. The fact that two different skin donors were used could have influenced the data due to differences such as age, skin condition and skin hydration (WHO, 2006:1). Although, the ratio between the two donors used for both of the studies was closely related and the data acquired was compared to the donors used, no confliction was found.

C.4.4.2 Tape stripping

Topical drug delivery studies were done to determine whether the encapsulated artemether found their way into the skin layers, i.e. the SCE and ED. The average concentration of artemether in the SCE and ED is tabulated in Table C.5.

Table C.5: Average concentration of artemether that diffused into the skin layers

	Average concentration in the SCE (µg/ml)	**Average concentration in the ED (µg/ml)**
Niosome (n = 10)[1]	0	2.04 ± 2.766
Proniosome (n = 8)[2]	0	2.32 ± 2.151

[1] The average concentration diffused was determined from all 10 Franz cells, but there were only artemether concentrations detected in 4 of the 10 Franz cells.

[2] The average concentration diffused was determined from 8 Franz cells, seeing that 2 Franz cells leaked and therefore had to be removed from the study. Artemether concentrations were only detected in 5 of the 8 Franz cells.

C.4.4.2.1 Stratum corneum-epidermis

During the skin diffusion studies, no artemether concentrations were found in the SCE (Table C.5) after application of both the niosomes and proniosomes, meaning the two vesicle dispersions cannot be compared to each other. Artemether, being lipophilic, raised the expectation that it would reside in the stratum corneum instead of diffusing through to the epidermis-dermis (Williams, 2013:680), but the fact that no artemether concentrations were

acquired in the SCE could mean the carrier vesicles were successful in their function of targeted drug delivery into the ED (Karim *et al*., 2010:374). This targeted drug delivery will be discussed in Section C.4.4.2.2.

C.4.4.2.2 Epidermis-dermis

Regarding the niosomes, the average concentration of artemether delivered to the ED was very low. Artemether concentrations were only found in four of the 10 Franz cells in the ED, but the average was calculated for ten Franz cells. This can explain the low average concentration of 2.04 ± 2.766 µg/ml found in the ED.

During the proniosome study, two Franz cells leaked and their data had to be removed from the study. Artemether concentrations were only detected in five of the eight Franz cells in the ED, although the average was determined on all eight Franz cells. The average concentration of artemether delivered from the proniosomes to the ED was 2.32 ± 2.151 µg/ml.

The average artemether concentration in the proniosomes was slightly higher than that of the niosomes, however it should be noted that the average artemether concentrations for both vesicle systems were below the LOQ (5.08 µg/ml) (Appendix A.4.8). Although the results obtained were quantified, they were not reliable. By increasing the concentration of artemether per volume in the formulation, the topical delivery of artemether can be improved since the topical drug doses are normally between 2 – 5 mg/cm^2 (Herkenne *et al*., 2008:87). Other methods which could help to improve the topical delivery of artemether include the addition of chemical, biochemical or physical enhancers (Prausnitz *et al*., 2012:2070; Prausnitz & Langer, 2008:1263).

C.5 Conclusion

The aim of this study was to determine whether encapsulating artemether in niosome and proniosome vesicles would help overcome the barrier properties of the SCE and lead to artemether penetrating the skin for a local effect (Leite-Silvia *et al.,* 2012:384).

The solubility of a drug plays an important role during absorption (York, 2013:14). The term aqueous solubility plays a significant role, not only in the choice of the receptor phase used during the diffusion studies, but also in the topical delivery of a drug. The aqueous solubility of artemether in water and PBS (pH 7.4) was determined as 0.11 ± 0.002 mg/ml and 0.09 ± 0.003 mg/ml, respectively, while for transdermal drug delivery the ideal aqueous solubility is approximately 1 mg/ml (Naik *et al*., 2000:319). The log D and log P values of artemether were determined as 2.35 ± 0.067 and 2.26 ± 0.117, respectively, indicating that artemether has an affinity for *n*-octanol, which is an organic solvent. The log D and log P values of a drug

should be between 1 and 3 to ensure the best results regarding permeation through the skin (Hadgraft, 2004:292; Mbah, 2011:680).

After completing four membrane release studies using PBS (pH 7.4) as the receptor phase, it was realised that the low concentrations obtained could be due to the artemether not being sufficiently soluble in PBS (pH 7.4). This resulted in only a small concentration of the artemether being diffused through the PVDF membranes. Consequently the solubility of artemether was determined in THF, isopropanol and ethanol (99.9%), as potential receptor phases; ethanol (99.9%) was selected as the receptor phase for the membrane release studies, while PBS (pH 7.4) was still used for the skin diffusion studies.

The membrane release studies indicated that artemether was released from the vesicle systems into the receptor phase. From the two vesicle systems tested, the niosomes released a higher concentration of artemether than the proniosomes. During the skin studies, artemether concentrations were only detected in the ED. The niosomes had an average artemether concentration of 2.04 ± 2.766 µg/ml, with only 40.0% (four out of ten) of the Franz cells having had artemether resided in their ED. The proniosomes had an average artemether concentration of 2.32 ±2.151 µg/ml, with only 62.5% (five out of eight) of the Franz cells having artemether resided in their ED. These concentrations of artemether found in the ED were lower than the LOQ and therefore the results are not reliable, however, the fact that concentrations were acquired in the ED indicates that targeted drug delivery occurred.

Some reasons for unsuccessful skin permeation during this study could be due to the physical characteristics of the vesicle system. The surface of the human skin is negatively charged, therefore it would repel any other negatively charged compounds. The zeta-potential of the niosome and proniosome dispersions (discussed in Section B.5) are both largely negative. Negatively charged vesicles have less efficiency in skin penetration than positively charged vesicles, but they accumulate in the ED. This could explain why no artemether concentrations were found in the SCE and receptor phase in contrast to the ED (Gillet *et al.*, 2011:223-224).

The solubility testing of artemether suggested that artemether is not water soluble and very lipophilic of nature. The reason for better artemether diffusion through the membranes than through the skin is due to the receptor phase being ethanol (99.9%) during the membrane release studies instead of PBS (pH 7.4). Ethanol is an organic solvent, while PBS (pH 7.4) is an aqueous solvent and with artemether having a much higher affinity for ethanol (99.9%) than for PBS (pH 7.4), artemether was drawn out of the vesicles by the ethanol (99.9%) in the receptor phase.

In conclusion, it can be said that the topical application of artemether encapsulated in niosome and proniosome vesicles were successful, as artemether was identified in the ED (target area). A higher bioavailability of artemether in the ED can most probably be achieved by increasing the concentration artemether. According to Morrow *et al.* (2007:39), improved drug penetration can be achieved by means of several approaches, some of which include alterations with regard to vehicle-drug interaction, vesicles and particles. By altering the artemether formulations, using these approaches, the topical delivery of artemether can be improved. A different nano-vesicle system can also be researched to carry artemether into the different skin layers.

References

DrugBank. 2013. Artemether (DB06697). http://www.drugbank.ca/drugs/DB06697 Date of access: 15 Jun. 2016.

Chandra, A. & Sharma, P.K. 2008. Proniosome based drug delivery system of piroxicam. *African Journal of Pharmacy and Pharmacology*, 2(9):184-190.

Gabriëls, M. & Plaizier-Vercammen, J.A. 2003. Densitometric Thin-Layer Chromatographic Determination of Artemisinin and its Lipophilic Derivatives, Artemether and Arteether. *Journal of Chromatographic Science*, 41(7):359-366.

Gillet, A., Compère, P., Lecomte, F., Hubert, P., Ducat, E., Evrard, B. & Piel, G. 2011. Liposome surface charge influence on skin penetration behaviour. *International Journal of Pharmaceutics*, 411(1-2):223-231.

Hadgraft, J. 2004. Skin deep. *European Journal of Pharmaceutics and Biopharmaceutics*, 58(2):291-299.

Herkenne, C., Alberti, I., Naik, A., Kalia, Y.N., Mathy, F-X., Préat, V. & Guy, R.H. 2008. In vivo methods for the assessment of topical drug bioavailability. *Pharmaceutical Research*, 25(1):87-103.

Human metabolome database. 2016. Showing metabocard for artemether (HMDB15643). http://www.hmdb.ca/metabolites/HMDB15643 Date of access: 15 Jun. 2016.

Joshi, V. 2012. In vitro diffusion studies in transdermal research: a synthetic membrane model in place of human skin. http://www.drug-dev.com/Main/Back-Issues/In-Vitro-Diffusion-Studies-in-Transdermal-Research-509.aspx Date of access: 4 Nov. 2016.

Karim, K.M., Mandal, A.S., Biswas, N., Guha, A., Chatterjee, S., Behera, M. & Kuotsu, K. 2010. Niosome: a future of targeted drug delivery systems. *Journal of Advanced Pharmaceutical Technology & Research*, 1(4):374-380.

Lachenmeier, D.W. 2008. Safety evaluation of topical applications of ethanol on the skin and inside the oral cavities. *Journal of Occupational Medicine and Toxicology*, 3(26):1-16.

Leite-Sylvia, V.R., de Almeida, M.M., Fradin, A., Grice, J.E. & Roberts, M.S. 2012. Delivery of drugs applied topically to the skin. *Expert Review of Dermatology*, 7(4):383-397.

Leo, A., Hansch, C. & Elkins, D. 1971. Partition coefficients and their uses. *Chemical Reviews*, 71(6):525-616.

Mbah, C.J., Uzor, P.F. & Omeje, E.O. 2011. Perspectives on transdermal drug delivery. *Journal of Chemical and Pharmaceutical Research*, 3(3):680-700.

More, S.B., Nandgude, T.D. & Poddar, S.S. 2016. Vesicles as a tool for enhanced topical drug delivery. *Asian Journal of Pharmaceutics*, 10(3):196-209.

Morrow, D.I.J., McCarron, P.A., Woolfson, A.D. & Donnelly, R.F. 2007. Innovative strategies of enhancing topical and transdermal drug delivery. *The Open Drug Delivery Journal*, 1:36-59.

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical Science &Technology Today*, 3(9):318-326.

Nasir, B., Shah, SNH., Murtaza, G., Shahid, N., Akhtar, M., Khan, B.A., Azhar, S., Mumtaz, A., Waseem, A., Khan, S.A., Ubaid, M., Noreen, S., Kamal, B. & Asad, M.H.H.B. 2012. New HPLC method for the determination of artemether in injections. *Scientific Research and Essays*, 7(10):1165-1168.

OECD (Organisation for Economic Co-operation and Development). 1995. OECD guideline for the testing of chemicals. Partition coefficient (n-octanol/water): shake flask method. http://www.oecd.org/chemicalsafety/risk-assessment/1948169.pdf Date of access: 4 Nov. 2016.

Pellet, M.A., Roberts, M.S. & Hadgraft, J. 1997. Supersaturated solutions with an in vitro SCE tape stripping technique. *International Journal of Pharmaceutics*, 151(1):91-98.

Prausnitz, M.R. & Langer, R. 2008. Transdermal drug delivery. *Nature Biotechnology*, 26(11):1261-1268.

Prausnitz, M.R., Elias, P.M., Franz, T.J., Schmuth, M., Tsai, J.C., Menon, G.K., Holleran, W.M.H. & Feingold, K.R. 2012. Skin barrier and transdermal drug delivery. http://drugdelivery.chbe.gatech.edu/Papers/2012/Prausnitz%20Derm%20Book%20Chapter%202012.pdf Date of access: 10 Nov. 2016.

Shatalebi, M.A., Mostafavi, S.A. & Moghaddas, A. 2010. Niosome as a drug carrier for topical delivery of N-acetyl glucosamine. *Research in Pharmaceutical Sciences*, 5(2):107-117.

Sunil, J., Nath, M.S. & Moorthy, U.S. 2010. HPLC method development and validation for simultaneous estimation of artemether and lumefantrine in pharmaceutical dosage forms. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2(4):93-96.

Ternullo, S., De Weerd, L., Flaten, G.E., Holsaeter, A.M. & Škalko-Basnet, N. 2017. The isolated perfused human skin flap model: a missing link in skin penetration studies. *European Journal of Pharmaceutical Sciences*, 96(1):334-341.

Uchechi, O., Ogbonna, J.D.N. & Attama, A.A. 2014. Nanoparticles for dermal and transdermal drug delivery. http://dx.doi.org/10.5772/58672 Date of access: 12 Sept. 2016.

Ueda, C.T., Shah, V.P., Derdzinski, K., Ewing, G., Flynn, G., Maibach, H., Marques, M., Rytting, H., Shaw, S., Thakker, K. & Yacobi, A. 2009. Topical and transdermal drug products. *Pharmacopeial Forum*, 35(3):750-764.

WHO see World Health Organization

World Health Organization. 2006. Environmental health criteria 235: dermal absorption. http://www.who.int/ipcs/publications/ehc/ehc235.pdf Date of access: 9 Nov. 2016.

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E. & Taylor, K.M.G, *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 675-697).

York, P. 2013. Design of dosage forms. (*In* Aulton, M.E. & Taylor, K.M.G. *ed*. Aulton's pharmaceutics: the design and manufacture of medicines. London: Elsevier. p. 7-19).

APPENDIX D

CYTOTOXICITY OF ARTEMETHER AND VESICLES

D.1 Introduction

The topical application of an API is a method designed for the administration of drugs through the skin to obtain local effects (Jepps *et al.,* 2013:154; Rahimpour & Hamishehkar, 2012:141). The topical delivery of artemether, which was encapsulated in niosomes and proniosomes, was determined in Appendix C. To determine whether artemether is toxic and could harm a human during topical application, the toxicity must be determined; a cytotoxicity screening of artemether, the empty niosome carriers and the artemether encapsulated in the niosome carriers were executed.

The human skin consists of three types of cells, i.e. keratinocytes, melanocytes and fibroblasts. HaCaT cell cultures were used during the cytotoxicity analysis because they are immortalised human keratinocyte cells, thus the results will be relevant to topical drug delivery (López-García *et al*., 2014:44). This being said, the *in vitro* cytotoxicity of a compound cannot be directly compared to the *in vivo* toxicity of a compound for the purpose of topical drug delivery, as other influences, such as metabolism, excretion, distribution and absorption with regards to *in vivo* toxicity, make this comparison difficult (Yoon *et al*., 2012:634).

D.2 Materials

Table D.1: Materials used during the LDH assay

Materials	Suppliers	Cat number#
50 ml Tubes	BD Falcon™	352070
15 ml Tubes	Corning®	430791
75 cm^2 cell culture Flask	Corning®	43146RU
96-Well plate	TPP®	92096
CytoTox 96® Non-Radioactive Cytotoxicity Assay kit	Promega™	PRG1780
Dulbecco's modified eagle's medium (DMEM) with high glucose, 4.0 mM L-glutamine, sodium pyruvate	HyClone™	SH30243.FS
Foetal Bovine Serum (FBS)	HyClone™	SV30160.03
L-Glutamine	HyClone™	SH30034.02
MEM Non-Essential Amino Acid (MEM NEAA) (100%)	HyClone™	SH30238.01
Penicillin/Streptomycin (Pen/Strep)	Lonza™	DE17-602E
Phosphate Buffered Saline (1x)	HyClone™	SH30256.01
Trypan Blue solution (0.4%)	Sigma-Aldrich®	T8154
Trypsin-Versene® (EDTA)	Lonza™	BE17-161F

D.3 Methods

During the *in vitro* cytotoxicity testing of artemether and the vesicle dispersions, HaCaT cells were used. The assay used during this analysis was the LDH assay.

D.3.1 Cell and tissue culture seeding

The method used for seeding the cell cultures was derived from the standard operating procedure (SOP): Pharmacen_CTC_SOP009_v01_Seeding mammalian cells (1).

Table D.2: Apparatus needed for the seeding of cell cultures

Cell culture apparatus
15 ml tubes
50 ml tubes
Calculator
Haemocytometer with cover slide
Microcentrifuge tubes (1.5 ml)
Paper towels
Pasteur pipettes
Pipettes: 10 µl, 20 µl, 200 µl and 1000 µl
Required cell culture flask or plate
Scrapers (if needed)
Spray bottle with 70% ethanol
Sterile, individually wrapped serological pipettes (5 ml; 10 ml; 25 ml)
Timer
Tube racks
Tips (for 10 µl, 20 µl, 200 µl and 1000 µl)
Waste containers (one for discarded liquid, one for plastic waste)
Yellow tips

D.3.1.1 Preparations of cell cultures

This procedure was performed in sterile conditions with sterile equipment and reagents. Contributing to the sterile conditions, all surfaces were cleaned with 70% ethanol before being used. Prior to harvesting the HaCaT cells, the quality of the cells was determined by confirming the absence of contamination and infection of unwanted growth by means of a light microscope. Between 12 and 24 h after seeding, prior to using the cells, it was confirmed the cells had attached to the growth surface, using a light microscope.

D.3.1.2 Toxicity determination procedure

Firstly, the growth media and Phosphate Buffered Saline were preheated. By decanting the growth media, it was removed from the flask following the cleaning of unwanted drops from the flask rim using 70% ethanol. The flask was rinsed twice, using 10 ml of the preheated Phosphate Buffered Saline and then removed by decanting, and the rim was wiped clean with 70% ethanol. The Trypsin-Versene® (EDTA) (3 ml) was transferred to the flask, ensuring equal distribution of the trypsin. The flask was placed back into the CO_2 incubator, set at 37 °C, for 10 to 15 min, while gently shaking the flask every 2 min to ensure detachment of the cells. After ensuring most of the cells were detached, they were pipetted after which, it was ensured that a single cell suspension was present using a light microscope. The preheated DMEM (6 ml) was transferred to the cells, ensuring the cells were rinsed from the sides of the flask gently with a pipette. All the cell suspensions were transferred to a 50 ml tube and vigorously pipetted with a Pasteur pipette to ensure thorough mixing.

D.3.1.3 Preparation of the haemocytometer and the counting mixture

A mixture of 25 µl Trypan Blue Solution (0.4%) and 15 µl Phosphate Buffered Saline were prepared in a microcentrifuge and mixed well. Using a micropipette, 10 µl of the cell suspension was transferred to the Trypan Blue-Phosphate Buffered Saline mixture, following the incubation of the final mixture at approximately 22 °C for 3 min. After incubation, the mixture was thoroughly mixed and 10 µl was transferred to the edge of a cover slide, ensuring that the mixture was drawn into the chamber by capillary forces. Another 10 µl of the mixture was transferred to the other side of the slide, ensuring the mixture was drawn into the chamber.

D.3.1.4 Counting of the cells using a light microscope

Each side of the counting chamber was stretched out to a total surface area of 9 mm^2, which presented nine large squares through the microscope. The number of clear and round cells that were present in the four corner squares and the middle square were counted. This step was repeated for the other side of the slide, counting up to 10 squares and then adding up all the live cells that were counted. The total number of live cells was divided by two and then five to obtain the number of cells per ml present in the cell suspension. By multiplying the cell concentration with the total cell suspension volume, the total number of cells present in the suspension was obtained.

The first reason for counting the cells is for a quick review to determine the cell viability after trypsinisation. Secondly, the cells are counted to determine the amount of cells that detached from the bottom of the flask after adding the Trypsin-Versene® (EDTA). The amount of cells that detached and were present in the DMEM would determine the volume of media that should be transferred to each well to obtain 20 000 cells per well.

D.3.1.5 Plating and maintaining

The HaCaT cultured cell lines were grown in a 96-well plate, at a density of 2 x 10^4 cells/well. The plate was incubated in a CO_2 incubator for no more than 24 h before the execution of the cytotoxicity assay.

D.3.2 Artemether stock solution preparation

The stock solution analysed using the LDH assay was prepared by weighing 50 mg of artemether and dissolving it in 10 ml of pure methanol. Three different concentrations of the active were prepared and analysed according to Table D.3.

Table D.3: Preparation of the different stock solution concentrations analysed

Concentration	Stock solution (µg//ml)	DMEM (µl)	Final volume per well (µl)	Final concentration (µg/ml)
0.5%	1.50	98.5	100.0	75.0
1.0%	3.00	97.0	100.0	150.1
2.0%	6.00	94.0	100.0	300.2

D.3.3 Niosome with active percentage calculations

The artemether encapsulated in the niosome dispersion was prepared according to the method discussed in Section B.4.3.6, with an artemether mass of 300.2 mg. The dispersion was diluted by adding 10 ml Milli-Q® water and mixing gently. The concentration of artemether in the final 20 ml vesicle dispersion was 15 010 µg/ml.

Table D.4: Preparation of the different loaded niosomes concentrations analysed

Concentration	Loaded niosome dispersion (µl)	DMEM (µl)	Final volume per well (µl)	Final concentration (µg/ml)
0.5%	0.50	99.5	100.0	75.0
1.0%	1.00	99.0	100.0	150.1
2.0%	2.00	98.0	100.0	300.2

D.3.4 The LDH-assay

An LDH-assay measures the activity of the stable cytosolic enzyme, LDH. This enzyme is present in all cells, but once the plasma membrane of a cell is damaged (cell lysis), the enzyme is released. During this assay, cell lysis will be initiated causing the immediate release of the LDH enzymes into the cell culture supernatant. The amount of LDH present in the supernatant is directly proportional to the %cell death.

Five different samples were analysed during the execution of this assay. The first sample was a positive control that contained only the grown cells and the added lysis, ensuring 100% cell death. The second sample was the negative control, which contained only DMEM and cells. Three wells in the 96-well plate were also filled with DMEM, since a No-cell control to determine the background of the growth medium was needed for the calculations. The absorbance measured from this sample would be used to calculate the %cell death caused by the niosomes and artemether. The remaining three samples were 1) the artemether stock solution, 2) the empty niosomes and 3) the artemether-loaded niosomes.

D.3.5 LDH-assay method

The CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit was used for the cytotoxicity analysis of artemether and the vesicle dispersions. A 96-well assay plate was prepared by placing 2×10^4 of the cells grown in DMEM in the wells. The 96-well plate containing the cells were incubated for 24 h at 37 °C to ensure proper attachment of the cells to the bottom of the wells. Every sample was analysed in triplicate. After 24 h incubation, the samples were added to the cells. For the analysis of the positive control, only the cultured cells were present in the wells, whereby a lysis solution would be added before the incubation/treatment period was finished. Regarding the negative control, 100 µl of DMEM was transferred to the cell cultures in the wells. To analyse the cytotoxicity of artemether and the niosome dispersions, these samples were added to the wells in the concentrations used in Tables D.3 and D.4. After transferring all the samples, the 96-well plate was incubated for 12 h at 37 °C. Forty-five min before the 12 h incubation period was finished, i.e. at 11 h and 15 min, 10 µl of the 10X Lysis Solution was added to the positive control wells to ensure total cell lysis, which would cause approximately 100% cell death. Following the incubation, 50 µl from all the samples were transferred to a clean 96-well flat clear bottom plate and 50 µl of the CytoTox 96® Reagent was added to all the wells. The plate was then covered with foil and incubated in darkness for 30 min at room temperature; after this incubation period, 50 µl of the Stop Solution was added to all the wells. Within an hour after the addition of the Stop Solution, the absorbance of the LDH enzyme was measured at a wavelength between 490 – 492 nm using a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA).

D.4 Results

An LDH assay is designed to determine whether LDH leaked from the damaged cells after 12 h of sample incubation by means of a microplate reader. In order to determine the concentration of LDH that leaked from the damaged cells, a tetrazolium salt was added to the samples, which lead to lactate being oxidised to pyruvate. This oxidation process was catalysed by LDH, which was then reduced to nicotinamide adenine dinucleotide (NADH). Using the NADH that formed,

the tetrazolium salt was converted to coloured formazan; the concentration formazan present was then measured using a microplate reader (Chan *et al*., 2013:66). According to ISO (2009:9), a compound is considered cytotoxic to cell cultures if it causes a percentage cell death larger than 30%.

After an incubation time of 12 h, the absorbance of the LDH enzyme was measured by means of a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA). The percentage cell death caused by the three samples was calculated by means of Equation D.1, where Maximum LDH release is the absorbance of the positive control.

$$\%\text{Cytotoxicity} = \frac{\text{Experimental LDH release } (OD_{490})}{\text{Maximum LDH release } (OD_{490})} \times 100 \qquad \textbf{Equation D.1}$$

$$\text{Experimental LDH release} = NC - S \qquad \textbf{Equation D.2}$$

Where NC is the absorbance LDH enzyme, measured from the No-cell control, which contained only the growth media DMEM imitating untreated cells, S is the absorbance LDH enzyme measured for the samples containing artemether or the vesicles. These absorbance values of the samples were subtracted from the absorbance of the No-cell control to obtain the experimental LDH release value, which was substituted in Equation D.1. The absorbance of the positive control, calculating to approximately 100% cell death, was substituted in Equation D.1 as the maximum LDH Release.

Table D.5: %HaCaT cell death caused after 12 h of incubation

Sample	Sample per well (µl)	%Cell death	%SD
Empty niosome dispersion	0.50	10.27	1.40
	1.00	12.08	1.94
	2.00	15.70	2.58
Artemether loaded niosome dispersion	0.50	13.34	2.56
	1.00	24.56	4.10
	2.00	71.94	6.25
Artemether stock solution	1.50	19.70	3.50
	3.00	42.44	7.74
	6.00	92.62	2.86
Positive control	10.00	100.00	0.00
Negative control	100.00	10.50	2.12

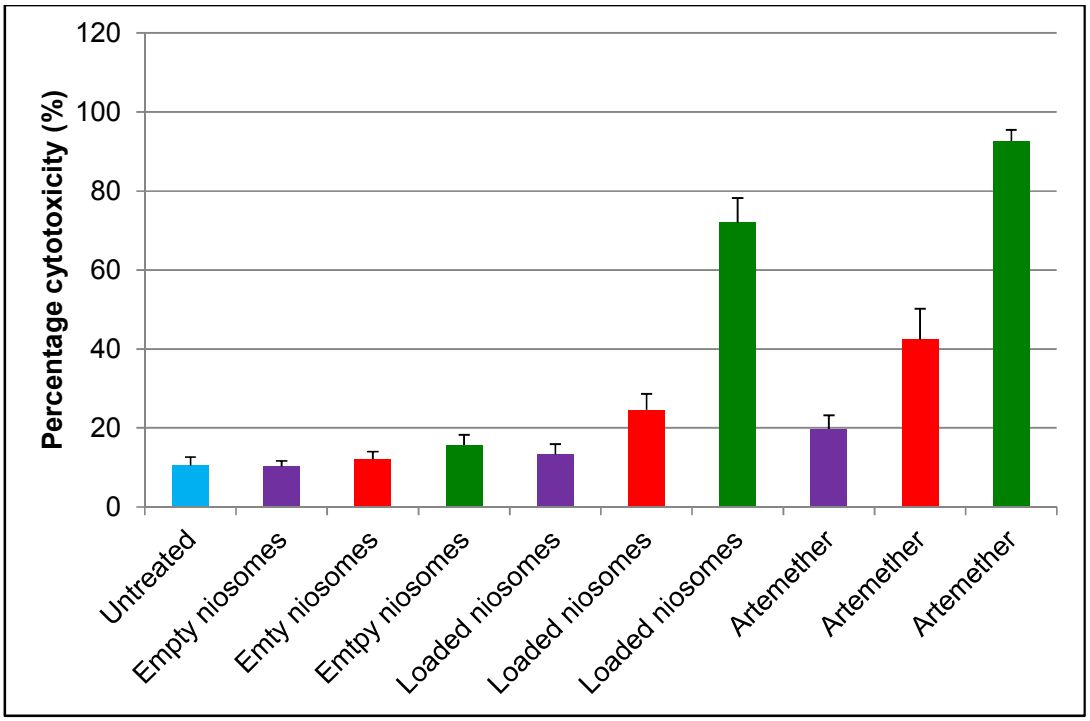


Figure D.2: %HaCaT cell death after 12 h of incubation during the LDH assay analysis. Each colour represents a different percentage of sample analysed; the blue bar represents the negative control, the purple bars represent 0.5% of the sample, the red bars represent 1.0% of the sample and the green bars represents 2.0% of the sample.

According to López-García *et al*. (2014:45), compounds causing a %cell viability greater than 80% is considered non-cytotoxic, a %cell viability between 60 and 80% is considered weak cytotoxic, a %cell viability between 40 and 60% is considered moderate cytotoxic and a %cell viability below 40% is considered strong cytotoxic to the cell cultures.

The results represented in Table D.5 and Figure D.2 indicates all three samples had a toxicity increase directly proportional to the increase in the dispersion concentration exposed to the cells. The 0.5%, 1.0% and 2.0% of loaded niosome dispersion and stock solution added to the DMEM represented an artemether concentration of 75 µg/ml, 150 µg/ml and 300 µg/ml, respectively.

The 0.5%, 1.0% and 2.0% empty niosomes resulted in 10.27 ± 1.40%, 12.08 ± 1.94%, 15.70 ± 2.58% %cell death, respectively. The empty niosome vesicles had a maximum %cell death of 15.70%, which caused a %cell viability of above 84.3%; therefore, all the empty niosome samples were considered non-cytotoxic to the HaCaT cells, according to López-García *et al*. (2014:45).

The loaded niosomes caused a %cell death of 13.34 ± 2.56%, 24.56 ± 4.10% and 71.94 ± 6.25% for the 0.5%, 1.0% and 2.0% concentrations, respectively. According to López-García *et al*. (2014:45), the 0.5% concentration of the loaded niosomes had a %cell viability of 86.66% and was considered as non-cytotoxic to the HaCaT cells. The 1.0% concentration loaded niosomes had a %cell viability of 75.44% and was considered weakly cytotoxic, while the 2.0% concentration loaded niosomes presented a 28.06% %cell viability and was considered strongly cytotoxic towards the HaCaT cell cultures (López-García *et al*., 2014:45).

During the analysis of the stock solution of artemether, the cytotoxicity of artemether to HaCaT cells were determined. At a 0.5%, 1.0% and 2.0% of the artemether stock solution, the %cell death caused was 19.70 ± 3.50%, 42.44 ± 7.74% and 92.62 ± 2.86%, respectively. The 0.5% concentration artemether stock solution had a %cell viability of 80.30% which was not considered as cytotoxic to the HaCaT cells, according to López-García *et al.* (2014:45). The 1.0% and 2% concentration artemether stock solution presented a %cell viability of 57.56% and 7.38%, respectively. Hence, the 1.0% concentration artemether stock solution was considered as moderately cytotoxic, while the 2% concentration artemether stock solution was regarded as strongly cytotoxic to the HaCaT cell cultures (López-García *et al*., 2014:45).

D.5 Conclusion

All the empty niosomes, the 0.5% concentration artemether loaded niosomes together with the 0.5% concentration artemether stock solution were considered as non-cytotoxic to HaCaT cells. However, the 1.0% and 2.0% concentrations of artemether loaded were considered weakly and strongly cytotoxic to HaCaT cells, respectively. The 1.0% and 2.0% concentrations artemether stock solution were regarded as moderately and strongly cytotoxic, respectively.

Artemether is known for its ability to inhibit the activation and proliferation of keratinocytes and T-cells (Wu *et al*., 2015:7072&7076). It was confirmed by Wu *et al*. (2015:7072) that artemether causes a decrease in the proliferation rate of HaCaT cells, directly proportional to the concentration of artemether present. These antiproliferative effects of artemether could explain why artemether was determined as cytotoxic to the HaCaT cells using the LDH assay.

In vivo and *in vitro* cell toxicity determination cannot be compared due to other processes involved during *in vivo* exposures. Consequently, even though artemether is toxic to cell cultures from the concentration 150 µg/ml and higher, it is not necessarily toxic when applied topically (Yoon *et al*., 2012:634).

References

Ahn, B-N., Kim, J-A., Kong, C-S., Seo, Y. & Kim, S-K. 2012. Protective effect of (2'S)-columbianetin from *Corydalis heterocarpa* on UVB-induced keratinocyte damage. *Journal of Photochemistry and Photobiology B:Biology*, 109:20-27.

Chan, F.K-M., Moriwaki, K. & De Rosa, M.J. 2013. Detection of necrosis by release of lactase dehydrogenase (LDH) activity. *Methods in Molecular Biology*, 979:65-70.

Gouws, C. 2014. Cell and tissue culture. Seeding mammalian cells. Potchefstroom: NWU, School of Pharmacy, Centre of Excellence for Pharmaceutical Sciences. (SOP no: Pharmacen_CTC_SOP009_v01_Seeding mammalian cells). (Unpublished).

ISO (International Organization of Standardization) 10993-5. 2009. Biological evaluation of medical devices – part 5: Test for *in vitr*o cytotoxicity. 3rd ed. Geneva: ISO.

Jepps, O.G., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. 2013. Modeling the human skin barrier – towards a better understanding of dermal absorption. *Advanced Drug Delivery Reviews*, 65(2):152-168.

López-García, J., Lehocký, M., Humpolíček, P. & Sáha, P. 2014. HaCaT keratinocytes response on antimicrobial atelocollagen substrates: extent of cytotoxicity, cell viability and proliferation. *Journal of Functional Biomaterials*, 5(2):43-57.

Stockert, J.C., Blázquez-Castro, A., Cañete, M. & Horobin, R.W. 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. *Acta Histochemica*, 114(8):785-796.

Rahimpour, Y. & Hamishehkar, H. 2012. Niosomes as carrier in dermal drug delivery. (*In* Sezer, A.D., *ed*. Recent advances in novel drug carrier systems. Rijeka: InTech. p. 141-164).

Wu, J., Li, H. & Li, M. 2015. Effects of artemether on the proliferation, apoptosis and differentiation of keratinocytes: potential application for psoriasis treatment. *International Journal of Clinical and Experimental Medicine*, 8(5):7069-7078.

Yoon, M., Campbell, J.L., Andersen, M.E. & Clewell, H.J. 2012. Quantitative *in vitro* to *in vivo* extrapolation of cell-based toxicity assay results. *Critical Reviews in Toxicology*, 42(8):633-652.

APPENDIX E

AUTHORS GUIDE: INTERNATIONAL JOURNAL OF PHARMACEUTICS

Introduction

The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

Types of paper

(1) Full length manuscripts

(2) Rapid communications

(a) These articles should not exceed 1500 words or equivalent space.

(b) Figures should not be included otherwise delay in publication will be incurred.

(c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

(3) Notes

Should be prepared as described for full length manuscripts, except for the following:

(a) The maximum length should be 1500 words, including figures and tables.

(b) Do not subdivide the text into sections. An Abstract and reference list should be included.

(4) Reviews and mini reviews

Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

Before you begin

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication see https://www.elsevier.com/editors/publishing-ethics and https://www.elsevier.com/authors/journal-authors/policies-and-ethics.

Human and animal rights

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed.

Declaration of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. More information at http://service.elsevier.com/app/answers/detail/a_id/286/supporthub/publishing/

Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an

electronic preprint, see 'Multiple, redundant or concurrent publication' section of our ethics policy for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service CrossCheck.

Contributors

Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and/or article preparation, so roles for all authors should be described. The statement that all authors have approved the final article should be true and included in the disclosure.

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service

This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal. More information at https://www.elsevier.com/authors/journal-authors/submit-your-paper/submit-and-revise/article-transfer-service.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of open access articles is determined by the author's choice of user license.

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. More information at https://www.elsevier.com/about/company-information/policies/copyright.

Elsevier supports responsible sharing

Find out how you can share your research published in Elsevier journals.

Role of the funding sources

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the Open Access Publication Fee. Details of existing agreements are available online.

Open access

This journal offers authors a choice in publishing their research:

Open access

- Articles are freely available to both subscribers and the wider public with permitted reuse.
- An open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Subscription

- Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.
- No open access publication fee payable by authors.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)

Let’s others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The open access publication fee for this journal is USD 3500, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form.

This journal has an embargo period of 12 months.

Elsevier Publishing Campus

The Elsevier Publishing Campus (www.publishingcampus.com) is an online platform offering free lectures, interactive training and professional advice to support you in publishing your research. The College of Skills training offers modules on how to prepare, write and structure your article and explains how editors will look at your paper when it is submitted for publication. Use these resources, and more, to ensure that your submission will be the best that you can make it.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

Note: The choice of general classifications such as "drug delivery" or "formulation" are rarely helpful when not used together with a more specific classification.

Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of at least four potential reviewers. Good suggestions lead to faster processing of your paper. Please note: Reviewers who do not have an institutional e-mail address will only be considered if their affiliations are given and can be verified. Please ensure that the e-mail addresses are current. International reviewers who have recently published in the appropriate field should be nominated, and their areas of expertise must be stated clearly. Note that the editor retains the sole right to decide whether or not the suggested reviewers are contacted.

To aid the editorial process when suggested reviewers are not chosen or decline to review, ensure that the classifications chosen as the field of your paper are as detailed as possible. It is not sufficient to state "drug delivery" or "nanotechnology" etc.

Preparation

Use of word processing software

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

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections

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

Introduction

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

Material and methods

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

Results

Results should be clear and concise.

Discussion

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

Conclusions

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

Appendices

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

Essential title page information

- ***Title.*** *Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.*

- ***Author names and affiliations.*** *Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.*
- ***Corresponding author.*** *Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication.* ***Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.***
- ***Present/permanent address.*** *If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.*

Abstract

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

The abstract must not exceed 200 words.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Keywords

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

Chemical compounds

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

Chemical compounds studied in this article:

Ethylene glycol (PubChem CID: 174); Plitidepsin (PubChem CID: 44152164); Benzalkonium chloride (PubChem CID: 15865).

More information at https://www.elsevier.com/books-and-journals/content-innovation/pubchem.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork

General points

- Make sure you use uniform lettering and sizing of your original artwork. • Embed the used fonts if the application provides that option.
- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Size the illustrations close to the desired dimensions of the published version.
- Submit each illustration as a separate file.

A detailed guide on electronic artwork is available at https://www.elsevier.com/authors/author-schemas/artwork-and-media-instructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

- EPS (or PDF): Vector drawings, embed all used fonts.
- TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
- TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
- TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

- Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork at https://www.elsevier.com/authors/author-schemas/artwork-and-media-instructions.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard

reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

A DOI can be used to cite and link to electronic articles where an article is in-press and full citation details are not yet known, but the article is available online. A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, http://dx.doi.org/10.1029/2001JB000884i. Please note the format of such citations should be in the same style as all other references in the paper.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be

automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link: http://open.mendeley.com/use-citation-style/international-journal-of-pharmaceutics.

When preparing your manuscript, you will then be able to select this style using the Mendeley plugins for Microsoft Word or LibreOffice.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/ (accessed 13.03.03).

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations found at http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 150 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of

the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Supplementary material

Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s). For more detailed instructions please visit our artwork instruction pages at https://www.elsevier.com/authors/author-schemas/artwork-and-media-instructions.

Research data

Data in brief

Authors have the option of converting any or all parts of their supplementary or additional raw data into one or multiple Data in Brief articles, a new kind of article that houses and describes their data. Data in Brief articles ensure that your data, which is normally buried in supplementary material, is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. Authors are encouraged to submit their Data in Brief article as an additional item directly alongside the revised version of their manuscript. If your research article is accepted, your Data in Brief article will automatically be transferred over to Data in Brief where it will be editorially reviewed and published in the new, open access journal, Data in Brief. Please note an open access fee is payable for publication in Data in Brief. Full details can be found on the Data in Brief website. Please use this template to write your Data in Brief.

Database in linking

Elsevier encourages authors to connect articles with external databases, giving readers access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). More information and a full list of

supported databases at https://www.elsevier.com/books-and-journals/content-innovation/data-base-linking.

Audio slides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at https://www.elsevier.com/books-and-journals/content-innovation/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Interactive plots

This journal enables you to show an Interactive Plot with your article by simply submitting a data file. Full instructions at https://www.elsevier.com/books-and-journals/content-innovation/iplots.

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review.

Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One Author designated as corresponding Author:

- E-mail address
- Full postal address
- Telephone and fax numbers

All necessary files have been uploaded

- Keywords
- All figure captions
- All tables (including title, description, footnotes)

Further considerations:

- Use continuous line numbering (every 5 lines) to facilitate reviewing of the manuscript.
- Manuscript has been "spellchecked" and "grammar-checked"

- References are in the correct format for this journal
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web)
- Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print or to be reproduced in color on the Web (free of charge) and in black-and-white in print
- If only color on the Web is required, black and white versions of the figures are also supplied for printing purposes

For any further information please visit our customer support site at service.elsevier.com.

After Acceptance

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is

sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

Author enquiries

Visit the Elsevier Support Center at http://service.elsevier.com/app/home/supporthub/publishing/ to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch. You can also check the status of your submitted article or find out when your accepted article will be published.

Gill Smithies

Proofreading & Language Editing Services

59, Lewis Drive, Amanzimtoti, 4126, Kwazulu Natal

Cell: 071 352 5410 E-mail: moramist@vodamail.co.za

Work Certificate

To	**Chantell van der Merwe**
Address	**Centre of Excellence for Pharmaceutical Studies, Faculty of Health Sciences, Potchefstroom Campus, North West University**
Date	**24/11/2016**
Subject	**Artemether loaded niosome and proniosome vesicles for topical delivery: Design, characterisation and skin diffusion.**
Ref	**GS/CvdM/01**

I, Gill Smithies, certify that I have proofed the following,

Chapters 1 to 4, Appendices A to D, the Abstract and Acknowledgements: Artemether loaded niosome and proniosome vesicles for topical delivery: design, characterisation and skin diffusion,

to the standard as required by NWU, Potchefstroom Campus.

Gill Smithies

24/11/2016

LINGUA Consultants (Pty) Ltd
PO Box 20802 · NOORDBRUG · 2522 · South Africa
Tel +27 18 290 6023 · Fax +27 86 610 0335 · Cell +27 72 142 2222
e-mail JacoCBreytenbach@gmail.com
e-mail LinguaConsultants@gmail.com
www LinguaConsultants.co.za

24 November 2016

TO WHOM IT MAY CONCERN

I hereby declare that I am a practising health sciences language practitioner and member of the South African Translators' Institute. I further declare that I have done the proofreading of the Afrikaans translation of the Abstract, called Uittreksel, of the MSc thesis of Ms Chantell van der Merwe from North-West University titled **Topical delivery of artemether, encapsulated in niosome and proniosome carriers.**

Prof JC Breytenbach PhD

LINGUA is always at your service