Topical delivery of clofazimine, artemisone and decoquinate utilizing vesicles as carrier system

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If we knew what we were doing, it would not be called research, would it?

-Albert Einstein

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ABSTRACT

Artemisone, clofazimine and decoquinate are part of the MALTBRedox MRC South African University Flagship Projects, which focus on oxidant-redox drug combinations for the treatment of tuberculosis and a few other diseases. These active pharmaceutical ingredients (APIs) were chosen as a possible treatment of cutaneous tuberculosis (CTB), an uncommon and undefined disease that is often misdiagnosed (Abdelmalek *et al.*, 2013; Baig *et al.*, 2014; Fader *et al.*, 2010). Currently CTB is only treated with regular oral anti-tuberculous medication, with occasional invasive procedures such as skin grafts (Yates, 2010).

Artemisone, clofazimine and decoquinate have a log P of 2.49, 7.7 and 7.8, respectively (Biamonte *et al.*, 2013; Dunay *et al.*, 2009; Nagelschmitz *et al.*, 2008; Steyn *et al.*, 2011). A high log P-value indicates that the API is highly lipophilic and therefore a delivery system, namely vesicles, was chosen to improve skin permeation. Many vesicles are currently being investigated all over the world as carriers for APIs in topical delivery, though for this study liposomes, niosomes and transferosomes were selected.

Dispersions containing a single API, a combination of all three APIs, as well as no API, were prepared for all three types of vesicles. Characterisation of dispersions containing 0.2%, 0.4% and 1% API was performed. Isothermal calorimetry indicated that no incompatibility occurred in the 1% API combination dispersions, except the niosome dispersion, which indicated a probable incompatibility. Encapsulation efficiency was above 85% for all 1% API dispersions. The empty vesicles depicted an average size of 154 nm, 167.5 nm and 106.3 nm for liposomes, niosomes and transferosomes, respectively. Vesicle sizes increased with increase in API concentration, whereas stability decreased. Clofazimine was found to have the most significant impact on vesicle size and stability when added as 1%, increasing the average niosome size to 2 461 nm. Viscosity was below 2 mPa.s for all 1% API dispersions, ensuring even spreadability when applied to the skin. The pH of all the dispersions were between 5–6, thus limiting skin irritation.

In vitro transdermal diffusion studies were conducted on black skin, using dispersions containing 1% of all three APIs. No APIs could be detected in the receptor phase. Artemisone was not detected in the skin by means of HPLC analysis, which might be due to the fact that the concentration was below the limit of detection (LOD). The LOD for artemisone was determined at 4.42 µg/ml, whereas it was 0.042 µg/ml for clofazimine and 0.703 µg/ml for decoquinate. Higher API concentrations were present in the stratum corneum-epidermis (SCE), compared to in the epidermis-dermis (ED) for all the dispersions. Transferosomes delivered the highest

concentration clofazimine into the SCE and ED, as well as the highest concentration decoquinate into the ED. The highest concentration decoquinate in the SCE, however, was obtained by the niosome dispersion.

Efficacy against tuberculosis of the APIs (1%) encapsulated in vesicles was tested on strain H37Rv. All dispersions were found to be effective to some degree against the tuberculosis strain tested, with clofazimine in niosomes being the most effective with 52% growth inhibition. The least effective was decoquinate in niosomes, with only 8% inhibition. The combination dispersions delivered inhibitions of 42%, 38% and 12% for liposomes, niosomes and transferosomes, respectively. Surprisingly, it was found that the vesicle dispersions containing no APIs also presented some efficacy against the tuberculosis strain tested.

New knowledge contributed to pharmaceutics by this study includes encapsulating the three APIs in liposomes, niosomes and transferosomes and successfully delivering them into the skin as proved by transdermal diffusion studies. Developing an HPLC method for the concurrent analysis of the three APIs and determining the activity of the vesicle dispersion against the specific tuberculosis strain tested also contributed new knowledge. Results indicated that decoquinate, an API never before considered for tuberculosis, does have anti-tuberculous activity. No significant increase in efficacy against the tuberculosis strain was noted when combining the three APIs in a vesicle dispersion, compared to when the APIs were incorporated separately into the vesicles, though the blank vesicles had surprisingly high activity against the specific tuberculosis strain tested.

Keywords: Clofazimine, artemisone, decoquinate, liposomes, niosomes, transferosomes, transdermal

REFERENCES

ABDELMALEK, R., MEBAZAA, A., BERRICHE, A., KILANI, B., OSMAN, A.B., MOKNI, M. & BENAISSA, H.T. 2013. Cutaneous tuberculosis in Tunisia. *Médecine et maladies infectieuses,* 43(9):374-378.

BAIG, I.A., MOON, J.Y., KIM, M.S., KOO, B.S. & YOON, M.Y. 2014. Structural and functional significance of the highly-conserved residues in *Mycobacterium tuberculosis* acetohydroxyacid synthase. *Enzyme and microbial technology,* 58-59:52-59.

BIAMONTE, M.A., WANNER, J. & LE ROCH, K.G. 2013. Recent advances in malaria drug discovery. *Bioorganic & medicinal chemistry letters,* 23(10):2829-2843.

DUNAY, I.R., CHI CHAN, W., HAYNES, R.K. & SIBLEY, L.D. 2009. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Journal of antimicrobial agents and chemotherapy,* 53(10):4450-4456.

FADER, T., PARKS, J., KHAN, N.U., MANNING, R., STOKES, S. & NASIR, N.A. 2010. Extrapulmonary tuberculosis in Kabul, Afghanistan: a hospital-based retrospective review. *International journal of infectious diseases,* 14(2):e102-e110.

NAGELSCHMITZ, J., VOITH, B., WENSING, G., ROEMER, A., FUGMANN, B., HAYNES, R.K., KOTECKA, B.M., RIECKMANN, K.H. & EDSTEIN, M.D. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial agents and chemotherapy,* 52(9):3085-3091.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W.C., HAYNES, R.K. & KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. *International journal of pharmaceutics,* 414(1-2):260-266.

YATES, V.M. 2010. Mycobacterial infections. (*In* Burns, T., Breathnach, S., Cox, N. & Griffiths, C., *eds*. Rook's textbook of dermatology. 8th ed. Vol 2. West Sussex, United Kingdom: Blackwell Publishing Ltd. p. 31.1-31.41.)

UITTREKSEL

Artemisoon, klofasimien en dekokwinaat is deel van die MALTBRedox MRC Suid-Afrikaanse Universiteit Flagship Projekte wat fokus op oksidasie-reduksie geneesmiddelkombinasies vir die behandeling van tuberkulose en ’n paar ander siektes. Hierdie geneesmiddels is gekies vir moontlike behandeling van kutaneuse tuberkulose (KTB), ’n ongewone en ongedefinieerde siekte wat dikwels verkeerd gediagnoseer word (Abdelmalek *et al.*, 2013; Baig *et al.*, 2014; Fader *et al.*, 2010). Tans word KTB slegs behandel met gewone orale anti-tuberkulose-medisyne, en soms met indringende prosedures soos veloorplantings (Yates, 210).

Artemisoon, klofasimien en dekokwinaat besit ’n log P van 2.49, 7.7 en 7.8, onderskeidelik (Biamonte *et al.*, 2013; Dunay *et al.*, 2009; Nagelschmitz *et al.*, 2008; Steyn *et al.*, 2011). ‘n Hoë log P dui op ’n sterk lipofiliese geneesmiddel en om hierdie rede is ‘n afleweringsisteem, naamlik vesikels, gekies om veldeurlaatbaarheid te verbeter. Baie vesikels word tans reg oor die wêreld ondersoek as draers van geneesmiddels vir topikale aflewering, maar vir hierdie studie is liposome, niosome en transferosome geselekteer.

Dispersies met ’n enkele geneesmiddel, ’n kombinasie van al drie geneesmiddels, sowel as geen geneesmiddel, is voorberei vir al drie tipes vesikels. Karakterisering van dispersies wat 0.2%, 0.4% en 1% geneesmiddel bevat, is uitgevoer. Isotermiese kalorimetrie-resultate het aangetoon dat geen onverenigbaarhede voorkom in die 1% geneemiddeldispersie nie. Resultate verkry vanaf die niosoomdispersie het egter op ’n moontlikheid van onverenigbaarheid gedui. Enkapsuleringeffektiwiteit was bo 85% vir alle 1% geneesmiddeldispersies. Die leë vesikels het ’n gemiddelde grootte van 154 nm, 167.5 nm en 106.3 nm gehad vir liposome, niosome en transferosomes, onderskeidelik. Vesikelgrootte het toegeneem met ’n toename in geneesmiddelkonsentrasie, terwyl stabiliteit afgeneem het. Dit is gevind dat klofasimien die grootste impak gehad het op vesikelgrootte en stabiliteit wanneer dit bygevoeg is in ’n 1% konsentrasie, met ’n gemiddelde vesikelvergroting tot 2 461 nm. Viskositeit was onder 2 mPa.s vir alle 1% geneesmiddeldispersies, wat eweredige spreibaarheid sal verseker tydens aanwending op die vel. Die pH van al die dispersies was tussen 5–6, wat vel-irritasie beperk.

In vitro transdermale-afleweringstudies is uitgevoer op swart vel, deur van dispersies gebruik te maak wat 1% van al drie geneesmiddels bevat. Geen geneesmiddel is waargeneem in die reseptorfase nie. Artemisoon kon nie in die vel opgespoor word met behulp van die HPLC-metode nie, wat moontlik verduidelik kan word deur die feit dat die konsentrasie onder die opsporingslimiet was. Die opsporingslimiet van artemisoon is bepaal as 4.42 µg/ml, terwyl dit

0.042 µg/ml vir klofasimien en 0.703 µg/ml vir dekokwinaat is. Hoër konsentrasies van die geneesmiddels was wel teenwoordig in die stratum korneum-epidermis (SKE) in vergelyking met die epidermis-dermis (ED) vir alle dispersies. Transferosome het die hoogste konsentrasie klofasimien afgelewer in die SKE en ED, sowel as die hoogste konsentrasie dekokwinaat in die ED. Die hoogste konsentrasie dekokwinaat in die SKE is egter verkry deur die niosoomdispersie.

Effektiwiteit van die geneesmiddels (1%) ingesluit in vesikels is getoets teen die spesifieke bakteriële stam van tuberkulose teen die H37RV variasie. Daar is gevind dat al die dispersies effektiwiteit toon, hoewel in 'n klein mate; met klofasimien in niosome die effektiefste met 52% groei-onderdrukking. Die laagste effektiwiteit teen die spesifieke tuberkulose-stam is getoon deur dekokwinaat in niosome met 8% onderdrukking. Die kombinasie-dispersies het onderdrukkings van 42%, 38% en 12% gelewer vir liposome, niosome en transferosomes, onderskeidelik. Verbasend is daar gevind dat die vesikeldispersies wat geen geneesmiddels bevat het nie, ook 'n mate van effektiwiteit getoon het.

Nuwe kennis wat bydra tot Farmaseutika deur hierdie studie, sluit in die enkapsulering van die drie geneesmiddels in liposome, niosome en transferosome, asook die suksesvolle aflewering daarvan in die vel soos bepaal deur transdermale afleweringsstudies. Ontwikkeling van 'n HPLC-metode vir die gesamentlike analise van die drie geneesmiddels, asook die getoetste aktiwiteit van die vesikeldispersies teen die spesifieke tuberkulose-stam, dra ook by tot nuwe kennis. Resultate het aangedui dat dekokwinaat, 'n geneesmiddel wat nooit voorheen oorweeg is teen tuberkulose nie, wel anti-tuberkulose-aktiwiteit besit. Geen merkwaardige toename in effektiwiteit teen tuberkulose is waargeneem wanneer die drie geneesmiddels gekombineer is in 'n vesikeldispersie, teenoor wanneer die geneesmiddels apart ingesluit is in die vesikels nie, alhoewel die blanko-vesikels verbasend hoë aktiwiteit teen die spesifieke tuberkulose-stam getoon het.

Sleutelwoorde: Klofasimien, artemisoon, dekokwinaat, liposome, niosome, transferosome, transdermaal

BRONNELYS

ABDELMALEK, R., MEBAZAA, A., BERRICHE, A., KILANI, B., OSMAN, A.B., MOKNI, M. & BENAISSA, H.T. 2013. Cutaneous tuberculosis in Tunisia. *Médecine et maladies infectieuses,* 43(9):374-378.

BAIG, I.A., MOON, J.Y., KIM, M.S., KOO, B.S. & YOON, M.Y. 2014. Structural and functional significance of the highly-conserved residues in *Mycobacterium tuberculosis* acetohydroxyacid synthase. *Enzyme and microbial technology,* 58-59:52-59.

BIAMONTE, M.A., WANNER, J. & LE ROCH, K.G. 2013. Recent advances in malaria drug discovery. *Bioorganic & medicinal chemistry letters,* 23(10):2829-2843.

DUNAY, I.R., CHI CHAN, W., HAYNES, R.K. & SIBLEY, L.D. 2009. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Journal of antimicrobial agents and chemotherapy,* 53(10):4450-4456.

FADER, T., PARKS, J., KHAN, N.U., MANNING, R., STOKES, S. & NASIR, N.A. 2010. Extrapulmonary tuberculosis in Kabul, Afghanistan: a hospital-based retrospective review. *International journal of infectious diseases,* 14(2):e102-e110.

NAGELSCHMITZ, J., VOITH, B., WENSING, G., ROEMER, A., FUGMANN, B., HAYNES, R.K., KOTECKA, B.M., RIECKMANN, K.H. & EDSTEIN, M.D. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial agents and chemotherapy,* 52(9):3085-3091.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W.C., HAYNES, R.K. & KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. *International journal of pharmaceutics,* 414(1-2):260-266.

YATES, V.M. 2010. Mycobacterial infections. (*In* Burns, T., Breathnach, S., Cox, N. & Griffiths, C., *eds*. Rook's textbook of dermatology. 8th ed. Vol 2. West Sussex, United Kingdom: Blackwell Publishing Ltd. p. 31.1-31.41.)

PREFACE

This thesis is submitted in an article format and written according to the requirements of the North-West University manual for postgraduate studies and conforms to the requirements preferred by the appropriate journals. The thesis is written according to UK English spelling, with the article chapters written according to each journal's Guide to Authors.

Chapter 2:

Article 1: Cutaneous tuberculosis overview and current treatment regimens

Article published in Tuberculosis.

This review publication was written in order to fulfil the requirement of the NWU that a complete literature overview should be included. No separate literature overview was thus included in this thesis as this review was seen as fulfilment of the above requirement.

Chapter 3:

Article 2: Development and validation of the simultaneous determination of artemisone, clofazimine and decoquinate with HPLC

Article accepted for publication in ^{DIE}Pharmazie.

Chapter 4:

Article 3: Topical delivery of artemisone, clofazimine and decoquinate encapsulated in vesicles and their *in vitro* efficacy against *Mycobacterium tuberculosis* H37Rv strain

Article for submission to the Journal of Pharmaceutical and Biomedical Analysis.

Chapter 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 INTRODUCTION AND PROBLEM STATEMENT

Human skin consists mainly of two layers, of which the epidermis is of most importance for this study. The epidermis can be divided into mainly four layers, of which the stratum corneum is the outermost layer. The stratum corneum regulates skin transport and is responsible for the skin's barrier function (Barry, 1983; Barry, 2001; Hadgraft, 2001; Suhonen *et al.*, 1999; Venus *et al.*, 2010; Williams, 2003).

Topical delivery of an active pharmaceutical ingredient (API) is subjected to various specifications due to the complicated structure of the skin and its excellent barrier function. For an API to be delivered into the skin it has to have both hydrophilic and lipophilic properties. The optimal partition coefficient (log P) range is between 1–3 and the optimal molecular weight is 500 g/mol or less (Karande & Mitragotri, 2009; Moser *et al.*, 2001; Niak *et al.*, 2000; Swart *et al.*, 2005).

The APIs chosen for this study are artemisone, clofazimine and decoquinate. These APIs were part of the MALTBRedox MRC South African University Flagship Projects, which focus on oxidant-redox drug combinations for the treatment of malaria, TB and related diseases. This study formed part of the topical and transdermal delivery of actives with the ultimate aim to treat dermal tuberculosis. The physicochemical properties of these APIs can be seen in Table 1.1.

Table 1.1: Physicochemical properties of the three chosen APIs

Property	**Artemisone**	**Clofazimine**	**Decoquinate**
Aqueous solubility (mg/L)	89	10	No data in literature
Molecular weight (g/mol)	401.5	473.4	417.5
Log P	2.49	7.6	7.8
pKa	No data	8.51	10.76
Half-life (h)	2.8	8	Only animal tested
Melting point	199.26	210-212	219.89
Peak plasma concentration (h)	0.875	8-12	Only animal tested

[References: Biamonte *et al.*, 2013; Bolla & Nangia, 2012; Brittain & Florey, 1992; Cholo *et al.*, 2011; Dunay *et al.,* 2009; Holdiness, 1989; Iglesias *et al.*, 2014; Nagelschmitz *et al.*, 2008; Nam *et al.*, 2011; Pharmacopeia online, 2014; Srikanth *et al.*, 2014; Steyn *et al.*, 2011]

This combination of APIs was chosen as a possible topical treatment for cutaneous tuberculosis (CTB). Tuberculosis is a significant public health threat, especially with co-infection of the human immunodeficiency virus (HIV). CTB is a rare and difficult disease to diagnose, consisting of only 1.5% of all extra-pulmonary manifestations (Abdelmalek *et al.*, 2013; Baig *et al.*, 2014; Fader *et al.*, 2010). Currently no topical treatment is available for this disease and only the regular oral treatment is done. In some cases more invasive procedures such as skin grafts are necessary (Yates, 2010).

Delivering APIs into and through the skin can be a complicated process since so many factors need to be considered. As seen in Table 1.1, only artemisone has a favourable log P for skin delivery, whereas decoquinate and clofazimine are both very lipophilic. To enhance permeability of the APIs, vesicles were chosen as a carrier system. Vesicles have been shown to enable topical delivery of difficult to deliver actives into the skin (Jain *et al.*, 2014). Very favourable characteristic of vesicles are their aqueous centre (where the artemisone can concentrate) and their lipid bilayer (where clofazimine and decoquinate can concentrate). There are many types of vesicles, each with its own advantages, but for this study liposomes, niosomes and transferosomes were chosen.

1.2. RESEARCH AIM AND OBJECTIVES

Research aim and objectives for this study included:

- Selecting the three different vesicles to be used as carrier systems for the three APIs chosen.
- Effectively entrapping the three APIs separately, as well as in combination in the different vesicles to be used.
- Determining the characteristics of the vesicle dispersions by means of transmission electron microscopy, pH, viscosity, zeta-potential, size, size distribution and entrapment efficiency.
- Investigating whether adding the APIs has an influence on the characteristics of the vesicle dispersions and how this changes with an increase in API concentration.
- Conducting transdermal skin diffusion studies on black skin and comparing the results obtained from the combination dispersions for the three types of vesicles, as well as a dispersion containing only the APIs and no vesicles.
- Comparing tape stripping data and skin diffusion data to determine whether the APIs permeate into/through the skin, and where the APIs prefer to accumulate.
- Investigating the activity of the different dispersions against tuberculosis to determine the *in vitro* efficacy of the encapsulated APIs.
- Determining whether any/all of the different APIs has activity against tuberculosis, and also what vesicle dispersion is found to be most effective.

REFERENCES

ABDELMALEK, R., MEBAZAA, A., BERRICHE, A., KILANI, B., OSMAN, A.B., MOKNI, M., *et al*. 2013. Cutaneous tuberculosis in Tunisia. *Médecine et maladies infectieuses,* 43(9):374-378.

BAIG, I.A., MOON, J.Y., KIM, M.S., KOO, B.S. & YOON, M.Y. 2014. Structural and functional significance of the highly-conserved residues in *Mycobacterium tuberculosis* acetohydroxyacid synthase. *Enzyme and microbial technology,* 58-59:52-59.

BARRY, B.W. 1983. Dermatological Formulations: Percutaneous Absorption. *Drugs and the pharmaceutical sciences*. Vol. 18. New York: Marcel Dekker. 480p.

BARRY, B.W. 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. *European journal of pharmaceutical sciences,* 14(2):101-114.

BIAMONTE, M.A., WANNER, J. & LE ROCH, K.G. 2013. Recent advances in malaria drug discovery. *Bioorganic & medicinal chemistry letters,* 23(10):2829-2843.

BOLLA, G. & NANGIA, A. 2012. Clofazimine Mesylate: A high solubility stable salt. *Crystal growth and design,* 12(12):6250-6259.

BRITTAIN, H.G. & FLOREY, K. 1992. Profiles of drug substances, excipients and related methodology. Vol 21. London: Academic Press Ltd. 696p.

CHOLO, M.C., STEEL, H.C., FOURIE, P.B., GERMISHUIZEN, W.A. & ANDERSON, R. 2011. Clofazimine: current status and future prospects. *Journal of antimicrobial chemotherapy,* 67(2):290-298.

DUNAY, I.R., CHI CHAN, W., HAYNES, R.K. & SIBLEY, L.D. 2009. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Journal of antimicrobial agents and chemotherapy,* 53(10):4450-4456.

FADER, T., PARKS, J., KHAN, N.U., MANNING, R., STOKES, S. & NASIR, N.A. 2010. Extrapulmonary tuberculosis in Kabul, Afghanistan: a hospital-based retrospective review. *International journal of infectious diseases,* 14(2):e102-e110.

HADGRAFT, J. 2001. Skin, the final frontier. *International journal of pharmaceutics,* 224(1-2):1-18.

HOLDINESS, M.R. 1989. Clinical pharmacokinetics of clofazimine: a review. *Clinical pharmacokinetics,* 16(2):74-85.

IGLESIAS, A., NEBOT, C., VÁZQUEZ, B.I., MIRANDA, J.M., ABUÍN, C.M.F. & CEPEDA, A. 2014. Detection of veterinary drug residues in surface waters collected nearby farming areas in Galicia, North of Spain. *Environmental science and pollution research*, 21(3):2367-2377.

JAIN, S., JAIN, V. & MAHAJAN, S.C. 2014. Lipid based vesicular delivery systems. *Advances in pharmaceutics,* 2014:1-12.

KARANDE, P. & MITRAGOTRI, S. 2009. Enhancement of transdermal drug delivery via synergistic action of chemicals. *Biochimica et biophysica acta (BBA) – Biomembranes,* 1788(11):2362-2373.

MOSER, K., KRIWET, K., NAIK, A., KALIA, Y.N. & GUY, R.H. 2001. Passive skin penetration enhancement and its quantification *in vitro*. *European journal of pharmaceutics and biopharmaceutics,* 52(2):103-112.

NAGELSCHMITZ, J., VOITH, B., WENSING, G., ROEMER, A., FUGMANN, B., HAYNES, R.K., KOTECKA, B.M., RIECKMANN, K.H. & EDSTEIN, M.D. 2008. First assessment in humans of the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial agents and chemotherapy,* 52(9):3085-3091.

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.

NAM, T., McNAMARA, C.W., BOPP, S., DHARIA, N.V., MEISTER, S., BONAMY, G.M.C., PLOUFFE, D.M., KATO, N., McCORMACK, S., BURSULAYA, B., KE, H., VAIDYA, A.B., SCHULTZ, P.G. & WINZELER, E.A. 2011. A chemical genomic analysis of decoquinate, a *Plasmodium falciparum* cytochrome *b* inhibitor. *ACS chemical biology,* 6(11):1214-1222.

PHARMACOPEIA ONLINE. 2014. http://www.uspbpep.com/usp29/v29240/usp29nf245O_m22310.html Date of access: 19 May 2014.

SRIKANTH, C.H., JOSHI, P., BIKKASANI, A.K., PORWAL, K. & GAYEN, J.R. 2014. Bone distribution study of anti leprotic drug clofazimine in rat bone marrow cells by a sensitive reverse phase liquid chromatography method. *Journal of chromatography B,* 960:82-86.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W.C., HAYNES, R.K. & KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. *International journal of pharmaceutics,* 414(1-2):260-266.

SUHONEN, M.T., BOUWSTRA, J.A. & URTTI, A. 1999. Chemical enhancement of percutaneous absorption in relation to stratum corneum structural alterations. *Journal of controlled release*, 59(2):149-161.

SWART, H., BREYTENBACH, J.C., HADGRAFT, J. & DU PLESSIS, J. 2005. Synthesis and transdermal penetration of NSAID glycoside esters. *International journal of pharmaceutics*, 301:71-79.

VENUS, M., WATERMAN, J. & McNAB, I. 2010. Basic physiology of the skin. *Surgery (Oxford),* 28(10):469-472.

WILLIAMS, A.C. 2003. Transdermal and Topical Drug Delivery. 1st ed. London: Pharmaceutical Press. 242p.

YATES, V.M. 2010. Mycobacterial infections. (*In* Burns, T., Breathnach, S., Cox, N. & Griffiths, C., *eds*. Rook's textbook of dermatology. 8th ed. Vol 2. West Sussex, United Kingdom: Blackwell Publishing Ltd. p. 31.1-31.41.)

Chapter 2

REVIEW ARTICLE PUBLISHED IN TUBERCULOSIS

This chapter contains the literature background for this study and is presented in article format as published in the journal *Tuberculosis* in 2015. This review publication was written in order to fulfil the requirement of the NWU that a complete literature overview should be included. No separate literature overview was thus included in this thesis as this review was seen as fulfilment of the above requirement.

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REVIEW

Cutaneous tuberculosis overview and current treatment regimens

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SUMMARY

Tuberculosis is one of the oldest diseases known to humankind and it is currently a worldwide threat with 8–9 million new active disease being reported every year. Among patients with co-infection of the human immunodeficiency virus (HIV), tuberculosis is ultimately responsible for the most deaths. Cutaneous tuberculosis (CTB) is uncommon, comprising 1–1.5% of all extra-pulmonary tuberculosis manifestations, which manifests only in 8.4–13.7% of all tuberculosis cases.

A more accurate classification of CTB includes inoculation tuberculosis, tuberculosis from an endogenous source and haematogenous tuberculosis. There is furthermore a definite distinction between true CTB caused by *Mycobacterium tuberculosis* and CTB caused by atypical *mycobacterium* species. The lesions caused by *mycobacterium* species vary from small papules (e.g. primary inoculation tuberculosis) and warty lesions (e.g. tuberculosis verrucosa cutis) to massive ulcers (e.g. Buruli ulcer) and plaques (e.g. lupus vulgaris) that can be highly deformative.

Treatment options for CTB are currently limited to conventional oral therapy and occasional surgical intervention in cases that require it. True CTB is treated with a combination of rifampicin, ethambutol, pyrazinamide, isoniazid and streptomycin that is tailored to individual needs. Atypical *mycobacterium* infections are mostly resistant to anti-tuberculous drugs and only respond to certain antibiotics. As in the case of pulmonary TB, various and relatively wide-ranging treatment regimens are available, although patient compliance is poor. The development of multi-drug and extremely drug-resistant strains has also threatened treatment outcomes. To date, no topical therapy for CTB has been identified and although conventional therapy has mostly shown positive results, there is a lack of other treatment regimens.

1. Introduction

Tuberculosis (TB) is one the oldest diseases of humankind. As humanity populated the earth, so did this disease spread as well. Typical tuberculous lesions, containing acid-fast bacilli (AFB), have been identified in Egyptian mummies [1–4]. The prevalence of TB increased dramatically during the seventeenth and eighteenth centuries, after which it declined over the next two-hundred years [5]. Later in the nineteenth century, TB again became a major health concern, although improved hygiene and immunisation caused the disease to wane again [6–8].

TB today continues to pose a significant public health threat. The World Health Organisation (WHO) estimates that approximately 20–40% of the world's population are affected, with 8–9 million new cases of active disease being reported every year [9–16]. TB is ultimately responsible for most deaths among patients infected with the human immunodeficiency virus (HIV) [8,17,18,19,20].

Despite TB being such a widespread disease, especially in developing countries, it manifests only as an extra-pulmonary disease in 8.4–13.7% of cases. The difference in data and the low values may also indicate how uncommon and undefined this disease truly is. This increases with co-infection of HIV. Cutaneous tuberculosis (CTB) is relatively uncommon and not a well defined disease, comprising only 1–1.5% of all extra-pulmonary manifestations [21–25,12,26,8,27–29,20,30]. Théophile Laennec [8], inventor of the stethoscope, described the first example of CTB in 1826. CTB is prevalent among women, mostly young adults. The most common site of CTB infection is the face, although it often appears on the neck and torso as well [31].

CTB has many different manifestations, which complicates diagnosis. The increase in multi-drug resistant TB has also resulted in an increase in the occurrence of CTB. Skin manifestations of infections caused by *Mycobacterium tuberculosis* are known as true CTB, but some of the other species of the *Mycobacterium* genus are also responsible for cutaneous manifestations, as summarised in

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Table 1. Mycobacteria can be sub-divided into two sub-genera, namely rapid/fast growers and slow growers. Slow growing organisms have a more than 7 days incubation period for mature growth, whereas rapidly growing organisms have a 7 days or less incubation period for mature growth [32,33,8,34,35].

To date, no topical therapy exists for any of the TB infections. Although most of the current treatment regimens have demonstrated positive results, they are not all completely effective, especially with the rise in multi-drug and extremely drug-resistant TB strains. The potential of using topical treatments to aid in treating TB thus need to be evaluated for improving therapeutic regimens.

2. Classification of cutaneous tuberculosis

In the past, the lack of an accurate classification of CTB has accounted for much of the confusion relating to the disease. In recent years, a more accurate classification system has been developed, using three criteria, i.e. pathogenesis, clinical presentation, and histologic evaluation [2,22,37,38,7,39–43,29]. Using these criteria, CTB can be classified as:

- Inoculation tuberculosis from an exogenous source.
- Tuberculosis from an endogenous source.
- Haematogenous tuberculosis.

These criteria and their symptoms are described next.

2.1. Inoculation of tuberculosis from an exogenous source

Primary inoculation TB (Figure 1), also known as tuberculous chancre, results from the entry of mycobacteria into the skin, or mucosa, through broken skin of a person not previously being infected with, or who has no immunity against *Mycobacterium tuberculosis* [36,44,39,41,45]. The access of mycobacteria through the skin barrier can be caused by inadequately sterilised needles, tattooing, circumcision, piercings, operations, wounds and post mouth-to-mouth resuscitation [37,8,35]. The lesions have often been reported as having a sporotrichoid appearance [46]. Inoculation can occur through various methods and persons working in a medical profession are most at risk of being infected. This was in fact how the first case of CTB was described by Théophile Laennec in 1826 [8], when he noted his own “prosector's wart”. Mucocutaneous contribution towards CTB accounts for approximately one-third of the total number of reported cases. These include infection through the oral cavity (after tooth extraction), or of the conjunctiva [37,47,8].

Exogenous inoculation can cause a warty lesion on the fingers, or other extremities, called *tuberculosis verrucosa cutis* (TVC), in patients previously infected with TB and who have moderate to high immunity [2,39,49,50]. TVC (also known as prosector's wart, lupus verricosus and warty tuberculosis) starts as a painful, small papule, surrounded by a purple, inflammatory corona that progresses into an asymptomatic warty lesion, as illustrated in Figure 2 [51,24,44,52,40,53]. TVC may, in 4.4–16% of cases, present in younger patients [41].

Table 1
Atypical *mycobacterium* species responsible for cutaneous infections [36,32].

Common	Uncommon
Slow growing	
M. haemophilum	*M. avium complex*
M. leprae	*M. kanasii*
M. marinum	*M. malmoense*
M. ulcerans	*M. scrofulaceum*
Fast growing	
M. chelonae	*M. abscessus*
M. fortuitum	–

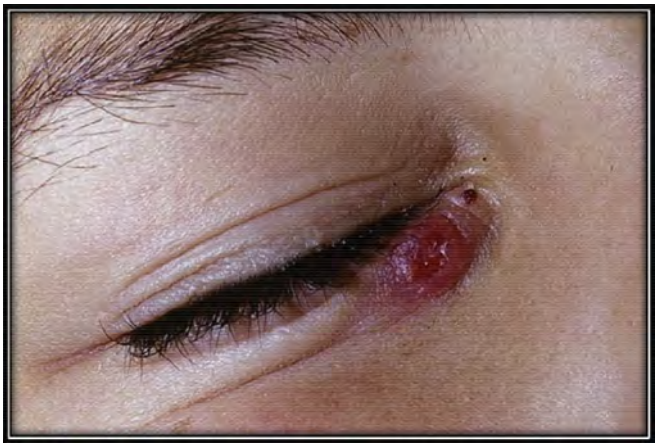

Figure 1. Inoculation tuberculosis in a child [48].

2.2. Tuberculosis from an endogenous source

CTB may also result from the involvement and breakdown of the skin covering a subcutaneous focus, usually a lymph gland (tuberculous lymphadenitis), or TB of the bones and joints, previously described as scrofuloderma (Figures 3 and 4). The lesions start as a subcutaneous, mobile nodule, which soon after attaches to the overlying skin. A discharge then starts and eventually a cutaneous abscess forms. These abscesses may heal spontaneously, although it takes years to completely cure [2,36,54,37,49,53,35]. Scrofuloderma is the most common form of CTB among children younger than ten years of age, with a prevalence of 36–48% [41]. Scrofuloderma suggests that the patient may have a systemic TB infection, particularly pulmonary TB, in 35% of cases. These lesions are more often seen in the axillae, neck, groin and chest [55,8,56].

Orifacial TB (Figure 5) is a rare form of CTB and results from the auto-inoculation of the mucous membrane that occurs when viable organisms are either expectorated, or spread in patients with low immunity. Tissue, normally resistant to infection, is invaded, usually in the nose, oral cavity, perineal and/or perirectal areas. Such

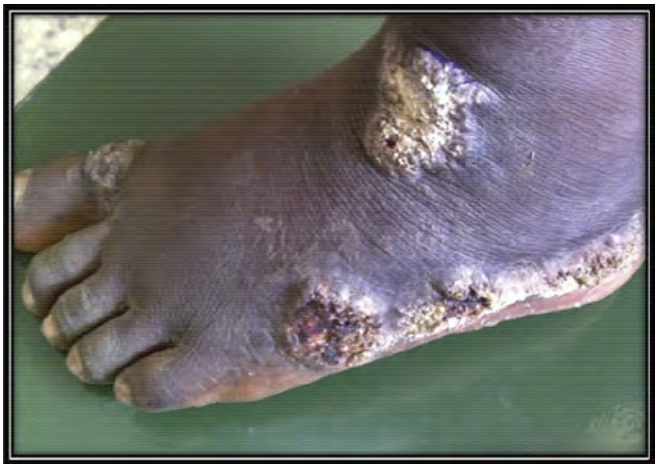

Figure 2. Tuberculosis verrucosa cutis [52].

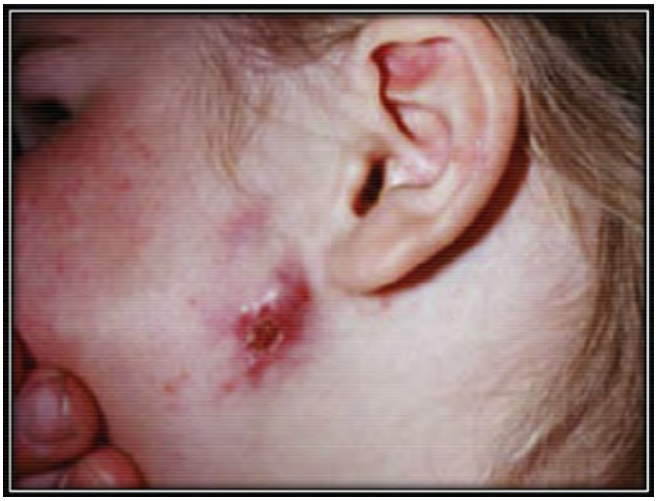

Figure 3. Scrofuloderma [48].

lesions are painful and ulcerative and do not heal naturally. Patients with these infections are likely to have progressive pulmonary, genital, urinary or intestinal TB. In some cases in China, caseation necrosis, visible in orifacial TB and scrofuloderma, has been reported [37,58,49,40,41,59,60].

2.2.1. Haematogenous tuberculosis

Haematogenous spread or lymphatic seeding, accounts for the majority of CTB cases. Haematogenous TB occurs when the AFB spread from a primary site of infection to the rest of the body. Also, it involves chronic CTB in a previously sensitised patient with a high level of TB sensitivity. The most common form of this infection is lupus vulgaris, which also has the highest potential for disfigurement [21,62,25,63,43].

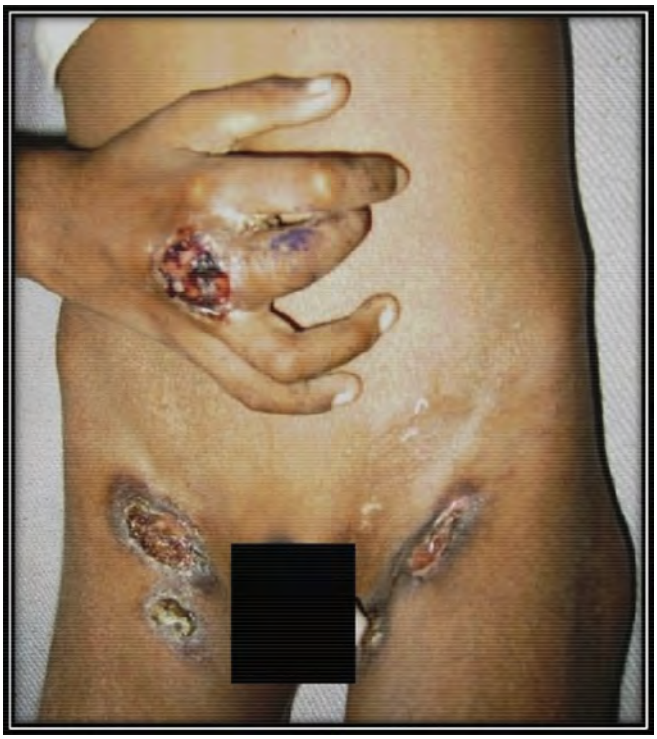

Figure 4. Scrofuloderma in a male patient showing lymph gland involvement [57].

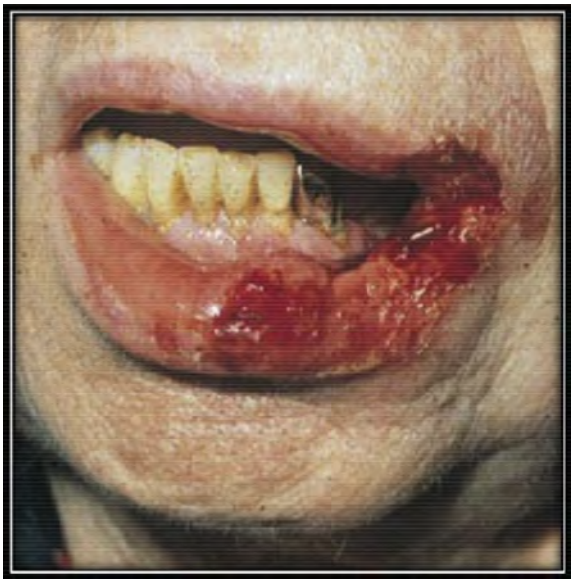

Figure 5. Orifacial tuberculosis [61].

Tuberculous gamma (Figure 6) is a rare form of haematogenous tuberculosis, with an incidence of only 1–2%. The lesions start as firm nodules, which later break down to form abscesses and ultimately ulcers. Tubercles and widespread caseation necrosis are often identifiable. These ulcers are frequently negative for AFB [64,40,60].

Lupus vulgaris (LV) (Figure 7) may develop after *Bacille Calmette Guérin (BCG)* vaccination, or from primary inoculation TB, or as a result of inoculation [25,65]. LV is also very common among younger children, with a prevalence of 41–68% in affected children and adolescents [41]. LV may present in mainly five general forms, of which the plaque form is the most common, representing approximately 32% of all cases. This form of LV starts as a flat, red-brown papule, which slowly expands into a light skin-coloured

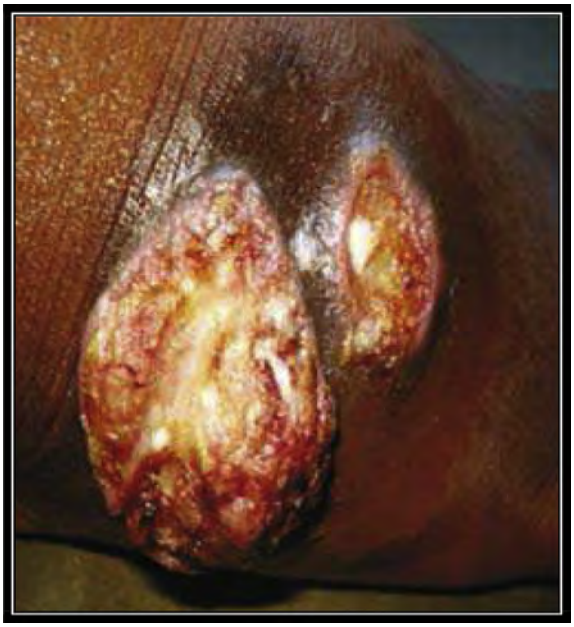

Figure 6. Tuberculous gamma on the dorsum of the right foot of an eight-year old boy [64].

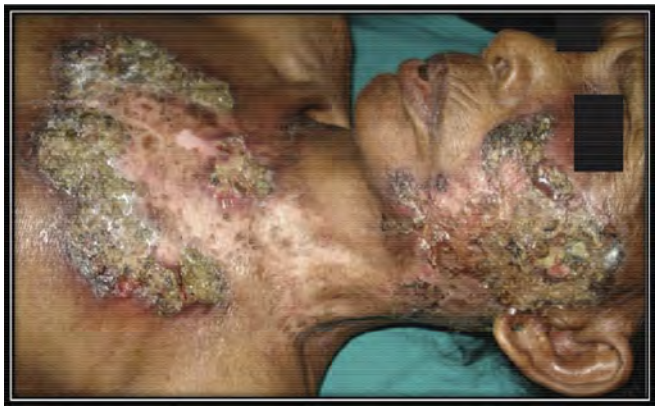

Figure 7. Lupus vulgaris plaque of the face, neck and chest [69].

plaque. It may show irregular areas of scarring and the edge of the plaque is often thickened and hyperkeratotic [66,63,8,53,67,35,68].

The ulcerative and mutilating form (Figure 8) of LV is the most destructive and deforming of all LV lesions. Underlying tissue is invaded and becomes ulcerative and necrotic, leaving an atrophic, crust-like scar [39,70,8,35]. The vegetative form of LV is also characterised by ulcers and necrosis, but with minimal scarring. Vegetative and ulcerative forms are especially destructive when the nasal, or auricular cartilage are involved [8,35].

Miliary, or disseminated TB (Figure 9), also known as *tuberculosis cutis miliaris disseminata*, is a life-threatening form of TB, resulting from the dissemination of tubercles, usually from a pulmonary source [44,42,43]. This disease primarily occurs in children and infants, following an infection such as measles or scarlet fever that reduces their immune response. This is a very rare form of TB, but re-emerges in patients infected with HIV and having a CD4 count lower than 100 cells/μL [44,39,40,35]. The lesions are initially papules (bluish to brownish-red in colour), which may be covered by small vesicles that eventually rupture, or dry with a crust that later develops into an ulcer. The lesions are often closely packed and are teeming with AFB [37,8,41,43].

2.2.1.1. Tuberculids. Tuberculids are not true CTB lesions, but rather arise as the result of hypersensitivity reactions to the TB organism, or its products present in the body of a patient with high immunity. All of the tuberculids show a positive response to anti-tuberculous

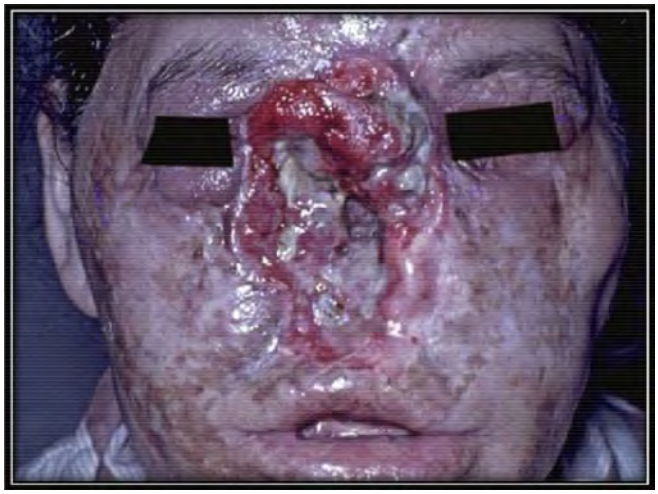

Figure 8. Deforming, ulcerative lupus vulgaris in a caucasian male [71].

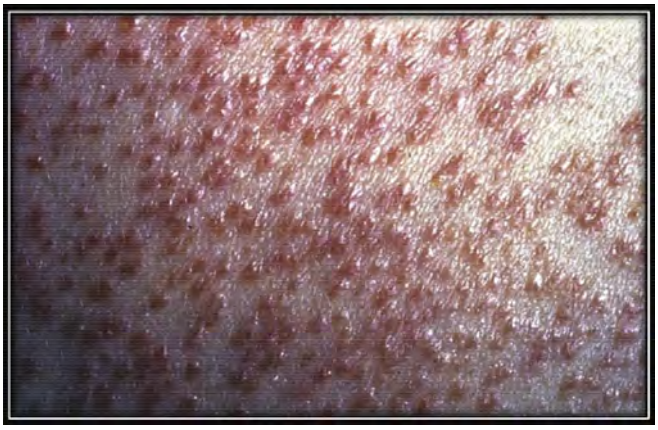

Figure 9. Cutaneous miliary TB before rupture of papules and crust formation [48].

therapy, though they are characterised by negative smears for AFB. Tuberculids may also occur as a result of BCG vaccination, and consequently the vaccination is now only recommended for certain high risk groups [21,72,73,37,25,74,8,35]. True tuberculids can be classified as follows:

- Micropapular: lichen scrofulosorum.
- Papular: papulonecrotic tuberculid.
- Nodular: erythema induratum of Bazin and nodular tuberculid [36,44,41,35].

Lichen scrofulosorum (LS) is a rare, asymptomatic skin eruption that primarily affects children and adolescents with high immunity. The lesions are closely grouped, lichenoid papules that are usually light skin-coloured, although they can also be yellowish or reddish-brown (Figure 10). The lesions are generally found on the chest, abdomen and back areas and are often reported after BCG vaccination. These lesions are also very common in children, with a prevalence among them in 23–33% of cases. The lesions have previously been misdiagnosed as psoriasis due to their inflammatory and scaly appearance [25,75,44,41,35].

Papulonecrotic tuberculids (Figure 11) present as an eruption of dusky-red, necrotising papules, with central crust that mainly affect the extremities of young adults, although it is also observed in infants and children (4% prevalence). The lesions are small and symmetrical and usually appear in clusters. The necrotic lesions leave behind a hyperpigmented atrophic scar and are essentially

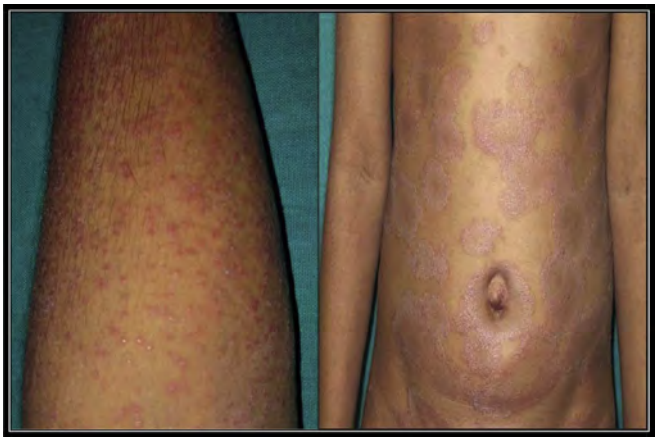

Figure 10. Lichen scrofulosorum of the forearm and abdomen [76,77].

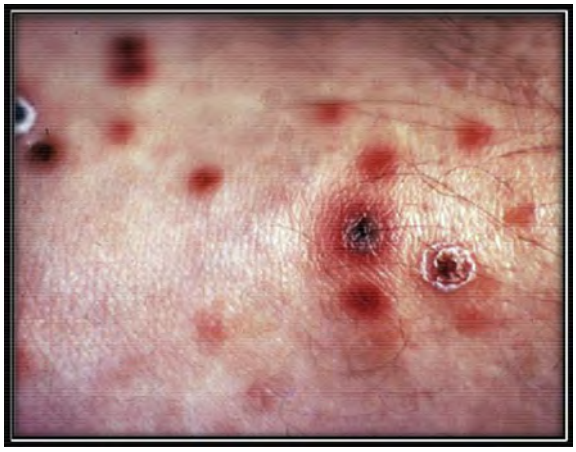

Figure 11. Papulonecrotic tuberculid [79].

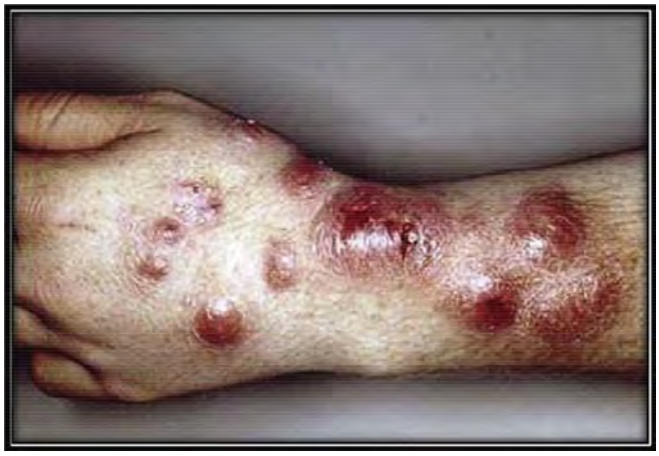

Figure 13. Infection with *Mycobacterium marinum* in the upper extremities [86].

found on the elbows, knees, legs, feet and hands [78,72,74,49,40,8,41,35].

Nodular tuberculids present as dusky-red, non-tender nodules of the lower extremities. The nodules progress into purple-red masses that have a tendency to ulcerate into asymmetrical, superficial ulcers that heal with an atrophic scar [49,40,41]. Erythema induratum of Bazin (EIB) (Figure 12) is accepted as the true nodular tuberculid in which the main pathology is located in the subcutaneous fat. Another pattern has also been identified where the pathology lies in the junction between the subcutaneous fat and the dermis, hence the term nodular tuberculid [44,8,35].

3. Atypical *mycobacterium* infections of the skin

More than 135 species of atypical *Mycobacterium* [or more recently known as non-tuberculous mycobacteria (NTM)] have been described [32,80,81,34], but only a few show cutaneous manifestations, as summarised in Table 1, of which only the most common ones are discussed in this article, namely:

- *Mycobacterium marinum.*
- *Mycobacterium ulcerans*, or Buruli ulcer.
- *Mycobacterium haemophilum.*
- *Mycobacterium fortuitum.*
- *Mycobacterium chelonae.*
- *Mycobacterium abscessus.*
- *Mycobacterium leprae*, or Hansen's disease [32].

NTM can be found in soil, water, flora and some fauna, almost anywhere in the world. The mode of transmission is not completely understood, but human-to-human transmission does not seem to occur. Infection predominantly occurs in immuno-compromised patients, or after skin trauma in immuno-competent patients. Some cases of cutaneous *Mycobacterium* infections have been reported to occur after individuals receive tattoos, subcutaneous insulin therapy, foot baths at nail salons and even acupuncture [82,80,81,49,83].

M. marinum infections are primarily localised at the site of inoculation, typically in the upper extremities [32,80]. These infections are also known as “swimming pool granulomas” or “fish tank granulomas”, since *M. marinum* is widespread in both fresh and marine water [84,85,32,33]. In 2008, an outbreak of *M. marinum* infections occurred on a fish farm in China that workers had contracted from abrasions, or trauma to the skin that had became infected. These infections are characterised by papular lesions (Figure 13) and cellulitis, also often as warty nodules, or plaques that may sometimes present with a sporotrichoid pattern. The nodules may contain a purulent fluid that is positive for AFB and that can ulcerate and become necrotic [85,32,33,86,35].

Mycobacterium ulcerans infections, also known as the Buruli ulcer, are prevalent in warmer climates in riverine areas, such as

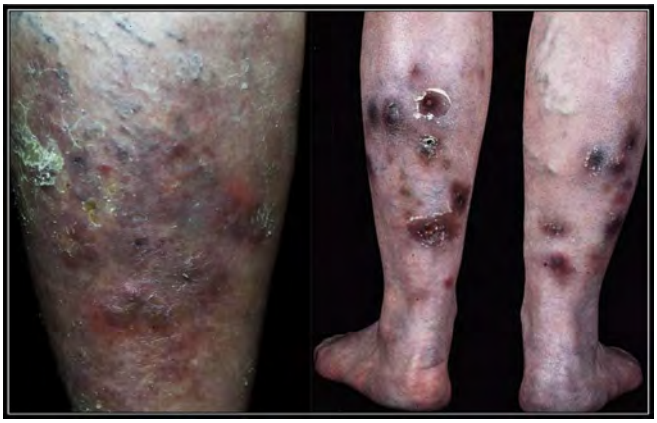

Figure 12. Erythema induratum of Bazin showing prevalence in the lower extremities [79].

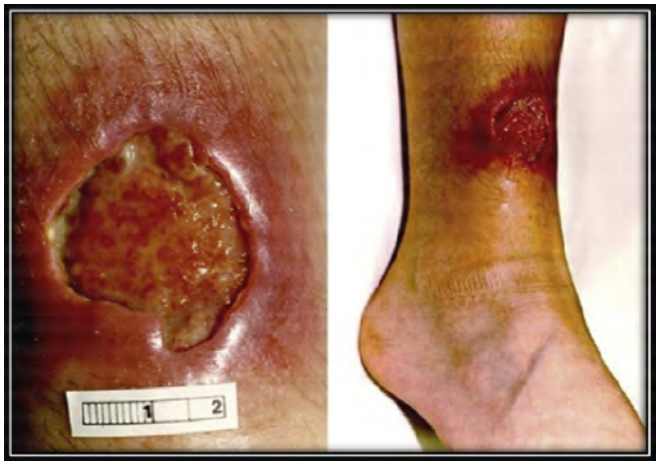

Figure 14. Buruli ulcer in an eleven-year old boy from Australia [90].

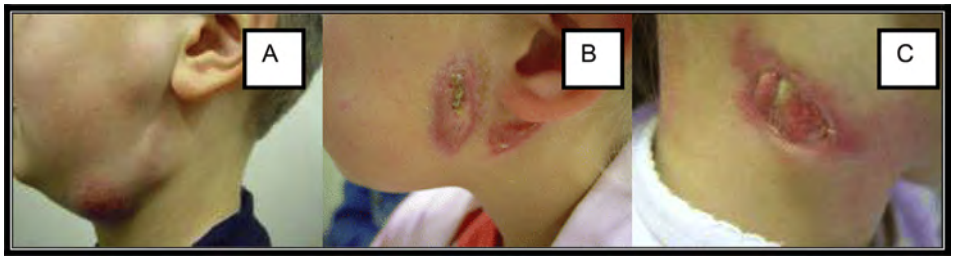

Figure 15. Cervicofacial *Mycobacterium haemophilum* lymphadenitis in a child, A: presenting as a red swelling of the skin, B: after skin breakdown, and C: ulcerating open wound [92]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lakes and swamps. The exact mode of transmission is unknown. Children under the age of fifteen are more prone to these infections. Because these ulcers are painless and the patients often live in remote areas, most patients receive treatment too late, when the damage is already extensive. The lesions start as small, subcutaneous nodules, which may be pruritic. Later, the nodules break and form a shallow necrotic ulcer (Figure 14) that may grow over massive areas of extremities and the body [87,88,33,89,35].

Mycobacterium haemophilum infections are commonly recognised in immuno-compromised patients, although the habitat of this organism and its means of acquisition are unknown [32,35]. Infections have been observed among patients who underwent organ transplants, who received long-term immuno-suppressive therapy and in immuno-competent children with lymphadenitis. *M. haemophilum* causes tender, nodular skin lesions, which may develop into ulcers, or abscesses, seeping a purulent exudate (Figure 15). These lesions may also present as annular plaques, or panniculitis, which typically occur on the extremities and are often located across joints [91,88,33,80,35].

Mycobacterium fortuitum infections also cause nodular skin lesions in immuno-compromised patients, like *Mycobacterium haemophilum* [33]. This organism can grow at a temperature of 45 °C [35]. The lesions are present as ulcers, abscesses, nodules, cellulitis and sinuses. Lesions may form a purulent discharge and may prompt extensive subcutaneous necrosis and pus formation [80].

Mycobacterium chelonae (Figure 16) and *Mycobacterium abscessus* (Figure 17) infections present as disseminated cutaneous diseases in immuno-compromised patients. Such infections present as multiple, nodular lesions in no particular pattern. Patients over than forty-five years of age are more often affected. *M. chelonae* infections are associated with corticosteroid therapy and often occur after direct inoculation. This organism causes community-acquired disease, for example after skin or soft-tissue infections and as sporadic, nosocomial infections (after surgery, injections, transplants and catheter use). *M. abscessus* more often than not causes abscesses at injection sites. This organism represents the most pathogenic and drug resistant mycobacteria of the fast growing group [83,35].

Mycobacterium leprae causes leprosy that mostly affects the skin, testes, upper respiratory passages, the superficial segment of peripheral nerves and anterior segments of the eyes. Leprosy, also known as Hansen's disease, has been widely discussed in the literature [95,96]. The disease starts as a few lesions that multiply and attack the peripheral nerves in susceptible patients. Transmission of *M. leprae* may be respiratorial, or via direct skin contact with the organism (e.g. through BCG vaccination) [73,32]. Early manifestation may present as a visible skin lesion, or an area of numbness on the skin. The most common initial lesions are that of indeterminate leprosy, which consist of one or more faintly hypopigmented, or erythematous macules, with poorly defined borders. Established leprosy (Figure 18) can be divided into tuberculoid leprosy, lepromatous leprosy and borderline leprosy, as summarised in Table 2 [95].

4. Current treatment regimens of cutaneous tuberculosis

Most cutaneous tuberculosis forms are sensitive to anti-tuberculous therapy taken orally [54,25,99,55,44,40,34,60]. *Mycobacterium tuberculosis* has the ability to create drug resistance and to avoid this, several anti-tuberculous drugs are administered

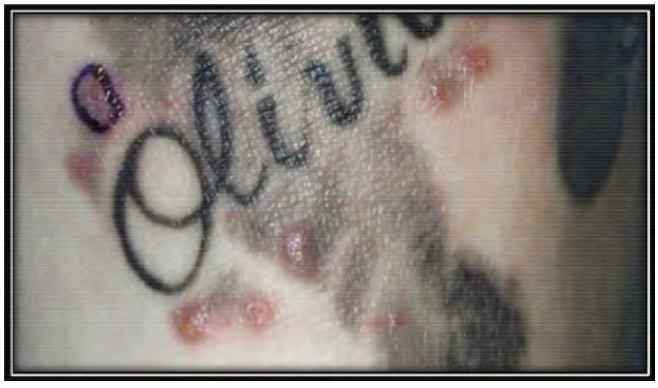

Figure 16. A fresh tattoo infected with *Mycobacterium chelonae* [93].

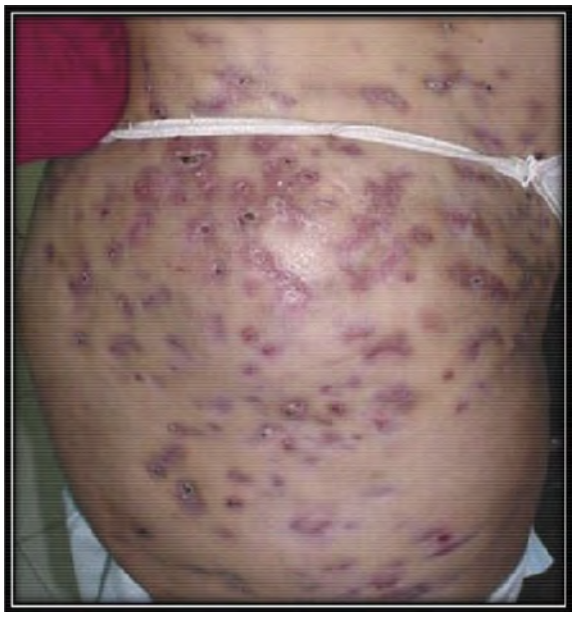

Figure 17. Lesions caused by *Mycobacterium* abscessus [94].

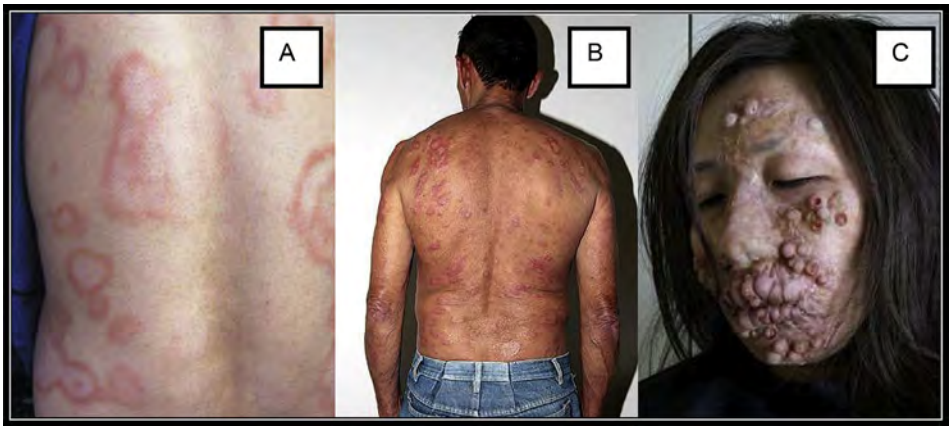

Figure 18. Established leprosy in order from A: tuberculoid leprosy, B: borderline leprosy, to C: lepromatous leprosy [97,98,79].

simultaneously. Frequent treatment is required (daily or every 3 days, according to individual need) in a combination of drugs and for a sufficiently long duration to ensure that the lesions are completely free of infection. Anti-tuberculous therapy usually stretches over a few months, which makes patient compliance difficult [100,20]. In 1993, the WHO launched the so called, directly observed treatment, short-term (DOTS) strategy, to improve patient compliance. Since 1995 to 2008, 83.7% of cases treated under DOT were cured and case fatalities had decreased from 8 to 4% [101,102,28].

The DOTS program, however, has not demonstrated adequate impact to eliminate TB by the targeted year 2050. The main setback in reaching this target has been the lack of resources to implement the Global Plan to Stop TB project, as launched in 2006 [103].

Table 2
The classification of established leprosy [95,49,96].

Types of leprosy	Clinical features
Tuberculoid leprosy (TT)	• Number of lesions is 1–10. • Nerve involvement is marked with anaesthesia localised to lesions. • Borders are well defined. • Lesion is typically a plaque that is erythematous, copper-coloured or purple and hypopigmented in the centre. • The surface is dry, hairless, insensitive and sometimes scaly.
Borderline leprosy (BB)	• More lesions are present, compared to TT. • Nerve involvement is common. • Borders are less defined than TT. • Lesions may take the form of macules, plaques, bizarre-shaped bands, or annular lesions. • BB is the most common type of leprosy and is unstable. It could down-grade to LL or upgrade to TT.
Lepromatous leprosy (LL)	• Multiple lesions are present. • Nerve involvement is common, with anaesthesia on dorsal areas of hands and feet. Blindness can result if corneal nerves are affected. • Borders may be vague. • Lesions can be a combination of macules, papules, nodules, or infiltration. Distribution is symmetrical and can also cause ulceration and bleeding in the nasal mucosa. • Nails become thin and brittle. Fingers may become crooked or short. Skin thickens and lines become deeper on the forehead. The voice becomes hoarse and teeth may become loose, or fall out.

Resistant TB has become a major public health challenge worldwide, since progress in TB treatment has declined, due to increasingly fewer patients being fully cured by pharmacotherapy [104,38,50,27,105]. This overall has led to increased research into the development of TB vaccines, and as a result promising vaccines are in the pipeline [106].

4.1. True cutaneous tuberculosis and tuberculids

The WHO recommends a drug regimen for the treatment of tuberculosis and thus also for true CTB. The regimen consists of two phases, i.e. firstly the intensive phase for 8 weeks, and secondly, the maintenance phase for 16 weeks. In HIV positive patients, phase two is administered for 28 weeks, instead of 16 [10,37,25,44]. The most useful first-line drugs for CTB treatment include isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (STR). Phase one treatment consists of INH, RIF, EMB and PZA for 2 months, followed by 4 months' treatment with INH and RIF in phase two. If INH resistance is suspected, EMB can also be given in phase two [22,6,47,38,13,14,29,65,56,34,20,35,30].

In cases where CTB is located around natural openings, additional treatment with 2% of lactic acid and with local anaesthetics is applied. Surgical excision of lesions and the correction of deformities can also be performed [66,25,44,107,20]. Since tuberculids are an allergic reaction to *M. tuberculosis*, present in the patient's system, anti-tuberculous therapy is also recommended for such lesions [62,74,35].

4.2. Atypical mycobacterium infections

Since non-tuberculous mycobacteria (NTM) is resistant to most anti-tuberculous drugs, the treatment of these organisms is long and difficult [33,81].

Studies have shown that rifampicin and rifabutin are the most active drugs against *Mycobacterium marinum* [35]. In immunocompetent patients, therapy consists of single or dual therapy with drugs, such as clarithromycin, minocycline, doxycycline, or trimethoprim-sulfamethoxazole, or combination therapy with EMB and RIF. Therapy should continue for at least 3–6 months. Deeper, more serious infections should primarily be treated with clarithromycin and RIF for at least 7 months [85,32,80,35].

Mycobacterium ulcerans infections are non-responsive to pharmacotherapy and extensive surgical management is the key treatment in virtually all cases. Skin grafting is also an option in some cases. Therapy with RIF and either STR, or amikacin has shown complete healing in 50% of patients after 8 weeks and has thus been adopted by the WHO. If the disease is diagnosed and treated at an

early stage, it may respond to a regimen including clarithromycin, RIF and EMB, although the optimal therapy duration is still unknown [87,32,80,89,35]

The *Mycobacterium haemophilum* organism is typically resistant to INH, EMB and STR. Multi-drug regimens, including rifabutin, RIF, clarithromycin, ciprofloxacin, amikacin, trimethoprim-sulfamethoxazole, levofloxacin, cefoxitin, doxycycline and cotrimoxazole have been found to be successful. Immuno-competent patients must continue with therapy for 6–9 months, whereas immuno-compromised patients have to continue with therapy indefinitely. Surgical excision has also been reported as being beneficial [91,108,32,80,35].

Typical treatments for the *Mycobacterium fortuitum* organism consist of clarithromycin and either doxycycline, trimethoprim-sulfamethoxazole, or ciprofloxacin for 2 months. Recently it has been found that this organism is susceptible to newer macrolides (e.g. roxithromycin, clarithromycin and azithromycin), doxycycline, minocycline, sulphonamides, cefoxitin, amikacin, ciprofloxacin and imipenem. More serious infections involving larger areas, deep tissue and extension into bony structures, are treated with intravenous amikacin, cefoxitin, or imipenem and treatment is recommended for 4–6 months. Surgical debridement is beneficial and the addition of clarithromycin to the treatment regimen has been suggested [32,80,34,35].

Combination therapy of at least two agents must be given to decrease the chances of resistance against *Mycobacterium chelonae*, although therapy with clarithromycin alone has shown effective results. Ciprofloxacin is for this reason combined with clarithromycin, whilst doxycycline has also shown effectiveness against this organism. More serious infections with deep tissue involvement can be treated with a combination of imipenem, amikacin, tobramycin, linezolid and macrolides for at least 6–12 months. *M. chelonae* usually shows resistance to cefoxitin [109,32,80,83,34].

Therapy for a mild infection of *Mycobacterium abscessus* may include clarithromycin/azythromycin, clofazimine and linezolid for 3–6 months. For a more serious infection, clarithromycin/azithromycin (oral) and either cefoxitin, imipenem, or amikacin (intravenously) may be given for a minimum of 6 months. The same course of treatment as for *M. fortuitum* may be followed, although the prognosis may be worse than for *M. fortuitum* infections [32,80,34,35].

Regimens recommended by the WHO for *Mycobacterium leprae* have been classified into three categories for simplicity:

- For few or singular lesions some clinicians prescribe RIF, ofloxacin and minocycline in combination or individually, depending on patient needs. Due to insufficient follow-up data, however, the WHO recommends that these patients can also be treated by the pauci-bacillary regimen.
- The pauci-bacillary regimen consists of rifampicin once a month and dapsone daily for 6 months.
- The multi-bacillary regimen is administered for more serious infections and includes RIF and clofazimine (300 mg) once a month, and dapsone and clofazimine (50 mg) once a day for at least 12 months. Clofazimine may be substituted by prothionamide, ethionamide, or minocycline in cases where communities disapprove of clofazimine, due to its adverse effect of darkening the skin, especially of the lesions [32,95,96].

5. Summary

TB poses a major health challenge worldwide [104,50,41,105]. CTB is an uncommon form of TB, with only a 1–1.5% prevalence among all reported extra-pulmonary TB cases [21,27,29,20,30]. CTB is difficult to diagnose, due to its rare nature and the fact that it may present in various clinical forms [55,110,65]. Such clinical presentations can vary from small papules, warty lesions, ulcers, or papules to highly deformative plaques that are thickened and hyperkeratotic [24,37,49,63,8,53,35]. It is very important to diagnose and treat TB as early as possible to avoid any complications, although it has often been misdiagnosed as tinea corporis and carcinoma, for example [2,70,41,59,65].

True CTB is caused by the *M. tuberculosis* organism and the BCG vaccine. It can be contracted through inoculation from an exogenous source (e.g. tuberculous chancre), from an endogenous source (e.g. scrofuloderma), or through haematogenous spread (e.g. lupus vulgaris). Atypical CTB infections are caused by other *Mycobacterium* species, such as *M. abscessus*, *M. chelonae* and *M. ulcerans* [22,32,38,80,81,43,65,34,45].

True CTB can be treated with the regular anti-tuberculous regimens, including INH, RIF, EMB and PZA. Surgical excision and reconstruction are often required in very serious cases. Atypical *Mycobacterium* infections can be treated with a variety of antibiotics, although treatment is difficult, due to these organisms often being resistant to regular TB medication [25,38,7,13,44,46,14,107,111,56,20].

CTB and other cutaneous mycobacterial infections are mostly unsightly and thus often also significantly impact on the patient's social and mental wellness. To date, no topical therapy has been identified for any of the discussed infections, and although most of the current regimens have demonstrated positive results, they are not all completely effective. There is furthermore a high probability of drug resistance formation, due to low patient compliance and ever evolving organisms.

In addition to the need for improving typical therapeutic regimens currently used, such as reducing the duration and adverse effects of TB treatments, the potential of topical treatments to aid in treating these infections need to be properly explored. No current topical treatment for CTB has been found in the literature. Although topical therapy would not be a substitute for conventional treatments, it may be used concurrently and possibly aid in improving treatment outcomes.

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References

[1] Djelouadji Z, Raoult D, Drancourt M. Palaeogenomics of Mycobacterium tuberculosis: epidemic bursts with a degrading genome. Lancet Infect Dis 2011;11(8):641–50.
[2] Banashankari GS, Rudresh HK, Harsha AH, Bharathi R, Kamble P. An unusual presentation of cutaneous tuberculosis for surgeons: review of literature. Indian J Surg 2012;74(4):314–7.
[3] Daniel TM. The history of tuberculosis. Respir Med 2006;100(11):1862–70.
[4] Shet A. Tuberculosis in the days of yore. Pediatr Infect Dis 2012;4(2):43–4.
[5] Daniel TM. Tuberculosis in history: did it change the way we live? In: Schlossberg D, editor. Tuberculosis and nontuberculous mycobacterial infections. 6th ed. Washington, DC: ASM Press; 2011. p. 3–10.

[6] Dandewate P, Vemuri K, Khan EM, Sritharan M, Padhye S. Synthesis, characterization and anti-tubercular activity of ferrocenylhydrazones and their ß-cyclodextrin conjugates. Carbohydr Polym 2014;108(1):135–44.
[7] Kar S, Krishnan A, Gangane N, Preetha K. Scrofuloderma: a case series from rural India. Indian J Tuberc 2011;58(4):189–95.
[8] Rullán J, Seijo-Montes RE, Vaillant A, Sánchez NP. Cutaneous manifestations of pulmonary disease. In: Sánchez NP, editor. Atlas of dermatology in internal medicine. 14th ed. San Juan, Puerto Rico: Springer; 2012. p. 17–30.
[9] Carman AS, Patel AG. Science with societal implications: detecting *Mycobacterium tuberculosis* in Africa. Clin Microbiol Newsl 2014;36(10):73–7.
[10] Hernandez C, Cetner AS, Jordan JE, Puangsuvan SN, Robinson JK. Tuberculosis in the age of biologic therapy. J Am Acad Dermatol 2008;59(3):363–80.
[11] Galagan JE. Genomic insights into tuberculosis. Nat Rev Genet 2014;15(5):307–20.
[12] Ilgazli A, Boyaci H, Basyigit I, Yildiz F. Extrapulmonary tuberculosis: clinical and epidemiologic spectrum of 636 cases. Arch Med Res 2004;35(5):435–41.
[13] Kaur IP, Singh H. Nanostructured drug delivery for better management of tuberculosis. J Control Release 2014;184(1):36–50.
[14] Petkova Z, Valcheva V, Momekov G, Petrov P, Dimitrov V, Doytchinova I, et al. Antimycobacterial activity of chiral aminoalcohols with camphene scaffold. Eur J Med Chem 2014;81:150–7.
[15] Singhal S, Jaiswa P. Presentation of tuberculosis in TB-HIV co-infection patients and the treatment outcome with directly observed short course therapy. Asian Pac J Trop Biomed 2011;1(2):S266–7.
[16] Ugarte-Gil C, Ponce M, Zamudio C, Canaza L, Samalvides F, Seas C. Knowledge about HIV prevention and transmission among recently diagnosed tuberculosis patients: a cross sectional study. BMC Public Health 2013;13:1237–46.
[17] Baig IA, Moon JY, Kim MS, Koo BS, Yoon MY. Structural and functional significance of the highly-conserved residues in *Mycobacterium tuberculosis* acetohydroxyacid synthase. Enzym Microb Technol 2014;58–59:52–9.
[18] Hanifa Y, Fielding KL, Charalambous S, Variava E, Luke B, Churchyard GJ, et al. Tuberculosis among adults starting antiretroviral therapy in South Africa: the need for routine case finding. Int J Tuberc Lung Dis 2012;16(9):1252–9.
[19] Saxena S, Karkhanis V, Joshi JM. Tuberculosis prevention: an enigma worth unravelling. Indian J Tuberc 2012;59(2):65–7.
[20] Wyrzykowska N, Wyrzykowski M, Zaba R, Silny W. Treatment of cutaneous infections caused by Mycobacterium tuberculosis. Adv Dermatol Allergol 2012;29(4):293–8.
[21] Abdelmalek R, Mebazaa A, Berriche A, Kilani B, Osman AB, Mokni M, et al. Cutaneous tuberculosis in Tunisia. Méd Mal Infect 2013;43(9):374–8.
[22] Bravo FG, Gotuzzo E. Cutaneous tuberculosis. Clin Dermatol 2007;25(2):173–80.
[23] Fader T, Parks J, Khan NU, Manning R, Stokes S, Nasir NA. Extrapulmonary tuberculosis in Kabul, Afghanistan: a hospital-based retrospective review. Int J Infect Dis 2010;14(2):e102–10.
[24] Ghosh S, Aggarwal K, Jain VK, Chaudhuri S, Ghosh E, Arshdeep. Tuberculosis verrucosa cutis presenting as diffuse plantar keratoderma: an unusual sight. Indian J Dermatol 2014;59(1):80–1.
[25] Ho SCK. Cutaneous tuberculosis: clinical features, diagnosis and management. Hong Kong J Dermatol Venereol Bull 2003;11:130–8.
[26] Puri N. A clinical and histopathological profile of patients with cutaneous tuberculosis. Indian J Dermatol 2011;56(5):550–2.
[27] Sankar MM, Singh J, Diana SCA, Singh S. Molecular characterization of *Mycobacteium tuberculosis* isolates from Indian patients with extrapulmonary tuberculosis. Tuberculosis 2013;93:75–83.
[28] Sevgi DY, Derin O, Alpay AS, Gündüz A, Konuklar AS, Bayraktar B, et al. Extrapulmonary tuberculosis: 7 year-experience of a tertiary center in Istanbul. Eur J Intern Med 2013;24:864–7.
[29] Stockamp NW, Paul S, Sharma S, Libke RD, Boswell JS, Nassar NN. Cutaneous tuberculosis of the penis in an HIV-infected adult. Int J STD AIDS 2013;24:57–8.
[30] Zouhair K, Akhdari N, Nejjam F, Ouazzani T, Lakhdar H. Cutaneous tuberculosis in Morocco. Int J Infect Dis 2007;11(3):209–12.
[31] Solis AH, González NEH, Cazarez F, Pérez PM, Diaz HO, Escobar-Gutierrez A, et al. Skin biopsy: a pillar in the identification of cutaneous *Mycobacterium tuberculosis* infection. J Infect Dev Ctries 2012;6(8):626–31.
[32] Gentry, C.A. http://www.accp.com/docs/bookstore/psap/p5b6sample02.pdf [accessed 18.03.14].
[33] Jones-Lopez EC, Ellner JJ. Tuberculosis and atypical mycobacterial infections. In: Guerrant RL, Walker DH, Weller PF, editors. Tropical infectious diseases. 3rd ed. London: Elsevier Inc.; 2011. p. 228–47.
[34] Walter N, Daley CL. Tuberculosis and nontuberculous mycobacterial infections. In: Spiro SG, Silvestri GA, Agustí A, editors. Clinical respiratory medicine. 4th ed. Philadelphia: Elsevier Ltd.; 2012. p. 383–405.
[35] Yates VM. Mycobacterial infections. In: Burns T, Breathnach S, Cox N, Griffiths C, editors. Rook's textbook of dermatology. 8th ed., vol. 2. West Sussex, United Kingdom: Blackwell Publishing Ltd; 2010. 31.1–31.41.
[36] Cardoso JC, Calonje E. Cutaneous infections presenting with granulomatous infiltrates: a review of histopathological patterns. Pathol Infect Dis 2013;19(2):54–61.
[37] Hill MK, Sanders CV. Cutaneous tuberculosis. In: Schlossberg D, editor. Tuberculosis and nontuberculous mycobacterial infections. 6th ed. Washington, DC: ASM Press; 2011. p. 409–14.
[38] Kansal HM, Goel S. Cutaneous manifestations in cases of pulmonary tuberculosis: a clinical profile. J Indian Acad Clin Med 2013;14(3–4):284–6.
[39] Macgregor RR. Cutaneous tuberculosis. Clin Dermatol 1995;13(3):245–55.
[40] Ramarao S, Greene JN, Casanas BC, Carrington ML, Rice J, Kass J. Cutaneous manifestations of tuberculosis. Infect Dis Clin Pract 2012;20(6):376–83.
[41] Sethuraman G, Ramesh V. Cutaneous tuberculosis in children. Pediatr Dermatol 2013;30(1):7–16.
[42] Sharma SK, Mohan A, Sharma A, Mitra DK. Miliary tuberculosis: new insights into an old disease. Lancet Infect Dis 2005;5(7):415–30.
[43] Steger JW, Barrett TL. Cutaneous tuberculosis. In: James WD, editor. The textbook of military medicine: military dermatology, vol. 4. Fort Sam Houston, Texas: Borden Institute; 1994. p. 355–89.
[44] Lai-Cheong LE, Perez A, Tang V, Martinez A, Hill V, Menagé HDUP. Cutaneous manifestations of tuberculosis. Clin Exp Dermatol 2007;32(4):461–6.
[45] Zadbuke S, Khan N, Set R, Shastri J. Primary inoculation tuberculosis following a vehicular accident. Indian J Med Microbiol 2011;30(1):98–100.
[46] Nakamura S, Hashimoto Y, Nishi K, Takahashi H, Takeda K, Mizumoto T, et al. Cutaneous tuberculosis simulating lymphocutaneous sporotrichosis. Australas J Dermatol 2012;53(4):136–7.
[47] Huang D, Yin H. Primary inoculation tuberculosis after accidental scalpel injury. Infect 2013;41(4):841–4.
[48] Ngan, V. http://www.dermnetnz.org/bacterial/tuberculosis.html [accessed 25.03.14].
[49] Paller AS, Mancini AJ. Hurwitz clinical pediatric dermatology: a textbook of skin disorders of childhood and adolescence. 3rd ed. Philadelphia: Elsevier Inc.; 2006. p. 737.
[50] Sahoo SP, Misra J, Subudhi BSK, Panda AK. Tuberculous lesion of the foot presenting as epithelioma. Singap Med J 2013;54(3):e59–61.
[51] Fukamachi S, Kawakami C, Kabashima R, Sawada Y, Sugita K, Nakamura M, et al. Tuberculosis verrucosa cutis with elevation of circulating T-helper 1 and 17 cells and their reductions after successful treatment. J Dermatol 2011;39(5):507–9.
[52] Rajan J, Mathai AT, Prasad PK, Kaviarasan PK. Multifocal tuberculosis verrucosa cutis. Indian J Dermatol 2011;56(3):332–4.
[53] Shimizu H. Shimizu's textbook of dermatology. 1st ed. Hokkaido University Press; 2005. p. 528.
[54] Das CK, Mahapatra A, Das MM, Sahoo D, Chayani N. Coexistence of cutaneous tuberculosis (scrofuloderma) and Hanseniasis: a rare presentation. J Clin Diagn Res 2014;8(2):141–2.
[55] Kim GW, Park JH, Kim HS, Kim SH, Ko HC, Kim BS, et al. Delayed diagnosis of scrofuloderma misdiagnosed as a bacterial abscess. Ann Dermatol 2012;24(1):70–3.
[56] Verma S, Thakur BK, Gupta A. Multifocal childhood cutaneous tuberculosis: report of two interesting cases from Sikkim, India. Pediatr Dermatol 2013;30(3):e1–4.
[57] Singal, A. & Sonthalia, S. http://www.ijdvl.com/text.asp?2010/76/5/494/69060 [accessed 25.03.14].
[58] Mehta PK, Raj A, Singh N, Khuller GK. Diagnosis of extrapulmonary tuberculosis by PCR. FEMS Immunol Med Microbiol 2012;66(1):20–36.
[59] Sun WL, Xu KL, Chen LL, Yu ZS. Tuberculosis cutis orificialis with both gingival involvement and underlying pulmonary tuberculosis. Aust Dent J 2011;56(2):216–20.
[60] Wang H, Wu Q, Lin L, Cui P. Cutaneous tuberculosis: a diagnostic and therapeutic study of 20 cases. J Dermatol Treat 2011;22(6):310–4.
[61] Wolf, K. http://mizzouderm.com/misc-bacteria.html [accessed 25.03.14]..
[62] Arora S, Arora G, Kakkar S. Cutaneous tuberculosis: a clinic-morphological study. Med J Armed Forces India 2006;62(4):344–7.
[63] Rhodes J, Caccetta TP, Tait C. Lupus vulgaris: difficulties in diagnosis. Aust J Dermatol 2013;54:e53–5.
[64] Dash M, Sarangi R, Panda M. Generalized tuberculous gamma. Indian Pediatr 2012;49(9):773.
[65] Tutanc M, Arica V, Basarslan F, Dogramaci AC, Ozgur T, Akcora B. The youngest patient of lupus vulgaris: a cutaneous tuberculosis case report. Pak J Med Sci 2012;28(3):533–5.
[66] Ezzedine K, Simonart T, Malvy D, Bourgoin C, Noel JC. Cutaneous verrucous carcinoma arising in lupus vulgaris. J Dermatol 2011;39(5):505–7.
[67] Turan E, Yurt N, Yesilova Y, Celik OI. Lupus vulgaris diagnosed after 37 years: a case of delayed diagnosis. Dermatol Online J 2012;18(5):13.
[68] Zaki SA, Sami SA, Sami LB. Cutaneous tuberculosis. Lung India 2011;28(3):229–30.
[69] Thakur BK, Verma S, Hazarika D. A clinicopathological study of cutaneous tuberculosis at Dibrugarh district, Assam. Indian J Dermatol 2012;57(1):63–5.
[70] Rahman MH, Ansari NP, Hadiuzzaman M, Nipa NI, Islam S, Mumu SA, et al. Lupus vulgaris on the buttock mimicking tinea corporis. J Pak Assoc Dermatol 2011;21(4):295–7.
[71] Derm-IS team. http://www.dermis.net/dermisroot/en/10368/image.htm [accessed 26.03.14].
[72] Dongre AM, Sanghavi SA, Khopkar US. Papulonecrotic tuberculid at the site of tuberculin test in a patient with concomitant erythema induratum and papulonecrotic tuberculid. Indian J Dermatol Venereol Leprol 2013;78(2):248–51.
[73] Gantzer A, Neven B, Picard C, Brousse N, Lortholary O, Fischer A, et al. Severe cutaneous bacillus Calmette-Guérin infection in immunocompromised children: the relevance of skin biopsy. J Cutan Pathol 2012;40(1):30–7.

[74] Kim GW, Park HJ, Kim HS, Chin HW, Kim SH, Ko HC, et al. Simultaneous occurrence of papulonecrotic tuberculid and erythema induratum in a patient with pulmonary tuberculosis. Pediatr Dermatol 2013;30(2):256–9.
[75] Kumar U, Sethuraman G, Verma P, Das P, Sharma VK. Psriasiform type of lichen scrofulosorum: clue to disseminated tuberculosis. Pediatr Dermatol 2011;28(5):532–4.
[76] Dandale, A., Gupta, N., Dhurat, R. & Ghate, S. http://www.ijdvl.com/text.asp?2013/79/3/436/110797 [accessed 27.03.14].
[77] Singhal, P., Patel, P.H. & Marfatia, Y.S. https://www.scienceopen.com/document/vid/4f2a39ce-172d-4047-983f-5d6e3f86c730;jsessionid=DUzhMU8RAVXFsCr8dpd3CUnN.master:so-app1-prd [accessed 27.03.14].
[78] Brinca A, Andrade P, Vieira R, Figueiredo A. Papulonecrotic tuberculid: report of a clinical case. Dermatol Rep 2011;3(e29):66–7.
[79] Jehad. http://dermaamin.com/site/atlas-of-dermatology.html [accessed 27.03.14]..
[80] Khan, F.A. & Khakoo, R. http://www.cutis.com/fileadmin/qhi-archive/ArticlePDF/CT/088040194.pdf [accessed 31.03.14].
[81] Kullavanijaya P. Atypical mycobacterial cutaneous infection. Clin Dermatol 1999;17(2):153–8.
[82] Juhas E, English JC. Tattoo-associated complications. J Pediatr Adolesc Gynecol 2013;26(2):125–9.
[83] Shaaban HS, Bishop SL, Menon L, Slim J. *Mycobacterium chelonae* infection of the parotid gland. J Glob Infect Dis 2012;4(1):79–81.
[84] Broutin V, Bañuls AL, Aubry A, Keck N, Choisy M, Bernardet JF, et al. Genetic diversity and population structure of *Mycobacterium marinum*: new insights into host and environmental specificities. J Clin Microbiol 2012;50(11):3627–34.
[85] Feng Y, Xu H, Wang H, Zhang C, Zong W, Wu Q. Outbreak of cutaneous *Mycobacterium marinum* infection in Jiangsu Haian, China. Diagn Microbiol Infect Dis 2011;71(3):267–72.
[86] Nguyen, C. http://www.nejm.org/doi/full/10.1056/ENEJMicm000083 [accessed 01.04.14].
[87] Bratschi MW, Tabah EN, Bolz M, Stucki D, Borrell S, Gagneux S, et al. A case of cutaneous tuberculosis in a Buruli ulcer-endemic area. PLOS Negl Trop Dis 2012;6(8):1–3.
[88] Groves R. Unusual cutaneous mycobacterial diseases. Clin Dermatol 1995;13(3):257–63.
[89] Meyers WM, Walsh DS, Portaels F. *Mycobacterium ulcerans* infection (Buruli ulcer). In: Guerrant RL, Walker DH, Weller PF, editors. Tropical infectious diseases. 3rd ed. London: Elsevier Inc.; 2011. p. 248–52.
[90] Johnson, P.D.R., Stinear, T., Small, P.L.C., Pluschke, G., Merritt, R.W., Portaels, F., et al. http://www.plosmedicine.org/article/info%3Adoi%2F10.1371%2Fjournal.pmed.0020108 [accessed 01.04.14].
[91] Aslam A, Green RL, Motta L, Ghrew M, Griffiths CEM, Warren RB. Cutaneous *Mycobacterium haemophilum* infection in a patient receiving infliximab for psoriasis. Br J Dermatol 2012;168(2):446–7.
[92] Lindeboom, J.A., Bruijnesteijn Van Coppenraet, L.E.S, Van Soolingen, D., Prins, J.M. & Kuijper, E.J. http://cmr.asm.org/content/24/4/701.full [accessed 01.04.14].
[93] Welsh, J. http://www.businessinsider.com.au/mysterious-tattoo-infections-outbreak-running-rampant-2012-8 [accessed 01.04.14].
[94] Jardin, O., Hernández-Pérez, R., Corrales, H., Cardoso-Leao, S. & De Waard, J.H. http://zl.elsevier.es/es/revista/enfermedades-infecciosas-microbiologia-clinica-28/seguimiento-un-brote-infeccion-tejido-blando-causado-13184022-originales-2010 [accessed 01.04.14]..
[95] Lockwood DNJ. Leprosy. In: Burns T, Breathnach S, Cox N, Griffiths C, editors. Rook's textbook of dermatology. 8th ed., vol. 2. West Sussex, United Kingdom: Blackwell Publishing Ltd; 2010. 32.1–32.20.
[96] Walsh DS, Meyers WM. Leprosy. In: Guerrant RL, Walker DH, Weller PF, editors. Tropical infectious diseases. 3rd ed. London: Elsevier Inc.; 2011. p. 253–60.
[97] Brayer, T. http://healthwise-everythinghealth.blogspot.com/2012/04/lepromatous-leprosy.html [accessed 02.04.14].
[98] Esfandbod, M. http://www.nejm.org/doi/pdf/10.1056/NEJMicm1011992 [accessed 02.04.14].
[99] Jean M, Masse V, Carlier R, Perronne C, Crémieux AC. Isolated subcutaneous tuberculous abscesses of the lumbar wall. J Travel Med 2012;19(5):329–30.
[100] Fernández GP. Tuberculosis infections of the head and neck. Acta Otorrinolaringol Esp 2009;60(1):59–66.
[101] Arora VK, Jaiswal AK, Jain V. Changing trends of cutaneous tuberculosis in the era of DOTS strategy. Indian J Tuberc 2012;59(2):116–8.
[102] Martins F, Santos S, Ventura C, Elvas-Leitão R, Santos L, Vitorino S, et al. Design, synthesis and biological evaluation of novel isoniazid derivatives with potent antitubercular activity. Eur J Med Chem 2014;81:119–38.
[103] Lawn SD, Zumla AI. Tuberculosis. Lancet 2011;378:57–72.
[104] Abubakar I, Zignol M, Falzon D, Raviglione M, Ditiu L, Masham S, et al. Drug-resistant tuberculosis: time for visionary political leadership. Lancet Infect Dis 2013;13(6):529–39.
[105] Zignol M, Dara M, Sean AD, Falzon D, Dadu A, Kremer K, et al. Drug-resistant tuberculosis in the WHO European region: an analysis of surveillance data. Drug Resist Updat 2013;16:108–15.
[106] Gröschel MI, Prabowo SA, Cardona PJ, Stanford JL, Van Der Werf TS. Therapeutic vaccines for tuberculosis: a systemic review. Vaccine 2014;32(26):3162–8.
[107] Ramesh V. Cutaneous tuberculosis and esthiomene. Int J Gynecol Obstet 2011;114(3):293–4.
[108] Cameselle D, Hernández J, Francès A, Montenegro T, Cañas F, Borrego L. Sporotrichoid cutaneous infection by *Mycobacterium haemophilum* in an AIDS patient. Actas Dermo-sifiliogr 2007;98(3):188–93.
[109] Curcó, N. http://www.actasdermo.org/en/cutaneous-infection-in-tattoo-due/articulo/90167655/?pubmed=true [accessed 31.03.14].
[110] Sultana A, Bhuiyan MSI, Haque A, Bashar A, Islam MT, Rahman MM. Pattern of cutaneous tuberculosis among children and adolescents. Bangladesh Med Res Counc Bull 2012;38:94–7.
[111] Sattinger E, Meyer S, Costard-Jäckle A. Cutaneous tuberculosis mimicking erysipelas of the lower leg in a heart transplant recipient. Transpl Int 2011;24(6):e51–3.

Chapter 3

ARTICLE ON THE VALIDATION OF THE ANALYTICAL METHOD ACCEPTED FOR PUBLICATION IN DIEPHARMAZIE

This chapter is written in article format as requested by the DIEPharmazie and was accepted for publication.

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Development and validation of the simultaneous determination of artemisone, clofazimine and decoquinate with HPLC

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Abstract

The aim of this study is to develop and validate a novel HPLC method for the simultaneous analysis of artemisone, clofazimine and decoquinate. Detection was obtained at two wavelengths; 284 nm (clofazimine) and 210 nm (artemisone and decoquinate). Gradient elution was used with mobile phase A (**A**) consisting of 0.005 M sodium octanesulphonic-acid (pH 3.5) and mobile phase B (**B**) of HPLC grade acetonitrile. The flow rate was set to 1.0 ml/min with (**A**) at 35% and (**B**) at 65% for 2 min, followed by a gradient shift of 10/90% ((**A**)/(**B**)) over a duration of 4 min. After 10 min, the initial gradient conditions were readjusted to 35/65% ((**A**)/(**B**)). Distinctive peaks were identified for clofazimine, artemisone and decoquinate, respectively. The proposed HPLC assay method was validated and found to be reliable, reproducible and accurate for simultaneous analysis of the three compounds.

1. Introduction

Tuberculosis (TB) poses a significant public health threat, with 20 - 40% of the world's population being affected. Less than 14% of TB cases are extra-pulmonary, of which only 1.0 - 1.5% manifests as cutaneous tuberculosis (CTB). CTB is quite an exceptional presentation of TB; resulting in it being undefined and often misdiagnosed [Bravo and Gotuzzo, 2007; Rullán et al. 2012; Carman and Patel, 2014; Galagan et al. 2014].

A fixed-dose combination of artemisone, clofazimine and decoquinate formulated in a topical dosage form was chosen as a possible therapy to effectively treat CTB. The combination of these three APIs (active pharmaceutical ingredients) was based on the combination strategy of oxidant and redox APIs for the treatment of malaria and TB. The combination of the three APIs formed part of an investigative study; since the effectivity of decoquinate against TB has not yet been established, but its lipophilicity renders it an attractive compound for assessment. Although clofazimine was considered ineffective against pulmonary TB, recent advances in technology have renewed the use thereof for TB treatment, and therefore it was included in this combination as an API with redox capabilities. Artemisone is effective against *Plasmodium falciparum* (malaria), but was included in this study as an oxidant API [Cholo et al. 2011; Steyn et al. 2011; Haynes, 2013].

Clofazimine ($C_{27}H_{22}Cl_2N_4$) (Fig. 1A) [modified from Cholo et al. 2011] has an aqueous solubility of 10 mg/L, a log P of 7.60, a pKa of 8.51, a melting point of 210 - 212°C and a molecular weight of 473.40 g/mol [Holdiness, 1989; Brittain and Florey, 1992; Cholo et al. 2011; Bolla and Nangia, 2012; Srikanth et al. 2014]. Artemisone ($C_{19}H_{31}NO_6S$) (Fig. 1B) [modified from Biamonte et al. 2013], in addition, has an aqueous

solubility of 89 mg/L (pH 7.2, water), a log P of 2.49, a melting point of ≈199°C and a molecular weight of 401.52 g/mol [Nagelschmitz et al. 2008; Dunay et al. 2011; Steyn et al. 2011]. Very little information is available for decoquinate; since it has mainly been used as a veterinary API. Decoquinate ($C_{24}H_{36}NO_5$) (Fig. 1C) [modified from Biamonte et al. 2013] has a log P of 7.80, a pKa of 10.76, a melting point of ≈219°C and a molecular weight of 417.54 g/mol [Nam et al. 2011; Iglesias et al. 2014]. Currently, no HPLC method for the simultaneous determination of these three compounds is available in literature. Therefore, this method was developed and validated to determine the concentration assays of the three compounds simultaneously for different routes of administration such as solid oral dosage forms or transdermal formulations. This method was developed and validated based on the International Conference on Harmonisation (ICH) and current Good Manufacturing Practice (cGMP) guidelines and parameters [ICH, 2005; FDA, 2011].

2. Investigations, results and discussion

The proposed method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), system suitability and robustness. Thereafter the solubility of all three APIs was determined in different solvents (Table 1). The solubility of decoquinate, when compared to the other two APIs, was the lowest in all investigated solvents, consequently the solubility thereof was used as the deciding parameter during the method development steps. The solubility of decoquinate in tetrahydrofuran (THF) was the highest, however, since it is considered a toxic solvent; it will not be the solvent of choice during pre-formulation, dosage form development or API release testing from either solid or semi-solid formulations. Hence, ethanol was chosen as the main solvent.

The linearity was determined by constructing a regression plot of API concentration versus peak area response, allowing the calculation of a regression equation for each API. The resulting equations are listed in Table 2. The correlation coefficient (r^2) was also determined, where the strongest linear relationship is indicated by a correlation coefficient of 1 [Krause, 2003; UNODC, 2009]. The accuracy of the three compounds can be seen in Table 2. The mean recovery percentages were all between 98 - 102% (%RSD < 15%), thus complying with validation requirements for accuracy parameters.

Precision was conducted during a three-day period at three concentration levels (Table 2). All the parameters mentioned adhered to the specifications and thus the method was found to be accurate and precise. The stability of artemisone, clofazimine and decoquinate was evaluated, and no significant instability was observed for at least 24 h (Table 2). None of the API concentrations deviated with more than 15% and were consequently found to be stable for at least 24 h after preparation. System suitability was determined with the injection precision for retention times and peak areas as depicted in Table 2. System suitability was determined from six replicate injections. The obtained peaks were analysed in terms of peak area and retention times. All %RSD values were less than 2% (Table 2) and as a result the method was found to be suitable for the HPLC system.

The LOQ is the lowest concentration of API that can be quantitatively ascertained, above which analysis is possible with the specified degree of accuracy and precision. LOQ is used particularly for determining impurities and/or degradation products [ICH, 2005; VICH, 2015]. To ensure whether LOQ is accurate, the API should be injected at the calculated LOQ concentration (n = 6) and the %RSD should be calculated for each concentration. %RSD variation should not exceed 20 [Krause, 2003; ICH, 2005; Westgard, 2008;

Huber, 2010; VICH, 2015; González et al. 2014]. The LOD, on the other hand, can be determined according to several methods, though the method used in this study is based on the standard deviation of the regression line or the y-intercept and the slope of the calibration curve. The calculated LOD was 1.98 µg/ml for artemisone, 3.11 µg/ml for clofazimine and 2.29 µg/ml for decoquinate. Confirmation of the LOD required injecting the samples at the calculated concentration six times. The experimental LOD obtained was thus 4.42 µg/ml (%RSD 19.68), 0.042 µg/ml (%RSD 17.73) and 0.703 µg/ml (%RSD 17.79) for artemisone, clofazimine and decoquinate, respectively. LOQ was subsequently determined as 13.39 µg/ml for artemisone, 0.13 µg/ml for clofazimine and 2.13 µg/ml for decoquinate. The experimentally determined LOD and LOQ values corresponded well; whereas the calculated LOD did not correlate with the experimental values. In our experience the calculated LOD rarely correlates with experimentally determined values and is of limited use.

Since there is currently no dosage form containing this combination of compounds available on the market, it was decided to make two solutions containing possible excipients that is normally included in a solid oral dosage form and a transdermal/topical delivery system, in order to test the sensitivity and selectivity of the method. The solution for solid oral dosage form contained the three APIs, talc, microcrystalline cellulose, polyvinylpyrrolidone (PVP 30), lactose, magnesium stearate, vinylpyrrolidone-vinyl acetate copolymer (Kollidon® VA64) and Pluronic® F-127 dissolved in absolute ethanol in unspecified concentrations. The solution prepared for transdermal/topical delivery contained the three APIs, polysorbate 20 (Tween® 20), polysorbate 80 (Tween® 80), sorbitan monostearate (Span® 60), phosphatidylcholine, cholesterol, safflower oil and olive oil dissolved in absolute ethanol in unspecified concentrations. As observed from Fig. 2 - 4 the three compounds delivered peaks at the determined retention times with little to no interference from the different excipients, which can possibly be used during formulation of solid oral dosage forms and transdermal/topical delivery systems. In addition to the novelty for the simultaneous quantification of artemisone, clofazimine and decoquinate the method is reliable and sensitive which complies with the ICH guidelines for method validation. Validation parameters such as linearity, limit of detection and quantitation, accuracy, precision, sample stability and system suitability were established. The reproducible method was also used to determine sensitivity of the method when applying it to an excipient mixture for a possible solid oral dosage form and for a transdermal/topical delivery system. The method was found to be adequate in quantifying the three APIs with virtually no interference.

3. Experimental

An Agilent® 1100 Series HPLC which was used during this study consisted of an Agilent® 1100 pump, diode array detector, and an autosampler injector module, and ChemStation Rev. A.10.02 software was utilised for data acquisition and analysis (Agilent Technologies, Palo Alto, CA). A Restek Ultra C_{18} fully endcapped reversed phase column (250 x 4.6 mm, 5 µm) was used with 100 Å pores, a 20% carbon load (Restek corporation, Bellefonte, US), a pH range of 2.5 – 8.0 and a temperature limit of 80°C. Gradient elution was used with mobile phase A (**A**) consisting of 0.005 M sodium octanesulphonic-acid (pH 3.5) and mobile phase B (**B**) comprising of HPLC grade acetonitrile. The flow rate was set to 1.0 ml/min with (**A**) at 35% and (**B**) at 65% for 2 min, followed by a gradient shift of 10/90% ((**A**)/(**B**)) over a duration of 4 min. After 10 min the initial gradient conditions were readjusted to 35/65% ((**A**)/(**B**)). An injection volume of 20 µl was used and the UV detection was set at 210 and 284 nm. Retention times were approximately 6.4 min, 7.5

min and 10.2 min for clofazimine, artemisone and decoquinate, respectively and the run time was set to 15 min. Ethanol absolute (99.7%) was used as the solvent throughout the method validation.

Acknowledgements

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Disclaimer

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

References

Biamonte MA, Wanner J, Le Roch KG (2013) Recent advances in malaria drug discovery. Bioorg Med Chem Lett 23: 2829 - 2843.

Bolla G, Nangia A (2012) Clofazimine Mesylate: A high solubility stable salt. Cryst Growth Des 12: 6250 - 6259.

Bravo FG, Gotuzzo E (2007) Cutaneous tuberculosis. Clin Dermatol 25: 173 - 180.

Brittain HG, Florey K (1992) Profiles of drug substances, excipients and related methodology. In: Brittain H (ed.) 1st ed., London, 696p.

Carman AS, Patel AG (2014) Science with societal implications: detecting Mycobacterium tuberculosis in Africa. Clin Microbiol Newsl 36: 73 - 77.

FDA (2011) Current good manufacturing process Guidance for Industry. Process validation: General principles and practices .CGMP [online]. Available from: https://www.fda.gov/ohrms/dockets/98fr/fda-2008-d-0559-gdl.pdf [Accessed 20 July 2016]

Cholo MC, Steel HC, Fourie PB, Germishuizen WA, Anderson R (2011) Clofazimine: current status and future prospects. J Antimicrob Chemother 67: 290 - 298.

Dunay IR, Chi Chan W, Haynes RK, Sibley LD (2009) Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. Int J Antimicrob Agents 53: 4450 - 4456.

Galagan JE (2014) Genomic insights into tuberculosis. Nat Rev Genet 15: 307e20.

González O, Blanco ME, Iriarte G, Bartolomé L, Maguregui MI, Alonso RM (2014) Bioanalytical chromatographic method validation according to current regulations, with special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. J Chromatogr A 1353: 10 - 27.

Haynes, R.K. (2013) MRC South African University Flagship Projects: Project Proposal. Development to the clinical phase of oxidant and redox drug combinations for treatment of malaria. TB and related diseases [online]. Available from: http://www-chem.ust.hk/Seminars/2016/201610/Haynes%20Abstract%20Chem%20Dep%20HKUST%20041016.pdf [Accessed 20 April 2017]

Holdiness MR (1989) Clinical pharmacokinetics of clofazimine: a review. Clin Pharmacokinet 16: 74 - 85.

Huber l (2010) Validation of Analytical Methods. A Primer, Agilent Technologies, Germany, (Publ. No. 5990-5140EN).

ICH (2005) International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use Validation of analytical procedures: Text and methodology Q2(R1). [online]. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [Accessed 20 April 2017]

Iglesias A, Nebot C, Vázquez BI, Miranda JM, Abuín CMF, Cepeda A (2014) Detection of veterinary drug residues in surface waters collected nearby farming areas in Galicia, North of Spain. Environ Sci Pollut Res 21: 2367 - 2377.

Krause SO (2003) Good analytical method validation practice: Deriving acceptance criteria for the AMV protocol – Part II. J Val Tech 9: 162 - 178.

Nagelschmitz J, Voith B, Wensing G, Roemer A, Fugmann B, Haynes RK, Kotecka BM, Rieckmann KH, Edstein MD (2008) First assessment in humans of the safety, tolerability, pharmacokinetics, and *ex vivo* pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone. Antimicrob Agents Chemother 52: 3085 - 3091.

Nam T, McNamara CW, Bopp S, Dharia NV, Meister S, Bonamy GMC, Plouffe DM, Kato N, McCormack S, Bursulaya B, Ke H, Vaidya AB, Schultz PG, Winzeler EA (2011) A chemical genomic analysis of decoquinate, a *Plasmodium falciparum* cytochrome b inhibitor. ACS Chem Biol 6: 1214 - 1222.

Rullán J, Seijo-Montes RE, Vaillant A, Sánchez NP. Cutaneous manifestations of pulmonary disease. In: Sánchez NP (ed.) Atlas of dermatology in internal medicine. 14th ed., San Juan, Puerto Rico, p. 17 - 30.

Srikanth CH, Joshi P, Bikkasani AK, Porwal K, Gayen JR (2014) Bone distribution study of antileprotic drug clofazimine in rat bone marrow cells by a sensitive reverse phase liquid chromatography method. J Chromatogr B 960: 82 - 86.

Steyn JD, Wiesner L, Du Plessis LH, Grobler AF, Smith PJ, Chan WC, Haynes RK, Kotzé AF (2011) Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. Int J Pharm 414: 260 - 266.

VICH: International cooperation on harmonisation of technical requirements for registration of veterinary medicinal products (2015) Guidance for industry. Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Validation of analytical methods used in residue depletion studies (VICH GL49(R)). 23p.

Westgard JO (2008) Basic Method Validation: training in analytical quality management for healthcare laboratories. In: Westgard WI (ed.) 3rd ed. Madison QC, 320 pp.

Table 1: Solubility **(µg/ml)** (37°C) determined for artemisone, clofazimine and decoquinate in nine different solvents

	Artemisone	Clofazimine	Decoquinate
Water	100.50	0.05	0.00
PBS (pH 7.4)	86.01	0.00	1.21
Trisaminomethane (pH 7.4)	99.07	0.00	0.00
Ethanol	44 600.00	1 824.70	181.70
Methanol	46 541.60	827.74	163.13
Isopropanol	26 351.00	1 018.53	160.08
Acetone	105 923.00	2 181.64	18.95
Acetonitrile	201 960.00	1 207.80	12.98
THF	194 190.00	143 410.00	241.13

Table 2: Obtained validation parameters for the three compounds

Linearity			
	Artemisone (n = 9)	**Clofazimine (n = 7)**	**Decoquinate (n = 9)**
Concentration range (µg/ml)	5.00 - 400.10	0.93 - 55.86	1.50 - 120.10
Regression equation	y = 1.079x - 0.375	y = 72.623x + 42.390	y = 23.261x + 51.866
Correlation coefficient (r^2)	0.9999	0.9937	0.9989
Accuracy			
Mean recovery (%) (%RSD)	99.9 (± 1.4)	100.0 (± 0.3)	99.3 (± 7.5)
Stability			
Max sample deviation from hour zero (%) (%RSD)	2.5 (± 0.8)	0.2 (± 0.5)	5.0 (± 1.1)
System suitability			
Retention time (min) (%RSD)	7.3 (± 0.1)	6.3 (± 0.3)	9.9 (± 0.1)
Peak area (%RSD)	23.19 (± 0.16)	430.76 (± 0.90)	187.10 (± 1.71)

Table 3: Precision data for artemisone, clofazimine and decoquinate

		Artemisone	**Clofazimine**	**Decoquinate**
Concentration (µl/ml)		393.0	74.0	128.0
Repeatability (intra-day)	**%RSD**	1.03	0.21	4.55
	Mean recovery (%)	99.86	99.99	99.57
Intermediate precision (inter-day)	**%RSD (day 2)**	0.78	0.17	3.68
	Mean recovery percentage (day 2)	99.96	100.01	99.67
	%RSD (day 3)	1.20	0.14	4.33
	Mean recovery percentage (day 3)	99.89	99.99	99.63
	p-value of ANOVA (for the three days)	0.938	0.958	0.862

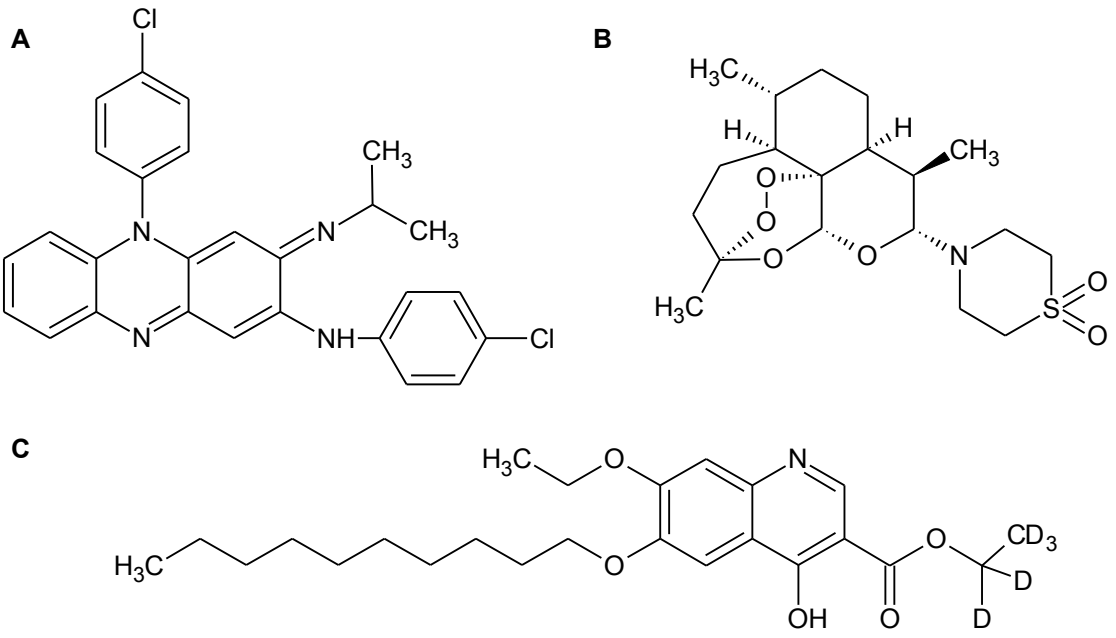

Fig. 1: Molecular structures of A) clofazimine, B) artemisone and C) decoquinate.

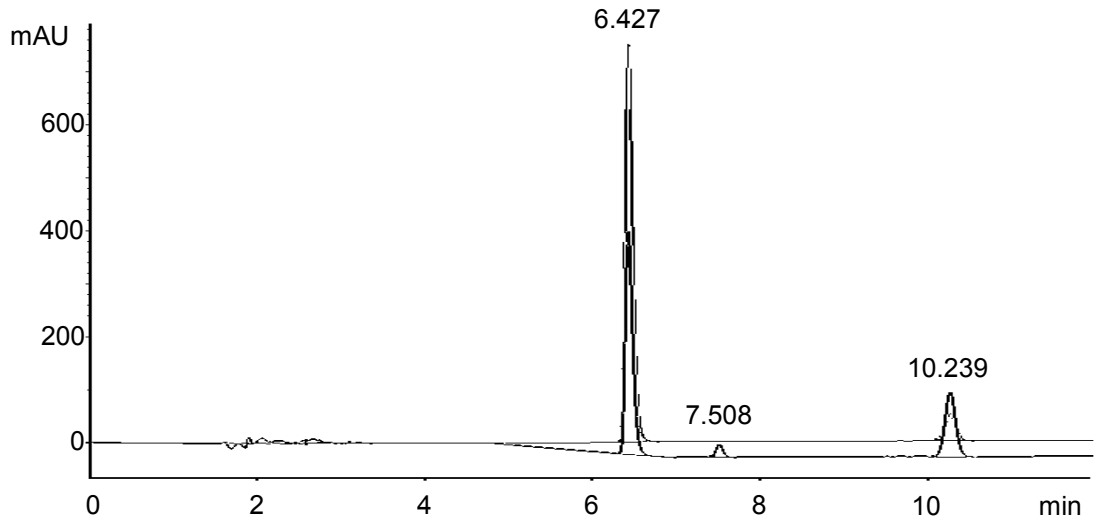

Fig. 2: Chromatographs of a standard solution containing clofazimine, artemisone and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

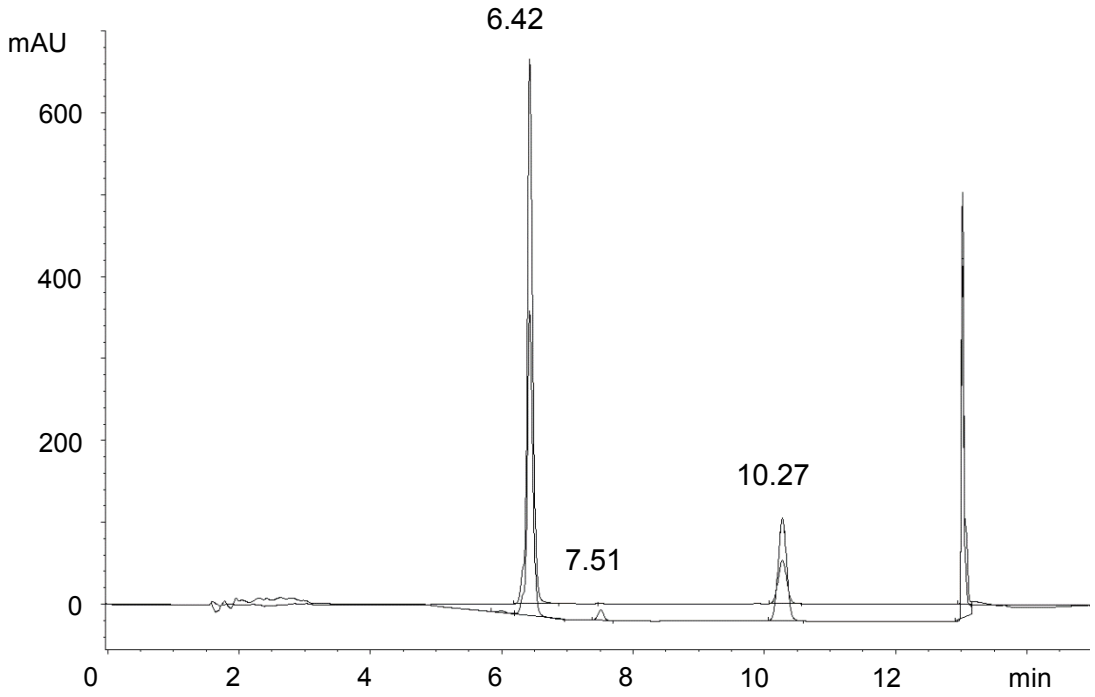

Fig. 3: Chromatographs obtained with a solution containing typical excipients used in formulation of solid oral dosage forms, observing clofazimine, artemisone and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

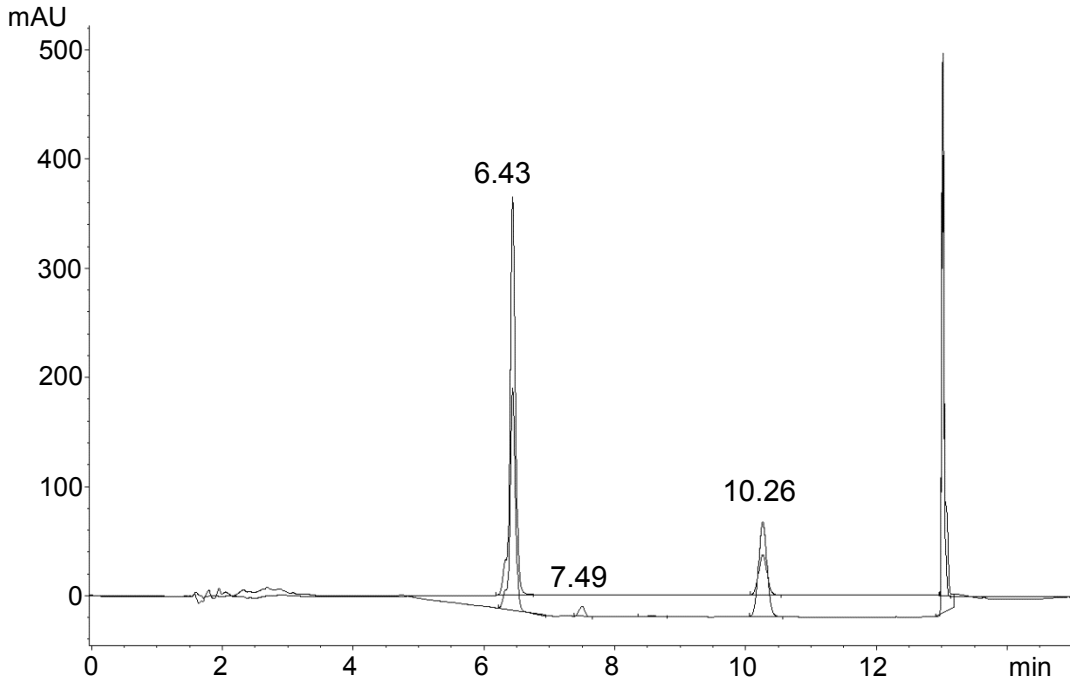

Fig. 4: Chromatographs of excipient solution for transdermal/topical delivery systems showing clofazimine, artemisone, and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

Chapter 4

MANUSCRIPT TO BE SUBMITTED TO THE JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS ON THE TOPICAL DELIVERY OF ARTEMISONE, CLOFAZIMINE AND DECOQUINATE ENCAPSULATED IN VESICLES AND THEIR IN VITRO EFFICACY AGAINST A TUBERCULOSIS CELL LINE

This chapter is written in article format as stipulated by the *Journal of pharmaceutical and biomedical analysis* according to the Guide to Authors in Annexure F.

Topical delivery of artemisone, clofazimine and decoquinate encapsulated in vesicles and their *in vitro* efficacy against *Mycobacterium tuberculosis* H37Rv strain

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Abstract

Vesicles are widely investigated as carrier systems for active pharmaceutical ingredients (APIs). For topical delivery they are especially effective since they create a “depot-effect” thereby concentrating the APIs in the skin. Artemisone, clofazimine and decoquinate were selected as a combination therapy for the topical treatment of cutaneous tuberculosis. Delivering APIs into the skin presents various challenges; however, utilising niosomes, liposomes and transferosomes as carrier systems may circumvent these challenges.

Vesicles containing 1% of each of the three selected APIs were prepared using the thin-film hydration method. Isothermal calorimetry, differential scanning calorimetry and hot-stage microscopy indicated no to minimal incompatibility between the APIs and the vesicle components. Encapsulation efficiency was higher than 85% for all vesicle dispersions. Vesicle stability decreased and size increased with an increase in API concentration. Niosomes were found to be most unstable.

Skin diffusion studies were conducted for 12 h on black human female skin utilising vertical Franz diffusion cells. Transferosomes and niosomes delivered the highest average concentrations of clofazimine and decoquinate into the skin, whereas artemisone was not detected. No APIs were present in the receptor phase.

Efficacy for antituberculosis was tested against *Mycobacterium tuberculosis* H37Rv laboratory strain. All the dispersions depicted some activity, surprisingly even the blank vesicles as well. The highest percentage inhibition (52%) against TB was obtained with niosomes containing 1% clofazimine.

Keywords

Vesicles, Artemisone, Clofazimine, Decoquinate, Tuberculosis, Topical delivery

Highlights

- Vesicle size increased and stability decreased with an increase in API concentration.
- Clofazimine had the largest impact on zeta-potential and vesicle size.
- Decoquinate and clofazimine were delivered topically in vesicle dispersions.
- Higher API concentrations were present in the stratum corneum compared to the concentrations in the epidermis-dermis.
- All of the dispersions displayed efficacy against tuberculosis, even vesicles containing no API.

1. Introduction

Delivery of active pharmaceutical ingredients (APIs) into the skin is a challenging endeavour that is mainly attributed to the barrier function of the stratum corneum [1, 2]. Vesicles, however, have been investigated as a delivery system to increase topical delivery of numerous APIs [3]. Vesicles were used in this study due to their unique ability to encapsulate both hydrophilic and lipophilic APIs, which is especially helpful when formulating a combination of artemisone (ART), clofazimine (CLF) and decoquinate (DQ) [4, 5]. Vesicles have the tendency to create a "depot-effect", concentrating the APIs in the skin, which is where cutaneous tuberculosis (CTB) normally resides [6, 7]. Focus was placed on liposomes, niosomes and transferosomes as topical carrier systems for the combination of ART, DQ and CLF. The combination of these three APIs was selected from a list of approved APIs supplied by the MALTBRedox MRC South African University Flagship Projects, for their redox-oxidant drug working mechanism to determine activity against tuberculosis when applied topically. The rationale behind this mechanism is explained by looking at each API closely and determining its purpose.

CLF is a lipophilic API which enables it to accumulate in the skin, whereas its anti-inflammatory action helps control harmful inflammation, supporting its efficacy in the treatment of leprosy. CLF is considered ineffective against pulmonary tuberculosis, though recent advances in technology and the rise in API resistance have renewed interest in the API as an anti-tuberculous agent. CLF has mainly two mechanisms of antimicrobial action, but for the benefit of the Flagship Project, the focus will be on the redox cycling. The outer membrane of the organism is the primary site of action. When CLF is added to the membrane of the organism in the presence of potassium cyanide [(KCN), terminal cytochrome (CYP) respiratory chain inhibitor] and the oxidisable cofactor reduced nicotinamide adenine dinucleotide (NADH), reduction and oxidation of CLF occur with the production of superoxides and H_2O_2 (hydrogen peroxide), also known as antimicrobial reactive oxygen species (ROS). The ROS interfere with the ATP (adenosine triphosphate) production which leads to bacterial death [8].

ART is a new derivative of artemisinin, which is active against all red blood cell stages of *Plasmodium falciparum*. There is limited resistance to this group of APIs and due to their short half-life it is recommended that they be administered in combination with other APIs [9]. ART is a 10-alkylaminoartemisinin developed as a highly effective antimalarial with very low or no neuro- and cytotoxicity due to its low log P value [10, 11, 12]. ART is metabolised primarily in the liver by CYP3A4 and bio-transformed into three main metabolites. These three metabolites (M1, M2, M3) possess intrinsic antimalarial activity [11]. The artemisinins are thought to generate toxic oxygen radicals through cleavage of the peroxide bond by Fe(II) (iron) found in heme proteins. This oxidant activity is of importance to the Flagship Project. The artemisinins will enhance the activity of CFZ against tuberculosis through acceleration of the cycling of the redox API [13].

DQ is a 4-hydroxy quinolone and an approved veterinary drug which has thus far only been tested in animals [13]. The Flagship Project is interested in the possible anti-tuberculous activity of DQ due to its high lipophilicity. The function of DQ in this combination of APIs will be to complement the activities of the above discussed APIs. DQ has anti-coccidial activity through disruption of the electron transport in the mitochondrial cytochrome system of coccidia [14, 15].

2. Materials and Methods

2.1. Materials

Clofazimine and artemisone were kindly donated by Cipla Pty Ltd (Mumbai, India), and decoquinate was purchased from Shanghai Hohance Chemicals, China, and used as received. The vesicles were manufactured using L-α-Phosphatidylcholine from egg yolk ~60% (Sigma-Aldrich, South Africa), cell culture suitable cholesterol (Sigma-Aldrich, South Africa), cell culture tested Tween® 20 (Sigma-Aldrich, South Africa) and absolute ethanol from Associated Chemical Enterprises (ACE) in South Africa. The water utilised was purified by a Milli-Q® water purification system from Millipore® (United States of America). Methanol (ACE, South Africa) and acetone (ACE, South Africa) were used as lipid solvents; and 2 mm diameter glass beads (Merck, Germany) were used during the thin film manufacturing method. All chemicals employed were of analytical grade.

2.2. Methods

2.2.1. Preparation of vesicles

A modified version of the thin-film hydration method was employed to prepare the vesicles [16, 17]. Vesicles were prepared as a 5% lipophilic phase in a phosphate buffer solution (pH 5.5), i.e. the aqueous phase. The lipophilic APIs were subsequently added to the lipophilic phase to promote accumulation in the vesicles. The lipophilic phase of the liposomes consisted of phosphatidylcholine (PC) and cholesterol in a 3:2 ratio; transferosomes comprised PC and absolute ethanol (4:1 ratio) and niosomes contained Tween® 20 and cholesterol (2:1 ratio). The lipophilic phase was dissolved in an acetone:methanol (2:1 ratio) mixture and the solvent evaporated over a heat of no more than 45°C. A thin lipid film was created to which the aqueous phase was added. Glass beads were transferred into the beaker, which was placed in a sonicator bath for 15 min. Thereafter, the glass beads were removed and the vesicle dispersion probe sonicated for 6 min at 2 min intervals. The vesicle dispersions were retained at 4°C for at least 2 h to ensure adequate vesicle formation.

2.2.2. Pre-formulation and characterisation

2.2.2.1. Isothermal calorimetry

Compatibility between the three APIs and the vesicle components was established with a 2277 Thermal Activity Monitor (TAMIII; TA Instruments, United States of America), equipped with an oil bath with a stability of ± 100 µK over 24 h. The calorimeter's temperature was maintained at 40°C. During the compatibility studies the heat flow was measured for the single components, as well as for the mixtures, since it is a powerful tool for detecting incompatibilities and instabilities between APIs and/or excipients.

2.2.2.2. Encapsulation efficiency

To determine the encapsulation efficiency, the centrifugation separation method was used. The selected dispersions were centrifuged at 2,000 *g* for 20 min for the unentrapped API to form a pellet in the tube [18]. An Eppendorf® 5804 R centrifuge equipped with an A-4-44 rotor was used. To determine entrapment efficiency, the unentrapped API was subtracted from the initial added API and a percentage calculated.

2.2.2.3. Zeta-potential, size distribution and vesicle size

Zeta-potential, as well as vesicle size and distribution of the vesicles, was determined by means of dynamic light scattering using a Zetasizer Nano® ZS (Malvern® Instruments Ltd., Worcestershire, United Kingdom) at 25°C. Zeta-potential is the electrostatic potential at interfaces and determines the electrostatic repulsion between particles in a system. The higher the zeta-potential, the stronger the repulsion and the more stable the colloidal system becomes [19].

The size and size distribution for these dispersions were also determined utilising the Zetasizer Nano® ZS. The different vesicles were also imaged and their sizes measured using the FEI Tecnai G2 20S-Twin TEM at 120 kV. A transmission electron microscope equipped with a Gatan® bottom mount camera using DigitalMicrograph® software for imaging and measuring vesicle size was employed.

2.2.2.4. pH and viscosity

The pH was determined for each of the selected dispersions using a Mettler® Toledo pH meter (Mettler® Toledo International Inc., United States of America) with a Mettler® Toledo Inlab® 410 NTC electrode 9823.

Viscosity was measured using a Brookfield® Viscometer model DV2T™ (Stoughton, United States of America) connected to a circulating water bath with a Brookfield® temperature controller to maintain the temperature in the water jacket at 25±1.0°C. A SC4-18 spindle was employed at 100 rpm, where the torque was approximately 20%.

2.2.3. Topical delivery

2.2.3.1. Skin preparation

Black skin, donated by anonymous female patients who underwent abdominoplastic surgery, was obtained for transdermal diffusion studies [NWU-00114-11-A5]. The donated skin was frozen at -20°C for no longer than 6 months. Prior to conducting the studies, the skin was left to thaw and visually inspected for defects such as stretch marks and/or holes/large hair follicles. The skin was cut into pieces of approximately 2 cm x 4 cm and 400 µm thick with a Zimmer® electric dermatome model 8821. The skin pieces were placed on Whatman® filter paper, cut in circles and covered in aluminium foil prior to storage in a freezer at -20°C until the studies were conducted (within 24 h).

2.2.3.2. Skin diffusion studies

In vitro skin diffusion studies were conducted utilising ten vertical Franz diffusion cells over 12 h [20]. Skin circles were mounted between a receptor and a donor compartment. The receptor phase consisted of phosphate buffer solution (pH 7.4) and a magnetic stirrer, whereas the donor compartment contained the vesicle dispersion. The Franz cells were placed in a water bath maintained at 37°C.

2.2.3.3. Tape stripping

After the 12 h diffusion studies, the skin circles were removed from the Franz cells and fixed onto a board with Whatman® filter paper. All excess dispersion was dabbed from the skin surface. For the tape stripping, fifteen strips of clear 3M Scotch® tape were used and an extra two strips for cleaning. The strips were used to remove the stratum corneum-epidermis, which was then put into a polytop containing 5 ml absolute ethanol and placed in the refrigerator for approximately 8 h. The remaining dermis of the diffusion area (circular imprint of the donor phase is indicative of this area) was cut into small pieces, to increase the surface area, and also transferred into a polytop with 5 ml absolute ethanol and stored between 2-8°C for approximately 8 h. Thereafter, the ethanol was filtered, using a 45 µm filter, and analysed by means of HPLC.

2.2.4. Efficacy against tuberculosis

Mycobacterium tuberculosis (*M. tb*) H37Rv was inoculated in a 1:10 dilution from stock cultures in a 25 cm^3 tissue flask for 4 days until the optical density at 600 nm (OD_{600}) reached 0.6. These cultures were sub-cultured at 1:5 dilutions into a 75 cm^3 tissue flask until OD_{600} reached 0.3 for the experiment. Aliquots of 10 ml from this culture were transferred into 25 cm^3 tissue flasks. The concentrations that showed 50% or less *M. tb* growth inhibition of CLF, ART and DQ ranged from 12.5, 10.5 and 11.9 µM, respectively, were used. Therefore the dispersions containing a single API were tested at 12.5 µM, correspondingly. During the experiment, CLF was the most active API of the selected APIs and thus its concentration [21] was utilised as a benchmark for the other APIs. Each culture flask was treated with the desired amount of API for 24 hrs as well as one control flask with no API.

After 24 h post-inoculation and incubation at 37°C, 200 µl samples from each culture flask were centrifuged, resuspended, washed twice in 7H9 broth and plated on 7H11 agar. After 12–15 days of incubation at 37°C the CFUs were enumerated and the percentage inhibition was calculated relative to the untreated control.

3. Results and discussion

3.1. Pre-formulation and characterisation

3.1.1. Isothermal calorimetry

It was established that ART, CLF and DQ were compatible (Figure 1) in the solid-state form. ART, CLF and the combination of all three APIs in combination with PC showed incompatibility. All three APIs in combination with Tween® 20 indicated incompatibility (Figure 2). Incompatibilities were seen when a difference between the measured heat flow and the theoretically calculated heat flow was observed. All other combinations tested were compatible.

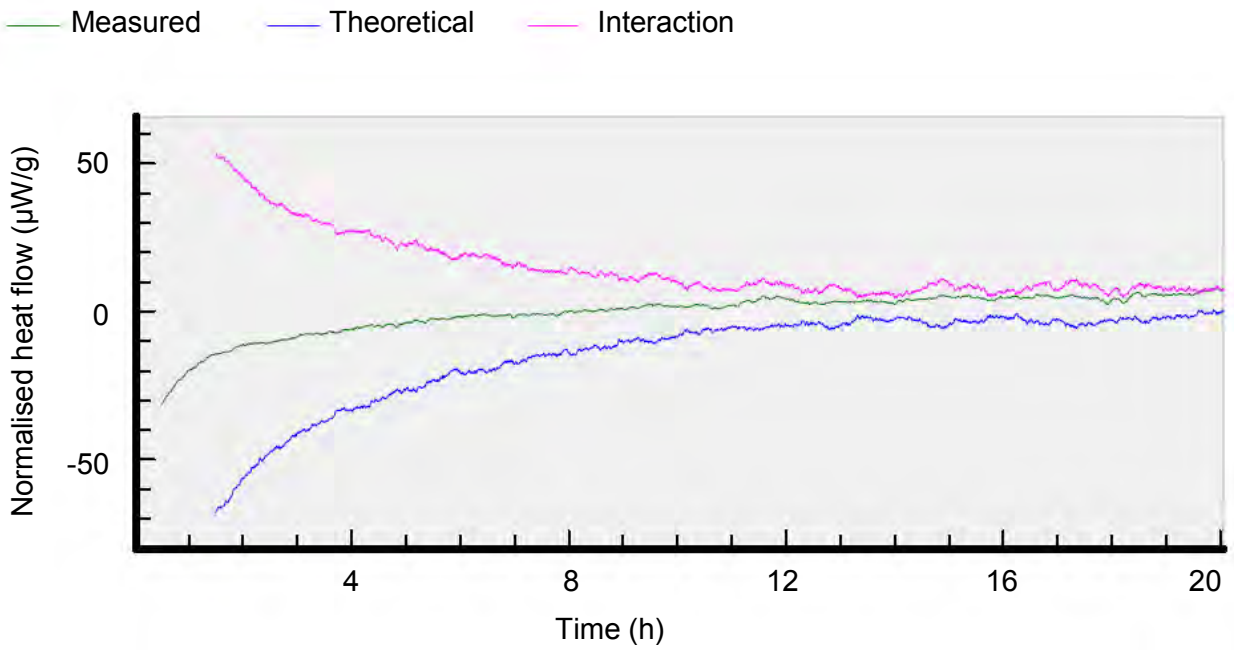

Figure 1: Heat flow versus time graph obtained for a combination of ART, CLF and DQ

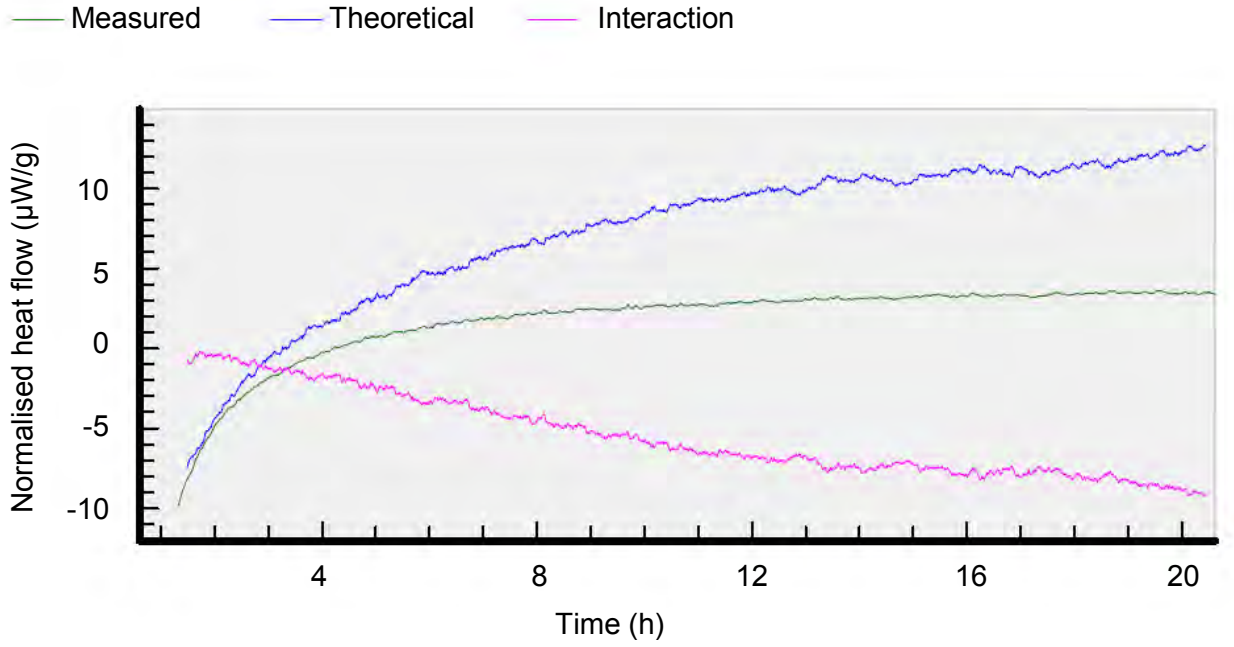

Figure 2: Heat flow data obtained for ART, CLF, DQ and Tween®20

Each of the liposome dispersions proved to be compatible, despite incompatibility of some of the individual ingredients. This may indicate that when phosphatidylcholine, the three APIs and cholesterol are combined, their individual incompatibilities are overcome. The transferosome dispersions displayed compatibility; however, the niosomes displayed the highest compatibility of all the tested dispersions with the lowest observed interaction errors.

3.1.2. Encapsulation efficiency

The vesicles containing 1% API depicted an encapsulation efficiency higher than 85% as can be seen is Table 1.

Table 1: Encapsulation efficiency (%) of vesicle dispersions containing 1% API(s)

	API	**Liposomes**	**Niosomes**	**Transferosomes**
Single API dispersions	ART	96.54	94.34	85.39
	CLF	94.04	97.94	87.33
	DQ	96.39	93.12	97.24
Combination	ART	96.87	93.77	87.24
	CLF	91.23	95.54	87.89
	DQ	94.32	92.71	98.50

DQ entrapped within the transferosome dispersion had the highest encapsulation efficiency (98.50%), whereas artemisone in the transferosome dispersion presented the lowest (85.39%) entrapment efficacy. Overall, liposomes (91.23–96.87%) and niosomes (93.77–97.94%) delivered relatively consistent encapsulation efficiencies.

3.1.3. Zeta-potential, size distribution and vesicle size

Blank dispersions containing no APIs were compared to dispersions containing 1% API(s). This was to determine the influence of increasing API concentration on the size and stability of the vesicles. The polydispersity index (PDI) is an indication of size distribution, where a dispersion is called uniform if the particles are similar in size, mass or shape. A PDI below 0.6 is usually indicative of a uniform dispersion [22]. Data collected for all the 1% API dispersion are presented in Table 2.

Table 2: Zeta-potential, size and size distribution (PDI) of the different dispersions

Vesicle	Dispersion	Zeta-potential (mV)	Size (nm)	PDI
Liposomes	Blank	-53.3 ± 5.9	154.0	0.306
	ART	-55.1 ± 4.5	146.6	0.239
	CLF	-31.5 ± 4.8	481.7	**0.618**
	DQ	-57.5 ± 5.3	252.7	**0.452**
	Combination	**-21.6 ± 4.6**	824.1	**0.673**
Niosomes	Blank	-43.3 ± 5.5	167.5	0.525
	ART	**-17.0 ± 3.8**	437.4	**0.481**
	CLF	**-8.5 ± 3.5**	2461.0	**0.939**
	DQ	**-11.5 ± 4.3**	670.1	**0.628**
	Combination	**-6.6 ± 4.8**	984.9	**0.925**
Transferosomes	Blank	-35.7 ± 5.3	106.3	0.257
	ART	-43.9 ± 8.9	123.7	0.394
	CLF	**-5.5 ± 3.3**	1222.0	**0.892**
	DQ	-44.7 ± 5.9	367.5	**0.482**
	Combination	**-15.5 ± 3.8**	524.7	**0.696**

The data shown in bold, indicate unstable or non-uniform dispersions. It is clear that CLF had the largest impact on the stability of the vesicles, with zeta-potentials below -10 mV for niosomes and transferosomes. The liposome dispersions were the most stable with zeta-potentials above -30 mV, though the combination dispersion was less stable at -21.6 mV. CLF furthermore significantly increased the vesicle size. Niosomes were found to be the most unstable dispersions, and liposomes the most robust. Blank vesicles were also viewed with TEM and can be seen in Figure 3. For TEM, a batch of vesicles was prepared with pure water as the aqueous phase, since the phosphate buffer solution crystallises, which may damage the instrument.

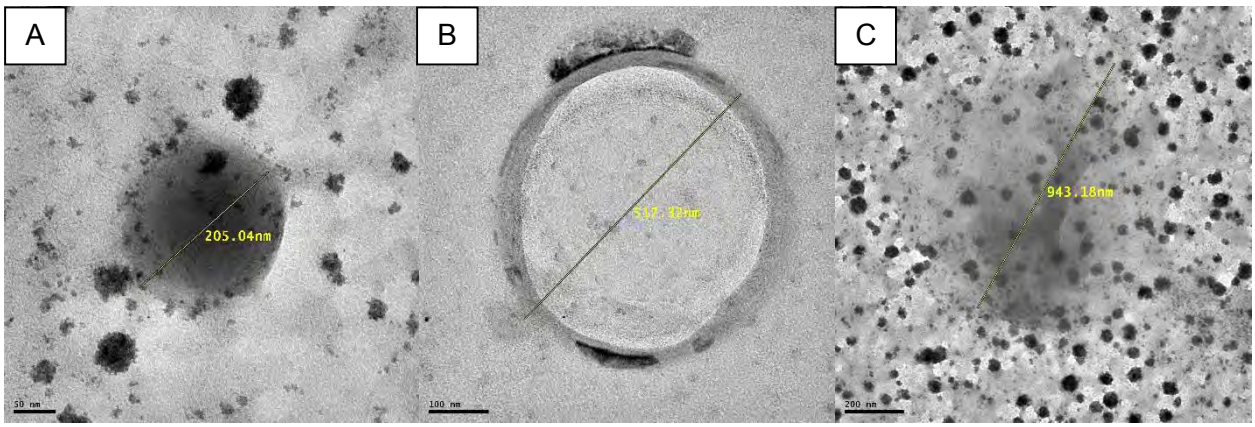

Figure 3: TEM imaging illustrating: A. Liposomes, B. Niosomes and C. Transferosomes prepared with pure water

3.1.4. pH and viscosity

The buffer used to formulate the vesicles was prepared at 1.5 standard strength, since a pilot study indicated that artemisone in liposomes lowers the pH to as low as 3.17 at 25°C. The dispersions were produced to be applied topically, thus the pH needs to be buffered between 4–9 in order to avoid skin irritation [23]. Viscosity readings were taken at 10 s intervals. Generally, it is assumed that more viscous formulations will inhibit or eliminate the skin partitioning and absorption of molecules within a formulation, partly due to poor skin distribution. This may not always be the case, since it was also found that thin layers of a more viscous formulation may enhance skin permeation due to the formation of an occlusive layer [24]. The viscosities of the dispersions were measured and the values obtained were relatively similar to that of water, which has a viscosity of 1 mPa.s at 20°C or 0.89 mPa.s at 25°C [25]. Data collected for the pH and viscosity of the dispersions are shown in Table 3.

Table 3: The pH and viscosity of 1% API dispersions at 25±1.0°C

Vesicle	Dispersion	pH	Viscosity (mPa.s)
Liposomes	Blank	5.50	1.88
	ART	5.51	1.89
	CLF	5.49	2.44
	DQ	5.51	1.87
	Combination	5.44	2.89
Niosomes	Blank	5.19	1.71
	ART	5.11	1.71
	CLF	5.24	1.66
	DQ	5.06	1.69
	Combination	5.75	1.99
Transferosomes	Blank	5.37	1.41
	ART	5.36	1.45
	CLF	5.53	1.54
	DQ	5.34	1.47
	Combination	5.43	1.78

The pH was kept above 5 and below 6, which is ideal for topical application. The dispersions were slightly more viscous than water, though not too viscous to form an occlusive layer; good spreadability was however still ensured.

3.2. Skin diffusion studies

During the skin diffusion studies, the samples tested were a combination of all three APIs in liposomes, niosomes and transferosomes, as well as a sample containing only the APIs with no carrier system included. This was to ascertain whether the carrier system had a significant impact on the topical delivery of the APIs. None of the samples tested, delivered any APIs in the receptor phase after 12 h, indicating that, were these samples applied to a patient, there would have been a high possibility that the APIs would not pass through the skin into the systemic circulation. Tape stripping data showed ART was not detected in the skin as indicated in Figure 4, though this may not mean that it was not present, but simply that the concentration may be too low to be detected.

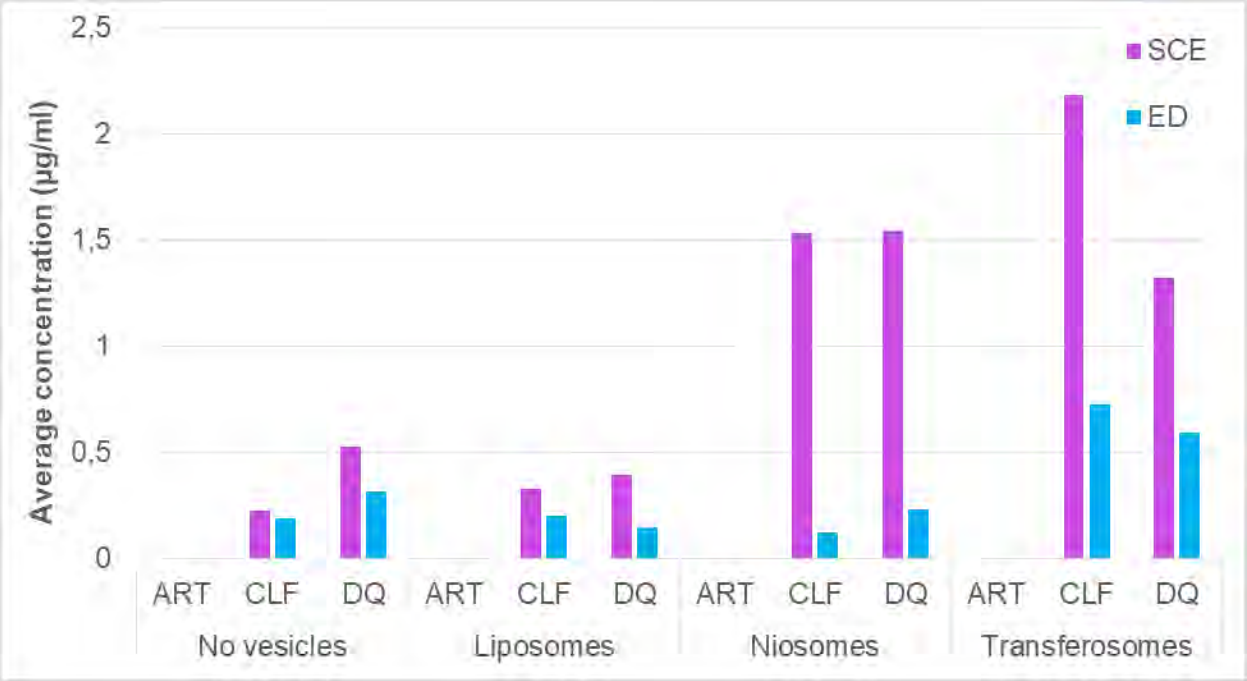

Figure 4: Average concentrations of APIs present in the stratum corneum-epidermis (SCE) and epidermis-dermis (ED) after tape stripping

The data clearly indicate that CLF and DQ can be delivered topically, with niosomes and transferosomes delivering the highest average concentrations. Decoquinate delivered higher concentrations in the ED with the no vesicle dispersion compared to both the liposome and niosome dispersions. The transferosome dispersion depicted the most favourable results overall, with a concentration CLF as high as 2.18 µg/ml present in the SCE and 0.73 µg/ml in the ED. DQ had a higher concentration in the SCE in the niosome dispersion (1.54 µg/ml) than in the transferosome dispersion (1.32 µg/ml), though the concentration DQ in the ED for the transferosome dispersion (0.59 µg/ml) was the highest of all the dispersions tested. This indicates that a higher concentration API will reach the deeper layers of the skin when applied as a transferosome dispersion, where the APIs are desired for activity. Even higher concentrations of

the APIs will possibly be delivered into the skin of a patient with CTB, since CTB is a disease that damages the skin and the stratum corneum will not be intact.

3.3. Efficacy against tuberculosis

A control culture was used as the positive control at 0% inhibition and isoniazid as the negative control (83% inhibition) since it is a known API against tuberculosis. Table 4 presents the data obtained from efficacy studies against tuberculosis for the 1% API dispersions evaluated.

Table 4: Growth inhibition (%) of the APIs in solid form, as well as in, the different dispersions

	API	**Liposomes**	**Niosomes**	**Transferosomes**
ART	28	31	20	38
CLF	42	42	52	38
DQ	28	29	8	35
Combination	-	42	38	12
Blank	-	12	36	42

All the dispersions and APIs tested depicted some activity against tuberculosis. Of the three APIs, CLF was the most active (42%). Comparing the single API dispersions, CLF in niosomes was the most active (52%) and DQ in niosomes the least active (8%). Of the combination dispersion, liposomes showed the highest activity against tuberculosis (42%) and transferosomes were least active (12%). Interestingly, even the empty vesicles displayed some activity against tuberculosis. This may be indicative that one or more of the vesicle components may have some degree of activity against tuberculosis. Transferosomes depicted the highest activity of the blank vesicles, which was similar to the CLF powder activity (42%). Transferosomes consist mainly of PC, though Acton [26] stipulated that PC was found to have no activity against tuberculosis. This is interesting and may indicate that either the combination of the buffer and the PC or the shape and size of the transferosomes have some activity against tuberculosis. Further investigation into the activity of blank transferosomes against tuberculosis will definitely be beneficial. Activity of the empty vesicles may also be caused by an occlusion effect caused by the vesicles over the tuberculosis cells, which may lead to cell death.

4. Conclusions

Liposomes, niosomes and transferosomes were prepared containing ART, DQ and CLF. It was established that an increase in API concentration had a definite influence on vesicles stability and size. The higher the concentration API in the vesicles, the higher the amount of API entrapped in the vesicle and thus an increase in size was obtained. This phenomenon, however, decreases

stability of the vesicle system. Size distribution and the zeta-potential indicated that CLF caused the most instabilities in the vesicles, with the biggest increase in size and the most unfavourable zeta-potential. Overall, niosomes were found to be most unstable and liposomes most robust.

In vitro skin diffusion studies indicated that DQ and CLF could be delivered into the skin, though no API was present in the receptor phase, which would represent the systemic circulation. Tape stripping data showed that transferosomes delivered the highest average concentration CLF and DQ into the skin. Transferosomes contain no cholesterol, unlike the niosomes and liposomes. This may suggest that transferosomes are less lipophilic, and thus, these highly lipophilic APIs are less likely to remain in the vesicles and would rather be inclined to penetrate more freely into the skin.

Though no definite conclusions can be made from the efficacy against tuberculosis data, further investigation into clofazimine would be beneficial, since clofazimine powder and clofazimine in the different vesicles, delivered the highest percentage inhibition. CLF is a documented anti-tuberculous API that was investigated in the 1990s, but which caused skin pigmentation due to ceroid lipofuscinosis [27, 28]. Further investigation is required of methods to deliver CLF locally, other than by means of vesicles, since CLF had the most influence on vesicle instabilities, while limiting the severe skin pigmentation might be advantageous for cutaneous tuberculosis. Further investigation into the reason why blank transferosomes also had such high anti-tuberculous activity, might produce some new insight into the way to combat tuberculosis and its ever increasing resistance.

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References

[1] Gillet, A., Lecomte, F., Hubert, P., Ducat, E., Evrard, B. & Piel, G, Skin penetration behaviour of liposomes as a function of their composition, *Eur. J. Pharm. Biopharm.* 79 (2011) 43-53.

[2] Tavano, L., Gentile, L., Rossi, C.O. & Muzzalupo, R, Novel gel-niosomes formulations as multicomponent systems for transdermal drug delivery, *Colloids. Surf. B: Biointerfaces.* 110 (2013) 281-288.

[3] El Maghraby, G.M.M., Williams, A.C. & Barry, B.W, Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration, *Int. J. Pharm.* 196 (2000) 63-74.

[4] Dragicevic-Curic, N. & Maibach, H.I, Percutaneous penetration enhancers - Chemical methods in penetration enhancement: Drug manipulation strategies and vehicle effects, first ed., New York, Springer, 2015.

[5] Mahale, N.B., Thakkar, P.D., Mali, R.G., Walunj, D.R. & Chaudhari, S.R, Niosomes: novel sustained release nonionic stable vesicular systems – an overview, *Adv. Colloid. Interface. Sci.* 183-184 (2012) 46-54.

[6] Jain, S., Jain, V. & Mahajan, S.C, Lipid based vesicular drug delivery systems, *Adv. Pharm.* 2014 (2014) 1-12.

[7] Kaur, I.P. & Singh, H, Nanostructured drug delivery for better management of tuberculosis, *J. Control. Release.* 18 (2014) 36-50.

[8] Cholo, M.C., Steel, H.C., Fourie, P.B., Germishuizen, W.A. & Anderson, R, Clofazimine: current status and future prospects, *J. Atimicrob. Chemother.* 67(2011):290-298.

[9] Steyn, J.D., Wiesner, L., du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W.C., Haynes, R.K. & Kotzé, A.F, Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology, *Int. J. Pharm.* 414(2011):260-266.

[10] Dunay, I.R., Chi Chan, W., Haynes, R.K. & Sibley, L.D, Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model, *J. Antimicrob. Agents. Chemother.* 53(2009):4450-4456.

[11] Nagelschmitz, J., Voith B., Wensing, G., Roemer, A., Fugmann, B., Haynes, R.K., Kotecka, B.M., Rieckmann, K.H. & Edstein, M.D, First assessment in humans of the

safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone, *Antimicrob. Agents. Chemother.* 52(2008):3085-3091.

[12] Vivas, L., Rattray, L., Stewart, L.B., Robinson, B.L., Fugmann B., Haynes, R.K., Peters, W. & Croft, S.L, Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone *in vitro* and *in vivo, J. Antimicrob. Chemother.* 59(2007):658-665.

[13] Biamonte, M.A., Wanner, J. & le Roch, K.G, Recent advances in malaria drug discovery, *Bioorganic Med. Chem. Lett.* 23(2013):2829-2843.

[14] Mikota, S.K. & Plumb, D.C, Elephant formulary. http://elephantcare.org/resources/formulary/drug-index/decoquinate/, 2017 (accessed 11 October 2017).

[15] Williams, R.B, The mode of action of anticoccidial quinolones (6-decyloxy-4-hydroxyquinoline-3-carboxylates) in chickens, *Int. J. Parasitol.* 27(1997):101-111.

[16] Agarwal, R., Katare, O.P. & Vyas, S.P, Preparation and in vitro evaluation of liposomal/niosomal delivery systems for antipsoriatic drug dithranol, *Int. J. Pharm.* 288 (2001) 43-52.

[17] Shaji, J. & Lal, M, Preparation, optimization and evaluation of transferosomal formulation for enhanced transdermal delivery of a COX-2 inhibitor, *Int. J. Pharm. Pharm. Sci.* 6 (2014) 467-477.

[18] New, R.R.C, Liposomes: a practical approach, first ed., New York, Oxford university press,1990.

[19] Zhao, C., Tong, K., Tan, J., Liu, Q., Wu, T. & Sun, D, Colloidal properties of montmorillonite suspensions modified with polyetheramine, *Colloids. Surf. A: Physicochem. Eng. Asp.* 457 (2014) 8-15.

[20] Van Zyl, L., du Preez, J., Gerber, M., du Plessis, J. & Viljoen, J, Essential fatty acids as transdermal penetration enhancers, *J. Pharm. Sci.* 105(2016):188-193.

[21] Lechartier, B. & Cole, S.T, Mode of action of clofazimine and combination therapy with benzothiazinones against *Mycobacterium tuberculosis*, *Antimicrob. Agents. Chemother.*, 59 (2015) 4457-4463.

[22] Malvern instruments Ltd, Zetasizer nano series: User manual (MAN0317; Issue 1.1), Malvern Instruments Ltd., England, 2004.

[23] Paudel, K.S., Milewski, M., Swadley, C.L., Brogden, N.K., Ghosh, P. & Stinchcomb, A, Challenges and opportunities in dermal/transdermal delivery, *Ther. Deliv.* 1 (2010) 109-131.

[24] Leite-Silva, V.R., De Almeida, M.M., Fradin, A., Grice, J.E. & Roberts, M.S, Delivery of drugs applied topically to the skin: Advanced formulation. http://www.medscape.org/viewarticle/771038_3 , 2012 (accessed 18 May 2016).

[25] Crittenden, J.C., Trussel, R.R., Hand, D.W., Howe, K.J. & Tchobanoglous, G, MWH's water treatment: Principles and design, third ed., New Jersey, John Wiley & Sons, Inc., 2012.

[26] Acton, A, Tuberculosis: New insights for the Healthcare Professional: 2011 Edition. https://books.google.co.za/books?id=HjllcI5deFUC&pg=PT115&lpg=PT115&dq=phosphatidylcholine+against+tuberculosis&source=bl&ots=0JoLR8YpCS&sig=OzipfIV7NY4ch0WKTsMs4f8MTeQ&hl=en&sa=X&ved=0ahUKEwiymNK9h4bWAhUDD8AKHVIiDO8Q6AEISjAE#v=onepage&q=phosphatidylcholine%20against%20tuberculosis&f=false, 2012 (accessed 2 September 2017).

[27] Job, C.K., Yoder, L., Jackobson, R.R. & Hastings, R.C, Skin pigmentation from clofazimine therapy in leprosy patients: A reappraisal, *J. Am. Acad. Dermatol.* 23 (1990) 236-241.

[28] Smith, M., Accinelli, A., Tejada, F.R. & Kharel, M.K, Chapter 28 – Drugs used in Tuberculosis and leprosy. *Side. Effect. Drug. Annual.* 38 (2016) 283-293.

Chapter 5

FINAL CONCLUSION AND FUTURE PROSPECTS

5.1. FINAL CONCLUSION

Artemisone, clofazimine and decoquinate were chosen as APIs for this study as part of the MALTBRedox MRC South African University Flagship Projects, which focus on oxidant-redox drug combinations for the possible treatment of malaria, tuberculosis and related diseases. These three APIs were chosen for this study for topical delivery by means of vesicles and effectivity against tuberculosis.

Cutaneous tuberculosis (CTB) is an uncommon disease, which manifests in only 1.5% of all extra-pulmonary cases; furthermore, this disease manifests in different forms, impeding diagnosis (Bravo & Gotuzzo, 2007; Fader *et al.*, 2010; Sankar *et al.*, 2013). Currently, the accepted oral anti-tuberculous therapy is employed for the treatment of CTB, but the duration of the treatment is long, resistance to the APIs occurs and patient compliance is unsatisfactory (Das *et al.*, 2014; Jean *et al.*, 2012; Ramarao *et al.*, 2012). The treatment is not completely effective and with these unsightly lesions, topical treatment might (concurrently with oral treatment) enhance treatment outcomes and possibly shorten treatment duration.

For topical delivery of APIs they have to adhere to certain criteria, since the skin has a remarkable barrier function (Hadgraft, 2001). The optimal partition coefficient (log P) is 1–3, with which only artemisone (log P 2.49) complies. Decoquinate (log P 7.8) and clofazimine (log P 7.6) are highly lipophilic APIs, rendering penetration of these APIs into the skin significantly more difficult as some form of hydrophilicity is necessary for skin permeation (Biamonte *et al.*, 2013; Dunay *et al.*, 2009; Nagelschmitz *et al.*, 2008; Steyn *et al.*, 2011). For this reason, different vesicle systems were chosen as delivery vehicles, since they can encapsulate both hydrophilic and lipophilic APIs, and they mostly create a “depot-effect”, increasing API concentrations at target organs (Dragicevic-Curic & Maibach, 2015; Jain *et al.*, 2014).

Currently, a variety of vesicles are employed as vehicles for topical delivery. For this study liposomes, niosomes and transferosomes were selected, and each system contained a final concentration of 1% API. The influence of API concentration on the vesicles was determined beforehand by preparing vesicles containing 0.2%, 0.4% and 1% API. The total vesicle concentration in phosphate buffer solution (10 ml as the aqueous phase) was retained at 5%. As part of the pre-formulation process, we used isothermal calorimetry in order to determine that none to minimal incompatibility existed between the APIs and vesicle components.

Encapsulation efficacy of the different 1% dispersions for all the vesicles was above 85%. At 0.2% API, the zeta-potentials for the formulations indicated mostly stable formulations, though an increase in API concentration increased vesicle size and the zeta-potential was below optimal. Zeta-potential and size distribution indicated complete instability of the vesicles from the 0.4% API concentration and higher. Thus, vesicles containing 0.6% and 0.8% API were not tested further. Niosomes were found to be most unstable with increasing vesicle size up to 2 461 nm in the 1% clofazimine formulation. Transferosomes delivered smaller vesicles (99.3 nm in the 0.2% combination API formulation) compared to liposomes (167.4 nm). Viscosity and pH were deemed acceptable for all formulations to ensure even spreadability and no skin irritation.

Skin diffusion studies were conducted on black skin using the 1% combination API dispersions as well as a dispersion containing no vesicles. For all four of the dispersions tested on skin, no APIs were present in the receptor fluid (representing human blood) and no artemisone were present in the epidermis-dermis (ED) or stratum corneum-epidermis (SCE). No APIs present in the receptor fluid were considered optimal, since the APIs should ideally be concentrated in the skin for local effectivity against CTB. However, as stated, artemisone was not detected in the skin and this finding is undesirable. This finding by no means suggests that no artemisone permeated the skin, it may simply be that the concentration of artemisone that did permeate the skin was below the minimum level of quantification, and this should be researched further. Tape stripping data depicted that the average concentrations of the APIs were higher in the SCE than in the ED. Decoquinate concentrations were higher than clofazimine in the no vesicle, liposome and niosome formulations, whereas the concentration of clofazimine in the transferosome formulation was higher than that of decoquinate. The transferosomes also delivered the highest average API concentrations in the skin.

Fifteen formulations, all containing 1% API, were sent to Stellenbosch University to be studied for activity against *Mycobacterium tuberculosis* (strain H37Rv). The empty vesicles presented with very low percentage inhibition, with the empty transferosomes (42%) displaying the highest percentage inhibition. Of the single API formulations, clofazimine in niosomes (52%) overall displayed the highest percentage inhibition. Between the combination API formulations, the transferosome formulation depicted the lowest (12%) and the liposome formulation the highest (42%) percentage inhibition. Clofazimine is a documented anti-tuberculous API (Smith *et al.*, 2016), though artemisone and decoquinate are not. The data showed that artemisone and decoquinate as APIs alone did have some activity against tuberculosis, though not significant. Niosomes and transferosomes containing no API demonstrated activity almost as high as pure clofazimine; this might possibly be explained by their ingredients, which mostly consisted of cholesterol and phosphatidylcholine (PC). Cholesterol and PC may possibly have some activity against TB, though according literature PC had no activity (Acton, 2012).

This study contributed to new knowledge in pharmaceutics, in that:

- Artemisone, clofazimine and decoquinate were successfully encapsulated in liposomes, niosomes and transferosomes.
- The influence of an increase in the APIs' concentration, as well as the effect of each API on the physical properties of the vesicles was determined as defined by measurement of the zeta-potential, size of the vesicles, size distribution, pH and viscosity.
- An HPLC method was developed for the concurrent determination of artemisone, clofazimine and decoquinate.
- Decoquinate and clofazimine were successfully delivered into the skin when utilising liposomes, niosomes and transferosomes as carrier system. When a suspension of APIs in phosphate buffer solution (no vesicles incorporated) was applied, clofazimine and decoquinate were detected in the skin.
- Results indicated that decoquinate had activity against tuberculosis, an API never before considered for tuberculosis.
- There was no significant increase in efficacy against tuberculosis when combining the three APIs in a vesicle dispersion, compared to the efficacy when the APIs were separately incorporated into the vesicles.
- Transferosomes and liposomes enhanced the activity of artemisone and decoquinate, whereas niosomes were found to enhance the activity of clofazimine. Niosomes, however, drastically decreased the activity of decoquinate.
- A combination of the APIs in transferosomes had significantly lower activity against tuberculosis when compared to the individual APIs in transferosomes, though the blank transferosomes were found to be most active against tuberculosis.

5.2. FUTURE PROSPECTS

Future prospects for additional investigations and aspects that need to be considered include:

- Efficacy against malaria, leprosy and relevant diseases.
- Different vesicles to enhance transdermal delivery.
- Different combinations of APIs, or substituting one or two of the APIs for another from the list of APIs investigated by the MALTBRedox MRC South African University Flagship Projects for activity against tuberculosis.
- Investigating the activity of the components of the vesicles used against tuberculosis.
- Exploring whether the 0.2% API formulations (depicting optimal characteristics and better stability) do have less activity against tuberculosis.
- Studying whether the 0.2% API formulations provided lower skin diffusion, since the 1% API formulation had a considerable amount of excess API that was not encapsulated, and which formed a sediment on the skin.

REFERENCES

ACTON, A. 2012. Tuberculosis: New insights for the Healthcare Professional: 2011 Edition. https://books.google.co.za/books?id=HjIlcl5deFUC&pg=PT115&lpg=PT115&dq=phosphatidylcholine+against+tuberculosis&source=bl&ots=0JoLR8YpCS&sig=OzipfIV7NY4ch0WKTsMs4f8MTeQ&hl=en&sa=X&ved=0ahUKEwiymNK9h4bWAhUDD8AKHVIiDO8Q6AEISjAE#v=onepage&q=phosphatidylcholine%20against%20tuberculosis&f=false Date of access: 2 September 2017.

BIAMONTE, M.A., WANNER, J. & LE ROCH, K.G. 2013. Recent advances in malaria drug discovery. *Bioorganic & medicinal chemistry letters,* 23(10):2829-2843.

BRAVO, F.G. & GOTUZZO, E. 2007. Cutaneous tuberculosis. *Clinical dermatology,* 25(2):173-180.

DAS, C.K., MAHAPATRA, A., DAS, M.M., SAHOO, D. & CHAYANI, N. 2014. Coexistence of cutaneous tuberculosis (scrofuloderma) and Hanseniasis: a rare presentation. *Journal of clinical and diagnostic research,* 8(2):141-2.

DRAGICEVIC-CURIC, N. & MAIBACH, H.I., eds. 2015. Percutaneous penetration enhancers - Chemical methods in penetration enhancement: Drug manipulation strategies and vehicle effects. 1st ed. New York: Springer. 341p.

DUNAY, I.R., CHI CHAN, W., HAYNES, R.K. & SIBLEY, L.D. 2009. Artemisone and artemiside control acute and reactivated toxoplasmosis in a murine model. *Journal of antimicrobial agents and chemotherapy,* 53(10):4450-4456.

FADER, T., PARKS, J., KHAN, N.U., MANNING, R., STOKES, S. & NASIR, N.A. 2010. Extrapulmonary tuberculosis in Kabul, Afghanistan: a hospital-based retrospective review. *International journal of infectious diseases,* 14(2):e102-e110.

HADGRAFT, J. 2004. Skin deep. *European journal of pharmaceutics and. biopharmaceutics*, 58(2):291-299.

JAIN, S., JAIN, V. & MAHAJAN, S.C. 2014. Lipid based vesicular drug delivery systems. *Advances in pharmaceutics*, 2014:1-12.

JEAN, M., MASSE, V., CARLIER, R., PERRONNE, C. & CRÉMIEUX, A.C. 2012. Isolated subcutaneous tuberculous abscesses of the lumbar wall. *Journal of travel medicine,* 19(5):329-330.

NAGELSCHMITZ, J., VOITH, B., WENSING, G., ROEMER, A., FUGMANN, B., HAYNES, R.K., KOTECKA, B.M., RIECKMANN, K.H. & EDSTEIN, M.D. 2008. First assessment in humans of

the safety, tolerability, pharmacokinetics, and ex vivo pharmacodynamics antimalarial activity of the new artemisinin derivative artemisone. *Antimicrobial agents and chemotherapy,* 52(9):3085-3091.

RAMARAO, S., GREENE, J.N., CASANAS, B.C., CARRINGTON, M.L., RICE, J. & KASS, J. 2012. Cutaneous manifestations of tuberculosis. *Infectious diseases in clinical practice,* 20(6):376-383.

SANKAR, M.M., SINGH, J., DIANA, S.C.A. & SINGH, S. 2013. Molecular characterization of *Mycobacteium tuberculosis* isolates from Indian patients with extrapulmonary tuberculosis. *Tuberculosis,* 93:75-83.

SMITH, M., ACCINELLI, A., TEJADA, F.R. & KHAREL, M.K. 2016. Chapter 28 – Drugs used in Tuberculosis and leprosy. *Side effects of drugs annual,* 38:283-293.

STEYN, J.D., WIESNER, L., DU PLESSIS, L.H., GROBLER, A.F., SMITH, P.J., CHAN, W.C., HAYNES, R.K. & KOTZÉ, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: potential application of Pheroid™ technology. *International journal of pharmaceutics,* 414(1-2):260-266.

Annexure A

ANALYTICAL METHOD VALIDATION FOR THE CONCURRENT DETERMINATION OF DECOQUINATE, ARTEMISONE AND CLOFAZIMINE BY MEANS OF HPLC

A.1. INTRODUCTION

Analytical method validation is an important on-going, regulatory requirement in pharmaceutical analysis. It provides documented, objective evidence which verifies that a method being used is adequate in measuring a performance parameter (BPCC, 2001; ICH, 2005; Shabir, 2004; Singh, 2013). The validation of a method is the process by which it is tested for accuracy, reliability, reproducibility, specificity, sensitivity and preciseness for its intended purpose (Ermer & Miller, 2005; McPolin, 2009; CGMP, 2011). Validation also establishes the performance limits of the measurement of a parameter; data is validated according to specific guidelines, though these are only recommendations (CGMP, 2011; Singh, 2013).

The scope and relevancy of an analytical method should be defined before the validation process is started. There are a few different types of validation, though only the most significant ones will be discussed in this annexure (Singh, 2013).

A.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD VALIDATION FOR DECOQUINATE, ARTEMISONE AND CLOFAZIMINE

A.2.1. CHROMATOGRAPHIC CONDITIONS

Analytical instrument: The analysis of artemisone, decoquinate and clofazimine was performed using an Agilent® 1100 Series HPLC system in a controlled environment (25°C). The instrument consisted of an Agilent® 1100 pump, diode array detector, autosampler injection mechanism and Chemstation Rev. A.10.02 software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA).

Column: A Restek Ultra C_{18} fully endcapped reversed phase column with a 5 µm particle size; 250 x 4.60 mm was used (Restek corporation, Bellefonte, US).

Mobile phase:	The mobile phase consisted of two containers, A and B. Container A consisted of 0.005 M octanesulphonic-acid Na in HPLC-grade water with added 2 ml of a 2 M ammonia solution adjusted to a pH of 3.5 with 2 M phosphoric acid. Container B contained acetonitrile.	
Solvent:	Ethanol.	
Run time:	15 min.	
Retention time:	Clofazimine:	Approximately 6.3 min.
	Artemisone:	Approximately 7.3 min.
	Decoquinate:	Approximately 9.8 min.
Flow rate:	1 ml/min with container A at 35% and container B at 65% for 2 min, then gradient to 10%/90% after 4 min and back to 35%/65% after 10 min.	
Injection volume:	20 µl.	
Detection wavelength:	UV at 210 nm and 284 nm.	

A.2.2. REFERENCE STANDARD AND SAMPLE PREPARATION

Approximately 40 mg artemisone, 7 mg clofazimine and 12 mg decoquinate were accurately weighed in a 100 ml volumetric flask and dissolved in absolute ethanol, after which it was filled to volume. The solution was diluted ten times by means of extracting 5 ml of the solution, placing it in a 50 ml volumetric flask, and filling it to volume with absolute ethanol. The diluted, as well as the original solution, was transferred to separate autosampler vials to be analysed by means of HPLC at different concentrations (Figure A.1).

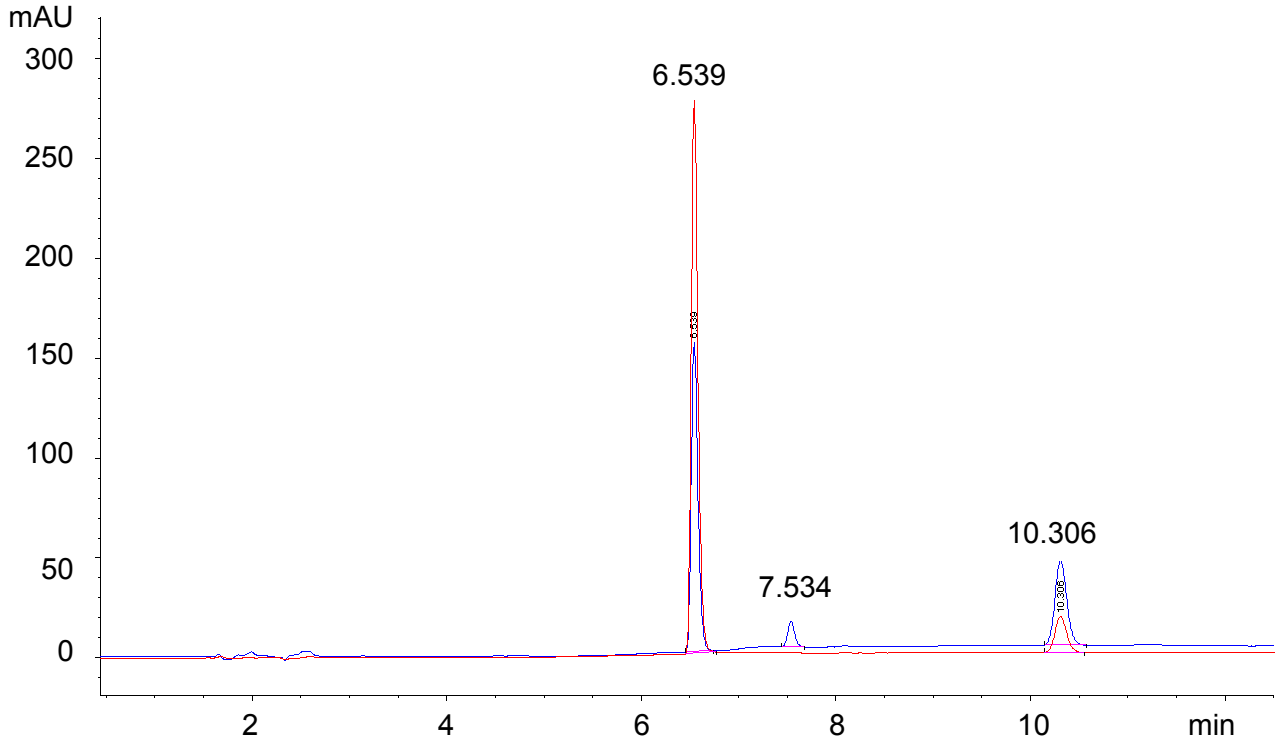

Figure A.1: Chromatogram of a reference standard injected into HPLC and the retention times of the three APIs

A.2.3. ANALYTICAL VALIDATION OF TEST PROCEDURE AND ACCEPTANCE CRITERIA

A.2.3.1. LINEARITY

The ICH (2005) defines linearity as the ability to obtain test results within a given range, which is directly proportional to the concentration of an analyte in a given sample, typically 25, 50, 75, 100, 150, and 200% of target concentration (McPolin, 2009). A correlation coefficient of $r^2 > 0.998$ is generally accepted as evidence of an adequate relation between the data and the regression line (Shabir, 2004; Singh, 2013). Linearity is usually obtained by plotting peak areas of the calibration standards on the Y-axis against the concentration on the X-axis. A series of three to six injections of a minimum of five or more standards is used for linearity determination (ICH, 2005; Singh, 2013). For assays, a minimum specified range of the target concentration is 80-120% (CDER, 1994; Shabir, 2004). Figure A.2 and Table A.1 demonstrate the linearity data obtained for artemisone.

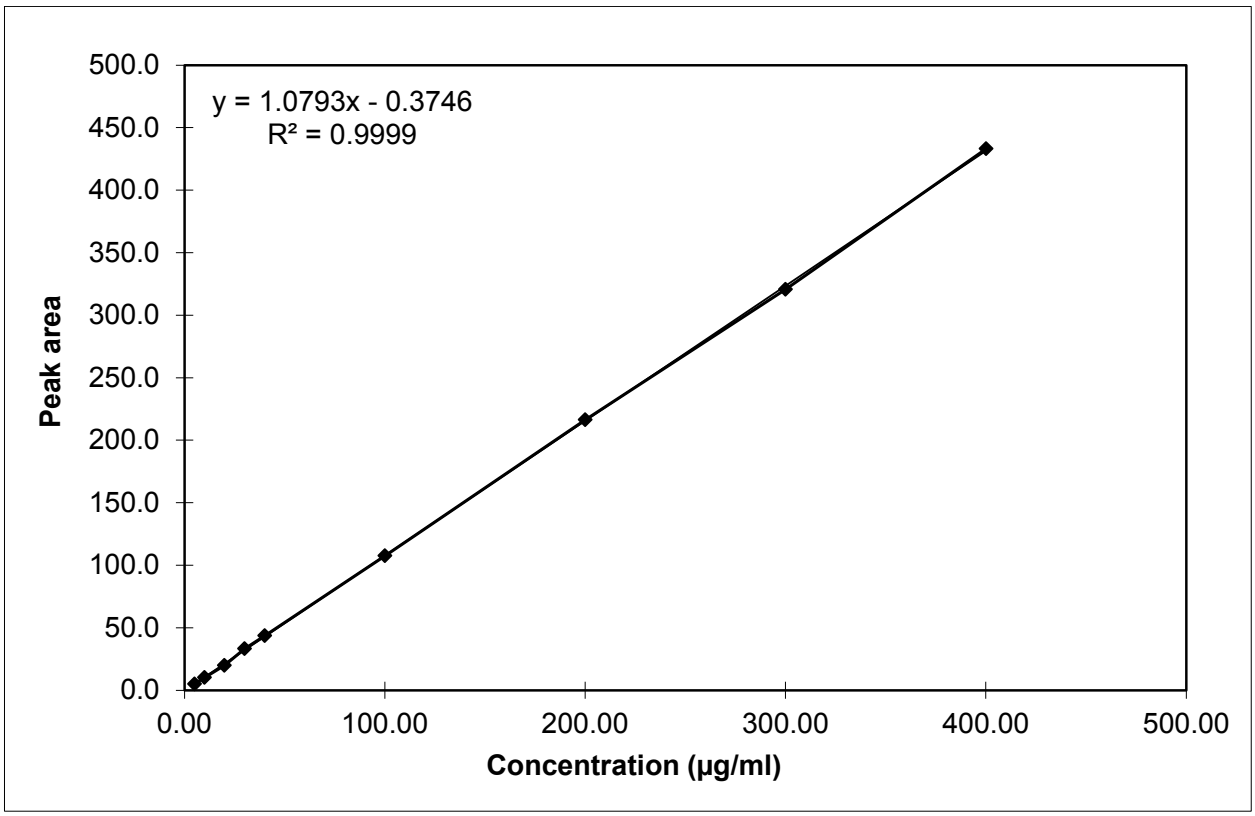

Figure A.2: Linear regression curve of artemisone

Figure A.2 displays that the correlation coefficient for artemisone was 0.999, which is acceptable according to ICH (2005) standards that require a value of more than 0.998.

Table A.1: Linear regression data obtained for artemisone

µg/ml	Peak area		Mean
5.00	5.1	5.1	5.1
10.00	10.3	10.0	10.2
20.01	19.9	19.9	19.9
30.01	33.2	33.1	33.2
40.01	43.7	43.6	43.7
100.03	107.6	107.5	107.6
200.05	216.5	216.0	216.3
300.08	321.1	320.4	320.8
400.10	432.4	433.8	433.1
R^2	0.99993	**Lower 95%**	**Upper 95%**
Intercept	-0.3746	-1.9050	1.1559
Slope	1.0793	1.0710	1.0877

In Table A.2 and Figure A.3 the linearity data of clofazimine are stipulated. Figure A.2 indicates a co-relation coefficient of 1 for clofazimine, which specifies perfect co-relation between the data and analyte concentration according to ICH (2005) guidelines.

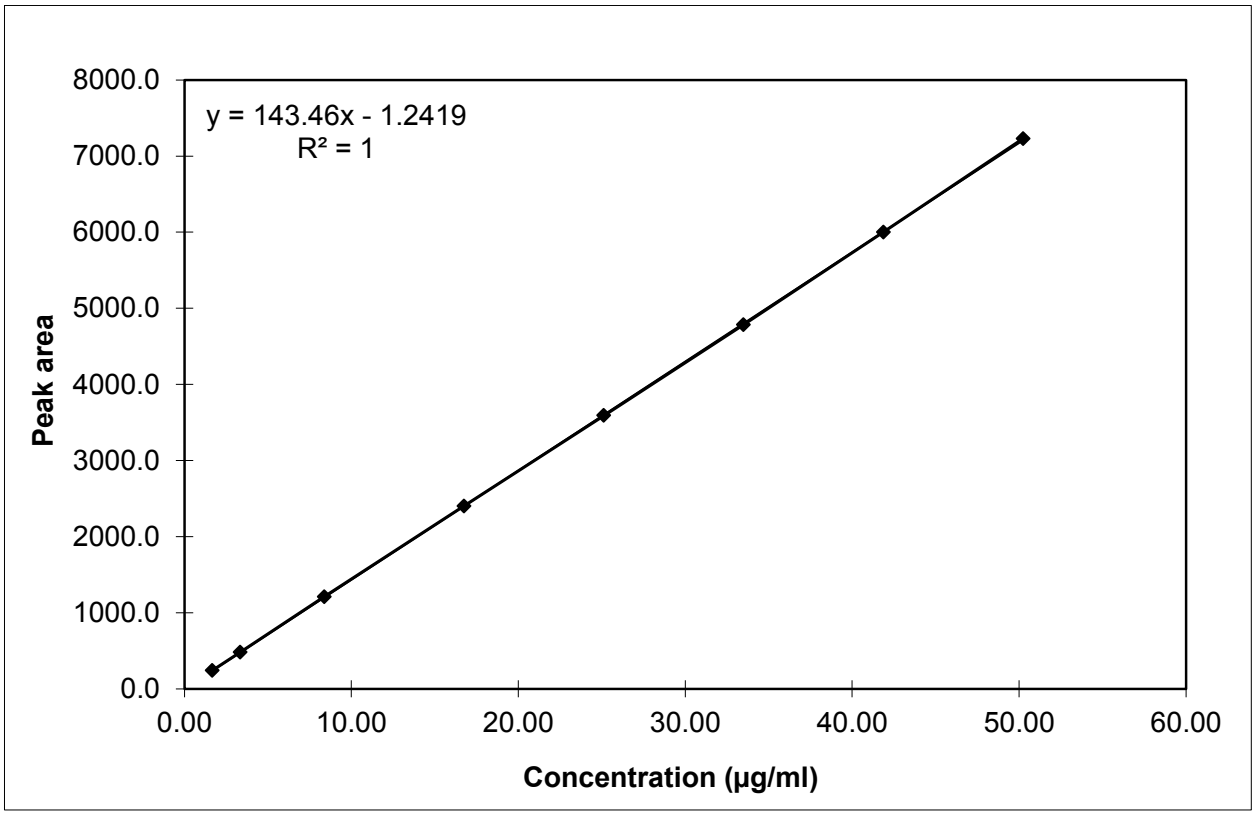

Figure A.3: Linear regression curve for clofazimine

Table A.2: Linearity data for clofazimine

µg/ml	Peak area		Mean
1.68	242.0	241.1	241.6
3.35	483.1	482.3	482.7
8.38	1217.8	1200.9	1209.4
16.75	2405.1	2399.7	2402.4
25.13	3590.0	3591.7	3590.9
33.50	4797.6	4772.5	4785.1
41.88	5993.1	6009.9	6001.5
50.25	7241.5	7216.4	7229.0
R^2	0.99998	**Lower 95%**	**Upper 95%**
Intercept	-1.2419	-20.8791	18.3954
Slope	143.4621	142.7676	144.1565

In Table A.3 and Figure A.4 the linearity data for decoquinate are noted. The data presented in Figure A.4 indicate an acceptable co-relation for decoquinate of 0.9989 (ICH, 2005).

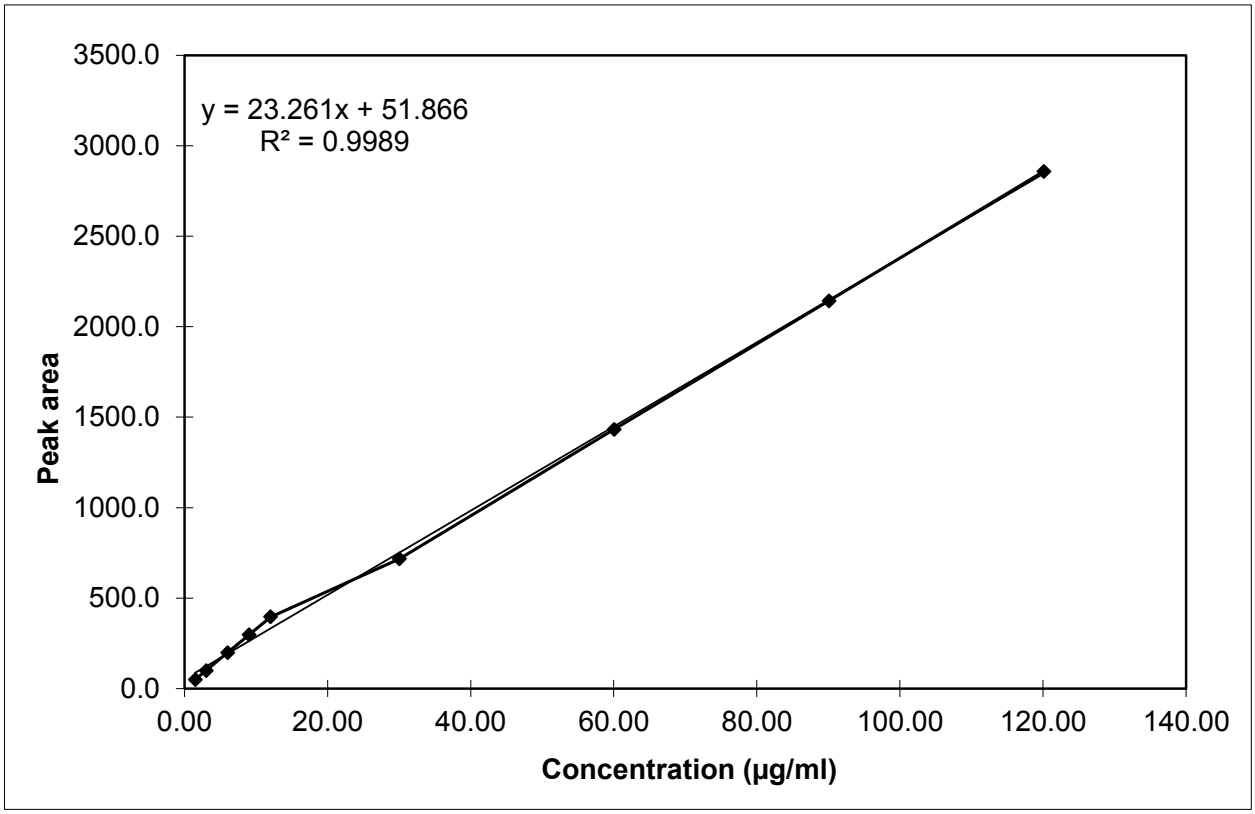

Figure A.4: Linear regression curve for decoquinate

Table A.3: Linearity data obtained for decoquinate

µg/ml	Peak area		Mean
1.50	47.0	49.9	48.5
3.00	98.2	98.6	98.4
6.01	197.4	197.9	197.7
9.01	296.1	296.2	296.2
12.01	395.6	395.6	395.6
30.03	713.3	718.9	716.1
60.05	1432.2	1431.2	1431.7
90.08	2140.7	2144.4	2142.6
120.10	2856.6	2858.9	2857.8
R^2	0.9989	**Lower 95%**	**Upper 95%**
Intercept	51.8663	13.7745	89.9582
Slope	23.2613	22.5699	23.9527

A.2.3.2. LIMIT OF DETECTION AND QUANTITATION

The detection limit (DL) of an analytical procedure is the lowest amount of API in a sample that can be detected, whereas the quantitation limit (QL) is the lowest amount of API in a sample that can be quantitatively determined with adequate accuracy and precision (ICH, 2005). Both the DL and QL can be estimated using Equation A.1 and Equation A.2 based on the standard deviation (SD) of the response and the slope used from the linearity from each API:

$$DL = \frac{3.3\sigma}{S}$$

Eq. A.1

and

$$QL = \frac{10\sigma}{S}$$

Eq. A.2

Where: σ = the SD of the response

S = the slope of the calibration curve (ICH, 2005).

The estimated limit of detection (LOD) was 1.979 µg/ml for artemisone, 3.107 µg/ml for clofazimine and 2.285 µg/ml for decoquinate. To ensure an accurate LOD, the sample was injected six times, starting at the estimated LOD concentration and the percentage relative standard deviation (%RSD) determined, which should be 20% or less (De Vito *et al.*, 2016; Shabir, 2004). The peak areas for the low concentrations can be seen in Table A.4, as well as the %RSD.

Table A.4: Limit of detection (LOD) determined for artemisone, clofazimine and decoquinate

	Artemisone	**Clofazimine**	**Decoquinate**
Concentration (µg/ml)	***4.42***	***0.042***	***0.703***
Peak area	4	4	16.2
	6.3	5.4	19.8
	5.4	3.9	21.3
	4.1	5.7	23.4
	4.2	5.6	25.8
	4.3	4.2	26.8
Average	4.71	4.80	22.21
SD	0.93	0.85	3.95
%RSD	19.68	17.73	17.79

The experimental LOD for artemisone, clofazimine and decoquinate was found to be 4.42 µg/ml, 0.042 µg/ml, and 0.703 µg/ml, respectively. The limit of quantitation (LOQ) was determined from the experimental LOD and estimated as 13.39 µg/ml for artemisone, 0.13 µg/ml for clofazimine and 2.13 µg/ml for decoquinate.

A.2.3.3. ACCURACY

The ICH (2005) defines the accuracy of an analytical procedure as the closeness of agreement between the test results obtained by the method and the true value of the API (BPCC, 2001; Shabir, 2004; Singh, 2013).

Accuracy should be assessed, using a minimum of nine determinations from a minimum of three concentrations covering the specified range. Accuracy should be recorded as percentage recovery by the assay of known analyte amount added in the sample; or as the difference between the mean and the accepted true value together with the confidence intervals (Ermer & Miller, 2005; ICH, 2005; Singh, 2013). An accuracy criterion for an assay method is a recovery percentage of 100 ± 2% (ICH, 2005; Shabir, 2004), though this is only a guideline and 100 ±15% is also acceptable when calculating difference to the mean or 100 ± 30% for individual recoveries to the reference (BPCC, 2011; Ermer & Miller, 2005; McPolin, 2009). It is recommended that the percentage relative standard deviation (%RSD) be equal to or less than 2% (Ermer & Miller, 2005). Table A.5, A.6 and A.7 represent the accuracy data for artemisone, clofazimine and decoquinate, respectively.

Table A.5: Accuracy of artemisone

Concentration spiked				**Recovery**	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
15.0	17.9	17.8	17.9	14.7	98.3
15.0	17.8	17.7	17.8	14.6	97.6
15.0	17.9	17.9	17.9	14.8	98.6
20.0	23.6	23.6	23.6	20.2	101.2
20.0	23.4	23.7	23.6	20.1	101.0
20.0	23.7	23.9	23.8	20.4	102.2
99.8	106.9	107.2	107.1	99.8	100.1
99.8	107.1	107.0	107.1	99.8	100.1
99.8	106.9	106.7	106.8	99.6	99.8
				Mean	99.871
				SD[1]	1.392
				%RSD[2]	1.394

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean value for the recovery percentage of artemisone was 99.871%, which is within the accepted range. The %RSD was 1.394, which is lower than 2% as recommended (BPCC, 2011; Ermer & Miller, 2005; McPolin, 2009).

Table A.6: Accuracy of clofazimine

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
1.8	265.3	265.4	265.4	1.8	99.8
1.8	265.9	265.5	265.7	1.8	99.9
1.8	264.5	263.6	264.1	1.8	99.3
3.7	530.9	532.5	531.7	3.7	100.5
3.7	528.7	529.3	529.0	3.6	100.0
3.7	530.9	530.1	530.5	3.7	100.2
9.1	1322.5	1318.7	1320.6	9.1	100.1
9.1	1317.9	1319.3	1318.6	9.1	100.0
9.1	1323.1	1311.4	1317.3	9.1	99.9
				Mean	100.0
				SD[1]	0.309
				%RSD[2]	0.309

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for clofazimine was exactly 100%. The %RSD was 0.309%, which is acceptable, since it is within the accepted range of less than 2% (Ermer & Miller, 2005; Shabir, 2004).

Table A.7: Accuracy of decoquinate

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
4.7	131.4	132.4	131.9	4.2	88.9
4.7	130.1	130.3	130.2	4.1	87.4
4.7	139.0	138.8	138.9	4.5	95.1
6.3	198.0	198.4	198.2	6.9	110.7
6.3	188.0	187.4	187.7	6.5	103.7
6.3	194.9	194.6	194.8	6.8	108.4
31.3	776.1	777.5	776.8	30.9	98.9
31.3	781.5	780.1	780.8	31.1	99.4
31.3	797.0	795.0	796.0	31.7	101.4
				Mean	99.326
				SD[1]	7.469
				%RSD[2]	7.519

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for decoquinate was 99.326%, which is within the acceptable range, whereas the %RSD was 7.519%, which is not less than 2% as required (Ermer & Miller, 2005; Shabir, 2004). This can be explained by the percentage recovery column, where individual recoveries are within the 100 ± 30% range (McPolin, 2009). Thus the %RSD is higher because the variation between individual recoveries was higher.

A.2.3.4. PRECISION

Precision is the closeness of agreement between a series of measurements acquired from multiple sampling of the same standardised sample (BPCC, 2001; CDER, 1994; ICH, 2005). Precision is expressed as %RSD for a number of samples; a minimum of five to nine measurements at a minimum of three concentrations covering the specified range (BPCC, 2001; McPolin, 2009; Singh, 2013). Precision may be performed at three levels, namely repeatability (intra-day assay precision), intermediate precision (inter-day assay variation) and reproducibility (CDER, 1994; Huber, 2015; ICH, 2005; Shabir, 2004; Singh, 2013).

A precision criterion for an assay method for drug substances is a %RSD ≤ 2% (Huber, 2015; McPolin, 2009; Shabir, 2004). CDER (1994) recommends a %RSD ≤ 1% for a minimum of five injections, though higher variations may be acceptable for low level impurities. The mean percentage recovery should be 100 ± 2%, and 100 ± 15% for individual recoveries, or even 100 ± 20% at the lower limit of quantification (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009).

A.2.3.4.1. Repeatability (Intra-day assay variation)

Shabir (2004) stated that for instrument precision the %RSD should be less than 1%, whereas at the limit of quantitation for the impurity assay, the %RSD could be ≤ 5%. Repeatabilities (intra-day variation or day 1 precision) of artemisone, clofazimine and decoquinate are stipulated in Tables A.8, A.9 and A.10, respectively.

Table A.8: Artemisone repeatability

Concentration spiked				**Recovery**	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
14.7	16.9	16.9	16.9	14.6	99.0
14.7	17.0	16.8	16.9	14.6	99.0
14.7	16.7	16.9	16.8	14.5	98.3
24.6	28.2	27.9	28.1	25.0	101.7
24.6	27.8	27.2	37.5	24.5	99.6
24.6	28.0	27.9	28.0	24.9	101.3
98.3	107.6	106.3	107.0	98.4	100.2
98.3	106.1	106.5	106.3	98.8	99.6
98.3	107.0	106.8	106.9	98.4	100.1
				Mean	99.860
				SD[1]	1.025
				%RSD[2]	1.026

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery was 99.860% for artemisone, which is within the accepted criterion. The %RSD was less than 2%, which is also within the accepted criteria (Huber, 2015; Shabir, 2004).

Table A.9: Clofazimine repeatability

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
2.8	398.2	397.9	398.1	2.8	99.9
2.8	397.9	398.9	398.4	2.8	100.0
2.8	396.9	398.3	397.6	2.8	99.8
4.6	667.1	668.3	667.7	4.6	99.9
4.6	669.7	668.5	669.1	4.6	100.1
4.6	670.2	668.8	669.5	4.6	100.2
18.5	2685.1	2683.0	2684.1	18.4	99.7
18.5	2690.3	2688.6	2689.5	18.5	99.9
18.5	2700.4	2708.0	2704.2	18.6	100.4
				Mean	99.989
				SD[1]	0.21
				%RSD[2]	0.210

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

Both the mean percentage recovery (99.989%) and the %RSD (0.21%) for clofazimine were within the accepted criteria stipulated above (Huber, 2015; McPolin, 2013; Shabir, 2004; Singh, 2013).

Table A.10: Decoquinate repeatability

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
4.7	154.4	153.9	154.2	4.4	93.5
4.7	155.1	155.3	155.2	4.4	94.6
4.7	153.7	155.0	154.4	4.4	93.8
6.2	198.6	197.3	198.0	6.4	103.4
6.2	202.0	201.8	201.9	6.6	106.4
6.2	199.5	199.7	199.6	6.5	104.6
31.0	711.3	714.5	712.9	30.6	98.8
31.0	720.4	720.9	720.7	31.0	100.0
31.0	726.7	728.7	727.7	31.3	101.0
				Mean	99.573
				SD[1]	4.526
				%RSD[2]	4.545

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for decoquinate was 99.573%, which is within the accepted range. The %RSD was 4.545%, which is higher than the preferred 2%, though lower than 5%, which is also acceptable (Huber, 2015; Shabir, 2004). The higher %RSD can be explained by the higher variation between individual percentage recoveries, which all ranged within 100 ± 15% (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009).

A.2.3.4.2. Intermediate precision (Inter-day assay variation)

Intermediate precision is the results from within lab variations, which include different days, analysts and equipment (ICH, 2005; McPolin, 2009; Shabir, 2004; Singh, 2013). For this study, the test results were acquired with the analytical method over three days, while the other conditions remained unchanged. The acceptance criterion for these results is also %RSD ≤ 2%, or ≤ 3.5-5% for instrument precision at LOQ (Ermer & Miller, 2005; Shabir, 2004). Tables A.11, A.12 and A.13 indicate the data obtained the second day of inter-day variation studies for artemisone, clofazimine and decoquinate, respectively.

Table A.11: Intermediate precision of artemisone

Concentration spiked				**Recovery**	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
15.49	17.5	17.6	17.6	15.5	100.3
15.49	17.3	17.5	17.4	15.4	99.4
15.49	17.3	17.3	17.3	15.3	98.8
20.65	23.1	22.9	23.0	20.7	100.1
20.65	23.2	22.8	23.0	20.7	100.1
20.65	23.3	23.1	23.2	20.9	101.0
103.25	112.1	111.6	111.9	104.4	101.1
103.25	109.6	111.8	110.7	103.3	100.0
103.25	109.4	109.4	109.4	102.1	98.9
				Mean	99.964
				SD[1]	0.778
				%RSD[2]	0.779

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for artemisone was 99.96% and is thus within the accepted range. The %RSD was less than 2%, which indicates good intermediate precision of the analytical method (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009).

Table A.12: Intermediate precision of clofazimine

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
2.51	368.4	368.5	368.5	2.5	100.2
2.51	367.8	367.5	367.7	2.5	100.0
2.51	368.2	368.0	368.1	2.5	100.1
3.35	490.3	491.1	490.7	3.3	99.9
3.35	489.5	489.6	489.6	3.3	99.7
3.35	492.4	491.3	491.9	3.4	100.2
16.75	2462.5	2459.2	2460.9	16.7	99.8
16.75	2466.7	2458.8	2462.8	16.7	99.9
16.75	2473.3	2467.9	2470.6	16.8	100.2
				Mean	100.007
				SD[1]	0.171
				%RSD[2]	0.171

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for clofazimine was 100.007%, which is within the acceptable range. Percentage relative standard deviation also fell within the accepted range, which is ≤ 2% (Huber, 2015; Shabir, 2004).

Table A.13: Intermediate precision of decoquinate

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
4.43	126.5	126.3	126.4	4.2	94.4
4.43	130.0	129.8	129.9	4.3	98.1
4.43	125.9	125.5	125.7	4.1	93.6
5.90	165.5	165.8	165.7	6.0	102.4
5.90	168.8	169.0	168.9	6.2	105.0
5.90	167.4	167.3	167.4	6.1	103.7
29.50	665.6	662.6	664.1	29.7	100.8
29.50	654.9	652.7	653.8	29.2	99.1
29.50	660.1	658.9	659.5	29.5	100.0
				Mean	99.671
				SD[1]	3.672
				%RSD[2]	3.684

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for decoquinate was 99.67%, which is well within the recognised range. The %RSD was higher than 2%, though still lower than 5% (Ermer & Miller, 2005; Shabir, 2004). The higher %RSD can be explained by the higher variation for individual percentage recoveries, though they were all within an acceptable range of 100 ± 15% (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009).

A.2.3.4.3. Reproducibility

Reproducibility is not generally expected if intermediate precision is established. This is determined by testing the same samples in multiple laboratories as part of inter-laboratory crossover studies. The %RSD is expected to be ≤ 2% of the primary testing lab, or ≤ 3.5-5% for an impurity method (CDER, 1994; Ermer & Miller, 2005; Shabir, 2004; Singh, 2013). Tables A.14, A.15 and A.16 represent the precision done on day three for artemisone, clofazimine and decoquinate, respectively.

Table A.14: Reproducibility of artemisone

Concentration spiked				**Recovery**	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
15.26	17.70	17.20	17.45	14.96	98.00
15.26	17.30	17.80	17.55	15.05	98.63
15.26	17.70	17.40	17.55	15.05	98.63
20.35	23.60	23.00	23.30	20.57	101.10
20.35	23.50	23.40	23.45	20.72	101.81
20.35	23.30	23.20	23.25	20.53	100.87
101.75	107.90	107.70	107.80	101.72	99.97
101.75	108.20	107.70	107.95	101.86	100.11
101.75	108.00	107.40	107.70	101.62	99.88
				Mean	99.888
				SD[1]	1.199
				%RSD[2]	1.201

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for artemisone was 99.888%, which is in the acknowledged range. The %RSD was 1.201%, which is less than 2% and thus adheres to the stipulated criterion (Shabir, 2004).

Table A.15: Reproducibility of clofazimine

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
2.63	384.60	384.40	384.50	2.62	99.87
2.63	383.70	384.10	383.90	2.62	99.72
2.63	384.90	386.30	385.60	2.63	100.16
3.50	513.90	513.70	513.80	3.50	100.09
3.50	513.20	512.60	512.90	3.50	99.92
3.50	514.20	514.40	514.30	3.51	100.19
17.50	2569.00	2560.40	2564.70	17.49	99.92
17.50	2566.80	2570.10	2568.45	17.51	100.06
17.50	2565.90	2568.40	2567.15	17.50	100.01
				Mean	99.994
				SD[1]	0.143
				%RSD[2]	0.143

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

Mean percentage recovery for clofazimine was 99.994%, which fell within the recognised range. The %RSD was less than 2% and thus also fell within the established criterion (Shabir, 2004).

Table A.16: Reproducibility of decoquinate

Concentration spiked				Recovery	
µg/ml	**Peak area**		**Mean**	**µg/ml**	**%**
4.31	127.20	127.20	127.20	4.07	94.35
4.31	126.70	126.90	126.80	4.05	93.96
4.31	128.90	129.20	129.05	4.15	96.14
5.75	175.80	175.40	175.60	6.09	105.83
5.75	171.30	171.50	171.40	5.91	102.79
5.75	172.90	172.60	172.75	5.97	103.77
28.75	713.60	711.70	712.65	28.46	99.00
28.75	696.80	696.70	696.75	27.80	96.70
28.75	749.00	747.40	748.20	29.94	104.15
				Mean	99.633
				SD[1]	4.312
				%RSD[2]	4.328

1. Standard deviation (SD)
2. Relative standard deviation (%RSD)

The mean percentage recovery for decoquinate was 99.633%, which is within the putative range, but the %RSD was 4.328%. The higher %RSD can be explained by the higher variation between individual percentage recoveries. All the individual recoveries are, however, still within the recognised criterion of 100 ± 15% (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009; Shabir, 2004).

A "between days" comparison was done for the three different compounds. This data is captured in Tables A.17, A.18 and A.19 for artemisone, clofazimine and decoquinate, respectively. For these tables, the three measurements were taken from the last value for each of the three concentrations used.

Table A.17: Precision of artemisone between three days

	Day 1	**Day 2**	**Day 3**	**Between days**
	98.3	98.8	98.6	
	101.3	101.0	100.9	
	100.1	98.9	99.9	
Mean	99.93	99.55	99.79	99.76
SD	1.21	1.03	0.92	1.07
%RSD	1.21	1.04	0.92	1.07

The mean percentage recovery for artemisone for the three days was within 100 ± 2% and the %RSDs were less than 2%. All of these data measurements thus adhere to the accepted criteria.

Table A.18: Precision of clofazimine between three days

	Day 1	**Day 2**	**Day 3**	**Between days**
	99.8	100.1	100.2	
	100.2	100.2	100.2	
	100.4	100.2	100.0	
Mean	100.13	100.17	100.12	100.17
SD	0.26	0.06	0.08	0.16
%RSD	0.26	0.06	0.08	0.16

The mean percentage recovery for clofazimine was 100.17%, which is within the accepted range. The %RSD was less than 2% and thus also adheres to the putative criteria.

Table A.19: Precision of decoquinate between three days

	Day 1	Day 2	Day 3	Between days
	93.8	93.6	96.1	
	104.6	103.7	103.8	
	101.0	100.0	104.2	
Mean	99.81	99.12	101.35	100.10
SD	4.53	4.19	3.69	4.25
%RSD	4.54	4.23	3.64	4.25

The mean percentage recovery for decoquinate over three days was 100.10%, which is within the standard criteria. The mean %RSD over three days was 4.25%, which is more than 2%, but still less than 5% as preferred. This higher %RSD can again be explained by higher individual recoveries for decoquinate. All individual recoveries were, however, still within the accepted range of 100 ± 15% (BPCC, 2001; Ermer & Miller, 2005; McPolin, 2009; Shabir, 2004).

A.2.3.5. RUGGEDNESS (STABILITY)

Ruggedness is the degree of stability and can be evaluated by freeze and thaw stability, short-term temperature stability, long-term stability, stock solution stability and post-preparative stability (BPCC, 2001). The accepted value for percentage relative standard deviation is 2% or lower (Ermer & Miller, 2005; Huber, 2015). For this study stock solution stability was performed. This stability testing should be done at room temperature (25°C) for at least six hours (BPCC, 2001). All the test parameters remained constant; and the time interval for injections was set to one injection every hour for 24 h. This was to observe the stability of the compound over a longer period. Stability testing is important since it estimates the time span allowed between sample collection and analysis (Huber, 2015). Shabir (2004) suggested injecting freshly prepared test solutions and then again after 24 h and 48 h, with a sample response within 2% of the freshly prepared standards. Tables A.20, A.21 and A.22 represent the ruggedness data obtained over 24 h for artemisone, clofazimine and decoquinate, correspondingly.

Table A.20: Stability of artemisone

Time	Peak area	Percentage (%)
0	23.20	100.00
1	23.50	101.29
2	23.20	100.00
3	23.30	100.43
4	23.30	100.43
5	23.20	100.00
6	23.20	100.00
7	23.40	100.86
8	23.40	100.86
9	23.30	100.43
10	23.30	100.43
11	23.00	99.14
12	22.90	98.71
13	23.00	99.14
14	23.00	99.14
15	23.00	99.14
16	23.10	99.57
17	23.00	99.14
18	23.20	100.00
19	23.30	100.43
20	23.20	100.00
21	23.50	101.29
22	23.20	100.00
23	23.00	99.14
24	23.10	99.57
Mean	23.19	99.97
SD	0.16	0.70
%RSD	0.70	0.70

The %RSD for artemisone was within the established range of less than 2%; and the percentage peak area compared to the first peak area was within 2%, thus indicating good sample stability for 24 h.

Table A.21: Stability of clofazimine

Time	Peak area	Percentage (%)
0	430.90	100.00
1	428.80	99.51
2	432.30	100.32
3	430.60	99.93
4	429.80	99.74
5	429.50	99.68
6	429.40	99.65
7	430.70	99.95
8	430.10	99.81
9	429.90	99.77
10	430.40	99.88
11	429.60	99.70
12	430.00	99.79
13	431.20	100.07
14	431.30	100.09
15	431.20	100.07
16	431.20	100.07
17	430.90	100.00
18	431.40	100.12
19	431.50	100.14
20	432.00	100.26
21	431.70	100.19
22	431.40	100.12
23	431.30	100.09
24	432.00	100.26
Mean	430.76	99.97
SD	0.90	0.21
%RSD	0.21	0.21

The %RSD for clofazimine was less than 2%, which is within the recommended range. The percentage peak area did not vary more than 2%, which indicates that the compound is stable during the 24 h analysis time.

Table A.22: Stability of decoquinate

Time	Peak area	Percentage (%)
0	179.10	100.00
1	186.80	104.30
2	186.90	104.36
3	186.60	104.19
4	186.70	104.24
5	186.90	104.36
6	187.10	104.47
7	187.50	104.69
8	186.70	104.24
9	186.90	104.36
10	186.60	104.19
11	187.60	104.75
12	187.80	104.86
13	187.80	104.86
14	187.50	104.69
15	187.70	104.80
16	187.80	104.86
17	188.10	105.03
18	187.60	104.75
19	188.00	104.97
20	187.80	104.86
21	188.00	104.97
22	188.40	105.19
23	187.80	104.86
24	187.70	104.80
Mean	187.10	104.46
SD	1.71	0.96
%RSD	0.92	0.92

The %RSD for decoquinate was less than 2%, which is within the recommended range. The percentage peak area between hour zero, and one, differed with 4.3%, though from hour one to twenty-four, the difference was no more than 2%. This may indicate that decoquinate was not stable during the 24 h analysis, though instability could only be observed within the first hour.

A.2.3.6. SYSTEM SUITABILITY

System suitability is a validation parameter used to determine whether the HPLC system and procedure are capable of providing sufficient data whenever they are used (Ermer & Miller, 2005; Snyder *et al.*, 1997). For system suitability the %RSD of the retention times and peak areas are presented for a minimum of six injections. The acceptance criteria for %RSD for both retention times and peak areas should be ≤1% for ≥ 5 samples (CDER, 1994; Shabir, 2004). Tables A.23, A.24 and A.25 depict the system suitability data of artemisone, clofazimine and decoquinate, respectively.

Table A.23: System suitability for artemisone

	Peak area	**Retention time (min)**
	105.10	7.32
	105.00	7.32
	104.80	7.32
	104.40	7.31
	103.70	7.31
	103.60	7.31
	103.80	7.31
	103.50	7.31
	103.70	7.32
	103.60	7.30
Mean	104.12	7.31
SD	0.60	0.01
%RSD	0.58	0.07

The recommended criteria of a %RSD ≤ 1% for both the retention times and the peak areas were met with a %RSD for artemisone of 0.58% for the peak area and 0.07% for the retention time.

Table A.24: System suitability for clofazimine

	Peak area	Retention time (min)
	2361.10	6.23
	2361.90	6.22
	2356.30	6.27
	2354.80	6.27
	2358.70	6.27
	2358.40	6.28
	2363.10	6.28
	2361.80	6.28
	2382.20	6.28
	2365.00	6.28
Mean	2362.33	6.26
SD	7.25	0.02
%RSD	0.31	0.34

Both the peak area and retention time percentage relative standard deviations for clofazimine were less than 1%, which is within the accepted range.

Table A.25: System suitability for decoquinate

	Peak area	Retention time (min)
	620.80	9.86
	624.50	9.88
	624.20	9.84
	623.30	9.84
	623.10	9.84
	623.70	9.84
	625.60	9.85
	629.20	9.85
	631.00	9.86
	632.70	9.82
Mean	625.81	9.85
SD	3.65	0.01
%RSD	0.58	0.14

Decoquinate data delivered a %RSD for the retention times of 0.14% and a %RSD for the peak areas of 0.58%, which are both less than 1%.

A.2.3.7. CONCLUSION

The HPLC method for the simultaneous analysis of artemisone, clofazimine and decoquinate was validated and it was found that this method is reliable and sensitive in determining the different concentrations of the APIs. Tables A.1 to A.25 show the validation parameters determined for the APIs. It was concluded that clofazimine, artemisone and decoquinate can be analysed by this method with all the acceptance criteria met. Decoquinate, however, displayed a little instability while conducting 24 h stability testing as seen in Table A.22. Decoquinate was also the compound with the most data variance when observing the accuracy and precision studies performed (Tables A.7; A.10; A.13 and A.16).

REFERENCES

BIOPHARMACEUTICS COORDINATING COMMITTEE (BPCC). 2001. Guidance for Industry. Bioanalytical method validation. 22p.

CENTER FOR DRUG EVALUATION AND RESEARCH (CDER). 1994. Reviewer guidance: Validation of chromatographic methods. 30p.

CURRENT GOOD MANUFACTURING PRACTICE (CGMP). 2011. Guidance for Industry. Process validation: General principles and practices. 19p.

DE VITO, V., SABA, A., LEE, H., OWEN, H., POAPOLATHEP, A. & GIORGI, M. 2016. Detection and quantification of the selective EP4 receptor antagonist CJ-023423 (grapiprant) in canine plasma by HPLC with spectrofluorimetric detection. *Journal of pharmaceutical and biomedical analysis*, 118:251-258.

ERMER, J. & MILLER, J.H., *eds*. 2005. Method validation in pharmaceutical analysis: A guide to best practice. Weinheim: Wiley-VCH Verlag. 403p.

HUBER, L. 2015. Validation of analytical methods. Agilent Technologies. http://www.fws.gov/aah/PDF/QI-Terms%20and%20Defs.pdf Date of access: 20 May 2015.

INTERNATIONAL CONFERENCE ON HARMONISATION (ICH) of technical requirements for registration of pharmaceuticals for human use. 2005. Validation of analytical procedures: Text and methodology Q2(R1). 17p.

McPOLIN, O. 2009. Validation of analytical methods for pharmaceutical analysis. Warren Point, UK: Mourne training services. 141p.

SHABIR, G.A. 2004. A practical approach to validation of HPLC methods under current good manufacturing practices. *Journal of validation technology,* 10:210-218.

SINGH, R. 2013. HPLC method development and validation – an overview. *Journal of pharmaceutical education and research,* 4(1):26-33.

SNYDER, L.R., KIRKLAND, J.J. & GLAJCH, J.L. 1997. Practical HPLC method development. 2nd ed. New York: Wiley -Interscience. p. 643-713.

Annexure B

FULL COMPATIBILITY REPORT OF CLOFAZIMINE, ARTEMISONE AND DECOQUINATE WITH VESICLE COMPONENTS

This chapter contains the full isothermal calorimetry report, conducted through thermal activity monitor (TAM) as received by Prof M. Aucamp. A compacted version of this data is reflected in Annexure C.

COMPATIBILITY STUDY OF ARTEMISONE, CLOFAZIMINE AND DECOQUINATE IN COMBINATION WITH CHOLESTEROL, PHOSPHATIDYL CHOLINE AND TWEEN® 20

Requested by:	Ms. Lindi van Zyl	**Date requested:**	August 2016
Company name:	Pharmacen	**Project number:**	TAM/2016/005
Project completed by:	Prof. M.E. Aucamp	**Project name:**	Compatibility study of clofazimine, artemisone and decoquinate in combination with cholesterol, phosphatidyl choline and Tween® 20

Report on compatibility project

1. Introduction

This compatibility study was performed by means of microcalorimetry. Firstly, to explain, *calorimetry* refers to measuring techniques that are used for direct determination of rate of heat production, heat and heat capacity as a function of temperature and time.

Microcalorimetry is a powerful tool for detecting incompatibilities and instabilities between active pharmaceutical ingredients (APIs) and/or excipients. The method of microcalorimetry is a trustworthy way of detecting incompatibilities due to the fact that practically all physical and chemical processes are accompanied by heat exchange. Therefore microcalorimetry is sensitive to all physical and chemical processes associated with heat flow. The high sensitivity of this method makes it possible to carry out measurements at temperatures close to real conditions and to detect very slow reactions. It should be mentioned though that heat flow data will contain contributions from either one process or several processes. To be able to distinguish specific contributions careful experimental planning will be necessary as well as sufficient background pertaining to the sample being analysed.

2. Method of analysis for compatibility

A 2277 Thermal Activity Monitor (TAMIII) (TA Instruments, USA) equipped with an oil bath with a stability of ±100µK over 24 hours was used during this study. The temperature of the calorimeters was maintained at 40°C. For compatibility studies the heat flow is measured for

the single components as well as the mixtures. The calorimetric outputs observed for the individual samples are summed to give a theoretical response. This calculated hypothetical response represents a calorimetric output that would be expected if the two materials do not interact with each other. If the materials interact the measured calorimetric response will differ from the calculated theoretical response.

3. Results

3.1 Combination of artemisone, clofazimine and decoquinate

The combination of the three drugs, artemisone, clofazimine and decoquinate was tested for compatibility. The mixture contained 23.6 mg artemisone, 20.2 mg clofazimine and 19.4 mg decoquinate. The mixture showed that the three compounds are compatible with one another with and interaction average heat flow of 14.57 µW/g and an interaction error of 17.97 µW/g. The lack of a slope on the interaction curve (Figure 1) is also indicative that no interaction between the three drugs exist. This signifies that there is only an insignificant difference between the measured heat flow and the theoretically calculated heat flow.

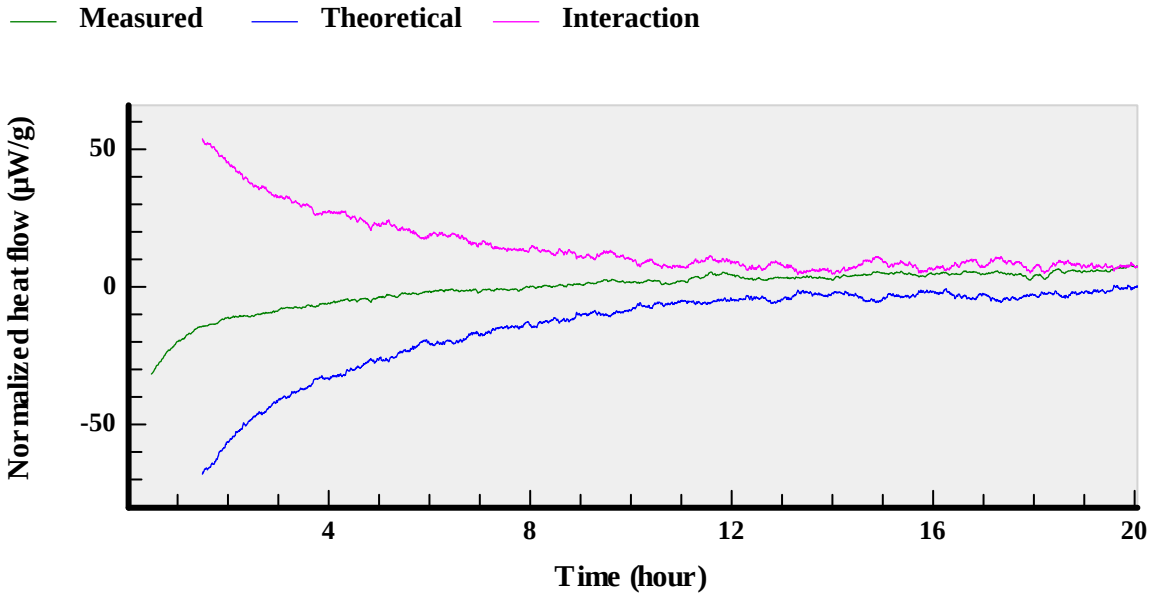

Figure 1: Heat flow *versus* time graph obtained for a combination of artemisone, clofazimine and decoquinate.

3.2 Artemisone in combination with phosphatidylcholine

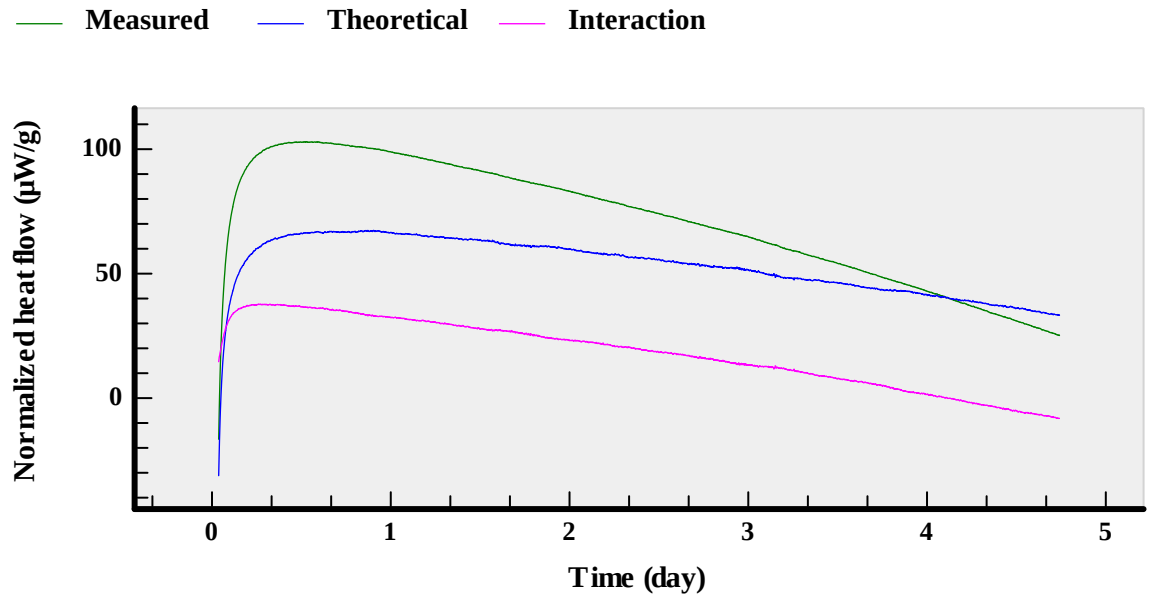

Figure 2: Graph depicting the heat flow data of artemisone combined with phosphatidylcholine.

Figure 2 depicts the heat flow *versus* time data obtained for artemisone in combination with phosphatidylcholine. The combination ratio was 21.7 mg artemisone and 307.2 mg phosphatidylcholine. The temperature was kept isothermal at 40°C. The average interaction heat flow was determined to be 18.19 ± 22.68 µW/g. From Figure 2 it is evident that there exist a difference between the measured heat flow and the theoretically calculated heat flow calculated from the heat flow of the individual compounds. This shows that an incompatibility between the combined compounds exists. This might be ascribed to the oxidative characteristic of phosphatidylcholine.

3.3 Clofazimine and phosphatidylcholine

Figure 3 depicts the heat flow data obtained with the combination of clofazimine and phosphatidylcholine. The combination contained 20.2 mg clofazimine and 307.2 mg phosphatidylcholine. The average interaction heat flow was calculated to be 32.71 ± 33.66 µW/g. The difference between the heat flow obtained during the actual measurement and the theoretical calculated heat flow obtained from the individual heat flow curves indicate that an incompatibility between clofazimine and phosphatidylcholine exist. Phosphatidylcholine is known to be oxidative in nature and this might cause the incompatibility. The difference between the heat flow curves is not as significant, though and it is also less significant than that observed with the combination of artemisone and phosphatidylcholine. This might be due to the fact that clofazimine is characterised as a redox drug, thereby the oxidation of phosphatidylcholine could possibly be minimised.

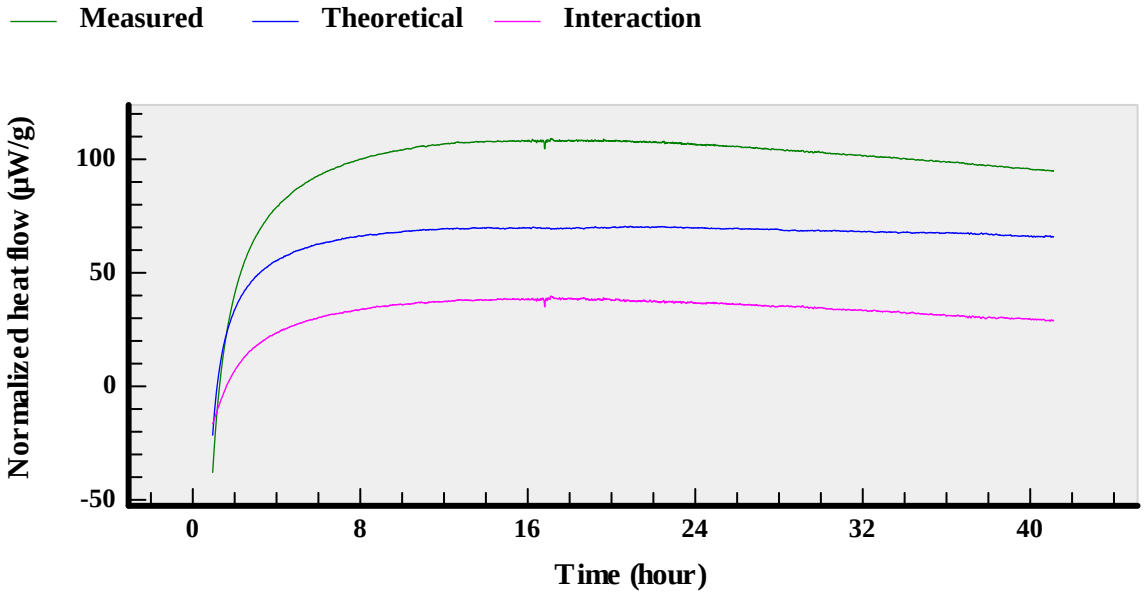

Figure 3: Heat flow *versus* time graph obtained for a combination of clofazimine and phosphatidylcholine.

3.4 Decoquinate and phosphatidylcholine

The compatibility of decoquinate and phosphatidylcholine was tested at 40°C with a mixture of 19.5 mg decoquinate and 198.9 mg phosphatidylcholine. The interaction average heat flow between the two compounds was calculated to be 79.89 ± 80.20 µW/g. The interaction curve shows that no incompatibility between decoquinate and phosphatidylcholine exist (Figure 4).

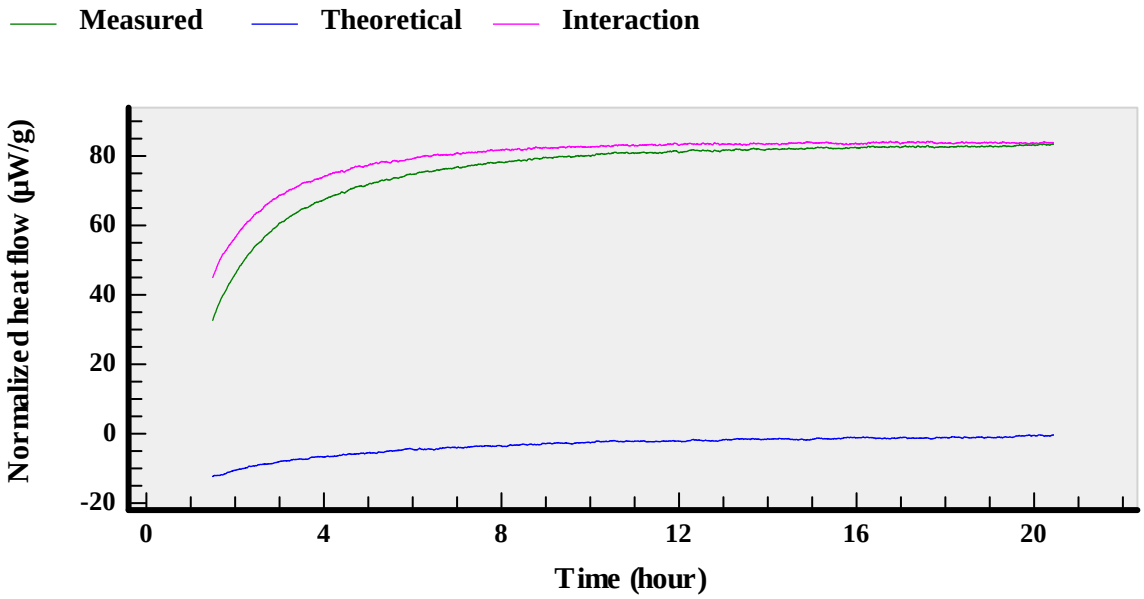

Figure 4: Heat flow data obtained for decoquinate and phosphatidylcholine.

3.5 Artemisone, clofazimine and decoquinate in combination with phosphatidylcholine

The combination of all three drugs in combination with phosphatidylcholine was tested for compatibility. The interaction average heat flow was calculated to be 34.36 µW/g and the interaction error 36.25 µW/g. From the results it is difficult to clearly identify compatibility of the four compounds. A difference can be seen from the interaction graph between the physically measured and the theoretically calculated heat flow. The fact that the difference between the two curves is larger than 100 nW/g shows that the four compounds do have a degree of incompatibility. This could also be explained due to the fact that both artemisone and clofazimine shows incompatibility with phosphatidylcholine.

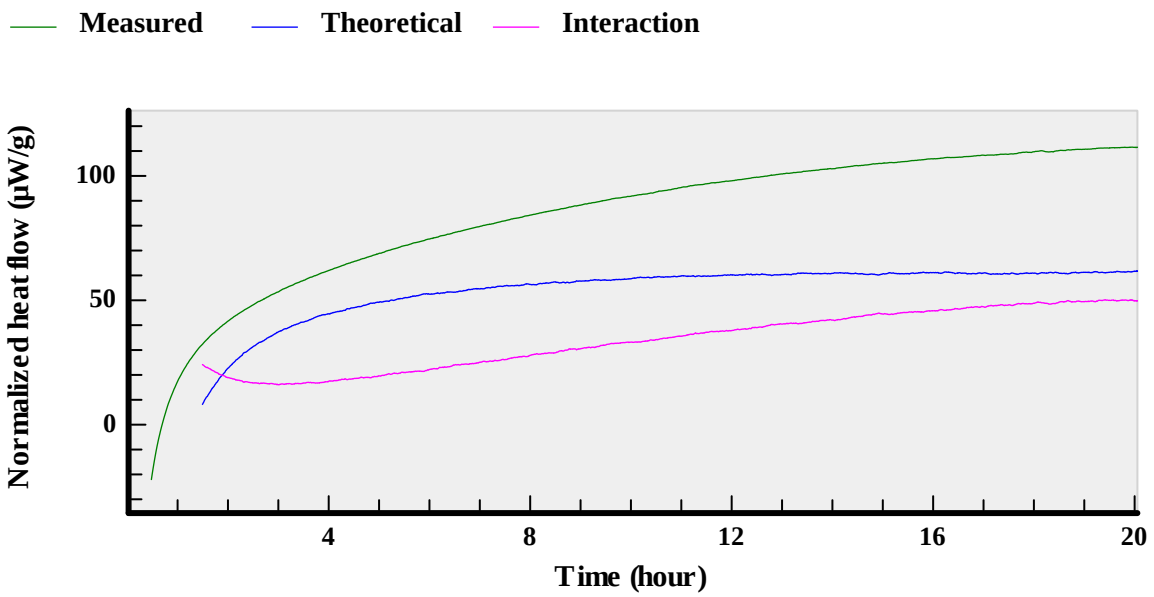

Figure 5: Heat flow data obtained for artemisone, clofazimine, decoquinate and phosphatidylcholine.

3.6 Artemisone in combination with cholesterol

Artemisone in combination with cholesterol was tested at 40°C and a combination of 203.6 mg cholesterol and 20.2 mg artemisone was used. Figure 6 shows the heat flow data obtained for this combination over time. The average interaction heat flow was calculated to be -15.71 ± 19.06 µW/g, which indicate that some degree of interaction exist between artemisone and cholesterol. Although this is not a significant interaction it should be noted that it might be enhanced when artemisone and cholesterol is combined with other compounds.

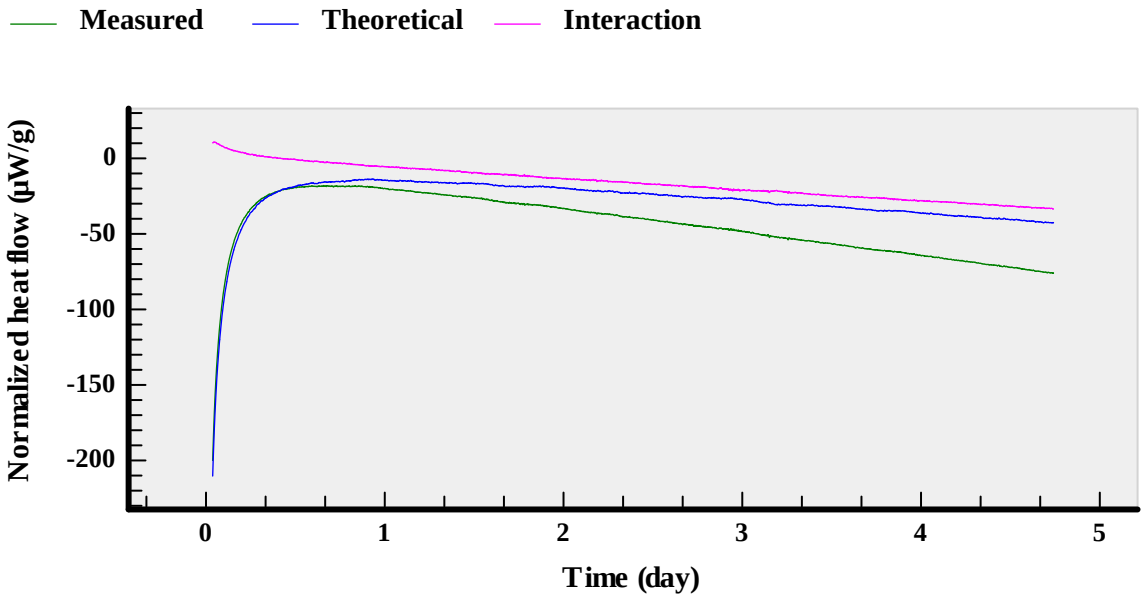

Figure 6: Heat flow *versus* time graph obtained for a combination of artemisone and cholesterol.

3.7 Clofazimine in combination with cholesterol

The compatibility of clofazimine with cholesterol was tested with a combination of the two compounds containing 20.8 mg clofazimine and 201.1 mg cholesterol. The interaction average heat flow was calculated to be 6.25 µW/g and the interaction error was determined to be 6.73 µW/g. The deviation between the measured heat flow and the theoretically calculated heat flow is considered to be insignificant and therefore it can be concluded that the two compounds are compatible.

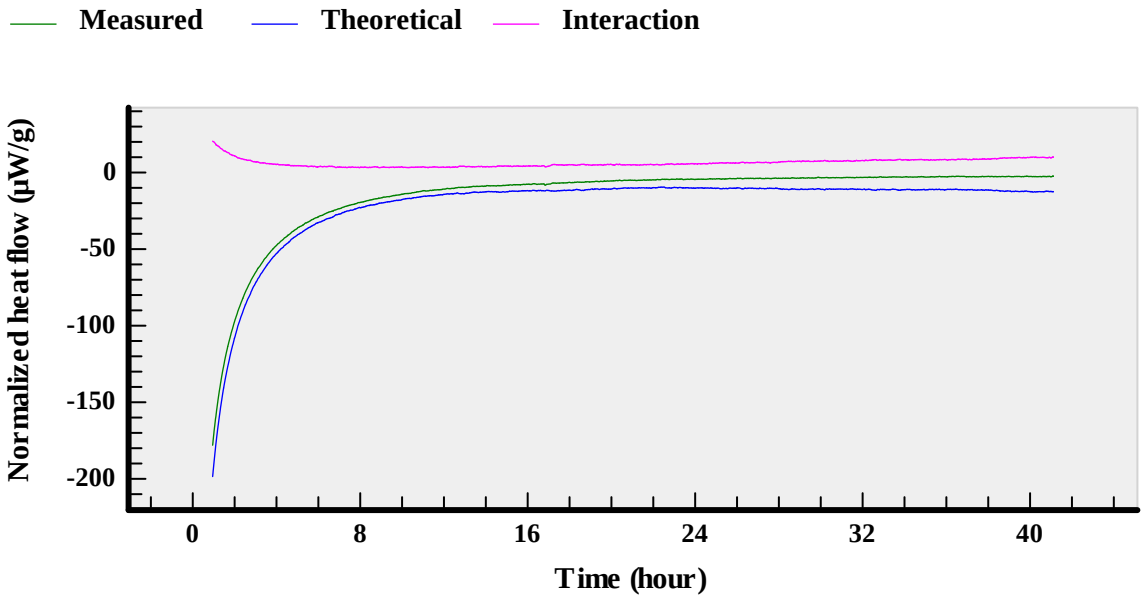

Figure 7: Heat flow *versus* time graph obtained for a combination of clofazimine and cholesterol.

3.8 Decoquinate and cholesterol

A combination of 24.9 mg decoquinate and 198.4 mg cholesterol was tested for compatibility. The average interaction heat flow with interaction error was calculated to be 13.81 ± 18.73 µW/g. Figure 8 shows a very small difference between the actual measured and theoretical calculated heat flow, thereby indicating that the two components are compatible with one another.

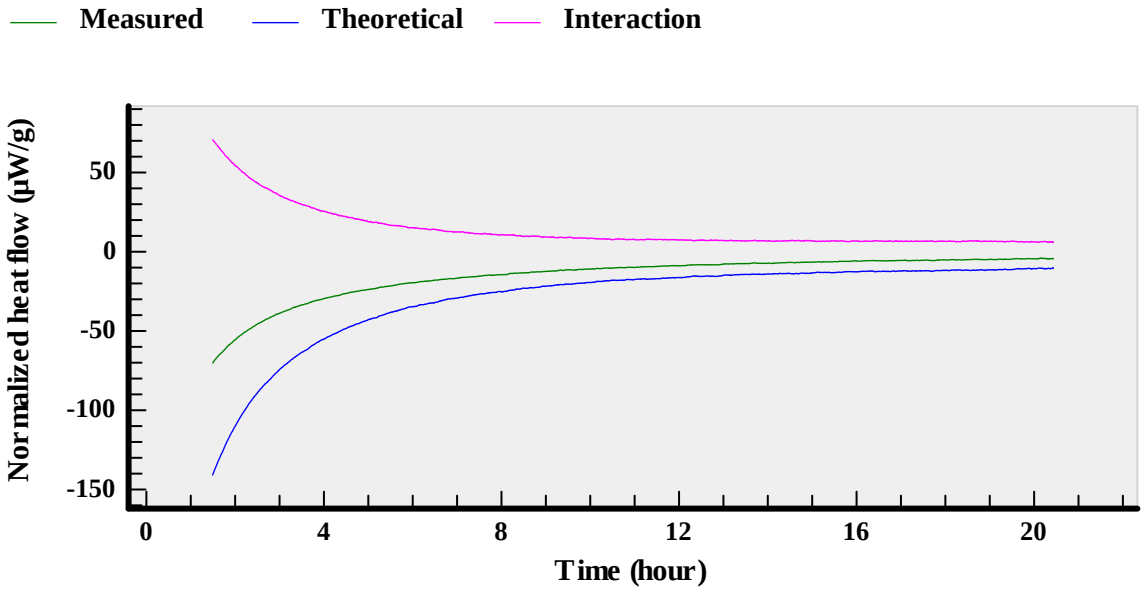

Figure 8: Heat flow data obtained for decoquinate and cholesterol.

3.9 Artemisone, decoquinate and clofazimine in combination with cholesterol

The combination of artemisone, decoquinate, clofazimine and cholesterol was tested for compatibility. The mixture contained 154.3 mg cholesterol, 24.5 mg artemisone, 20.5 mg decoquinate and 19.2 mg clofazimine. The interaction average heat flow was calculated to be 11.44 µW/g and the interaction error was determined to be 12.76 µW/g. From Figure 9 it can also be clearly seen that the deviation between the measured and theoretically measured heat flow is minimal and therefore the four compounds are compatible with one another.

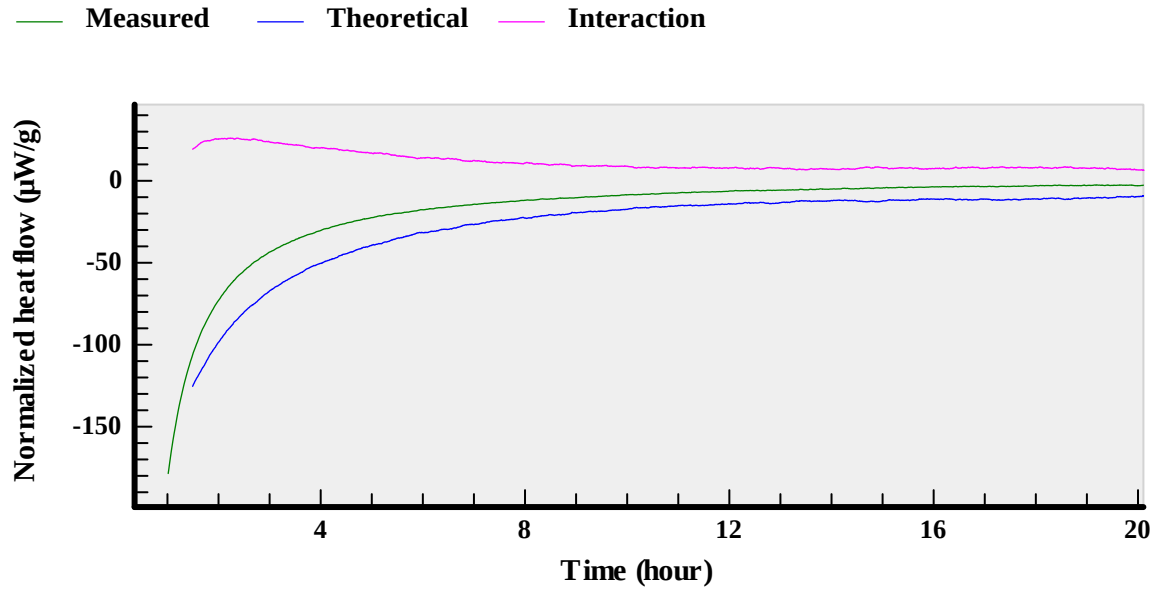

Figure 9: Heat flow data obtained for artemisone, decoquinate, clofazimine and cholesterol.

3.10 Phosphatidylcholine and cholesterol

Phosphatidylcholine and cholesterol were tested for compatibility with one another. The mixture consisted of 186.0 mg phosphatidylcholine and 181.8 mg cholesterol. The interaction average heat flow was calculated to be 3.68 µW/g and the interaction error 26.2 µW/g. The difference between the measured heat flow and the theoretically calculated heat flow seems to be significant due to the upwards slope both curves follow. However, upon looking at the graph, Figure 10, it is apparent that the difference between the 2 curves does not signify an incompatibility.

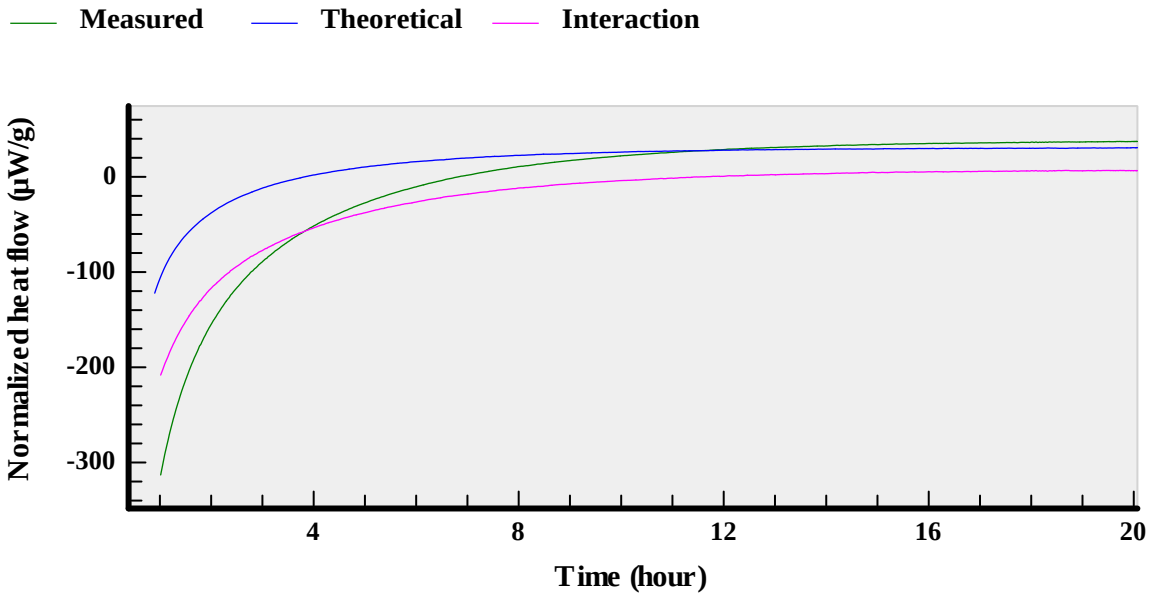

Figure 10: Heat flow data obtained for phosphatidylcholine and cholesterol.

3.11 Artemisone and Tween® 20

The combination of artemisone and Tween® 20 was tested for compatibility. A combination of 20.4 mg artemisone and 330.8 mg Tween® 20 was tested. The difference between the measured heat flow and the theoretically calculated heat flow showed that an incompatibility exist between artemisone and Tween® 20. The average interaction heat flow was measured to be -7.99 µW/g and the interaction error was determined to be 8.34 µW/g.

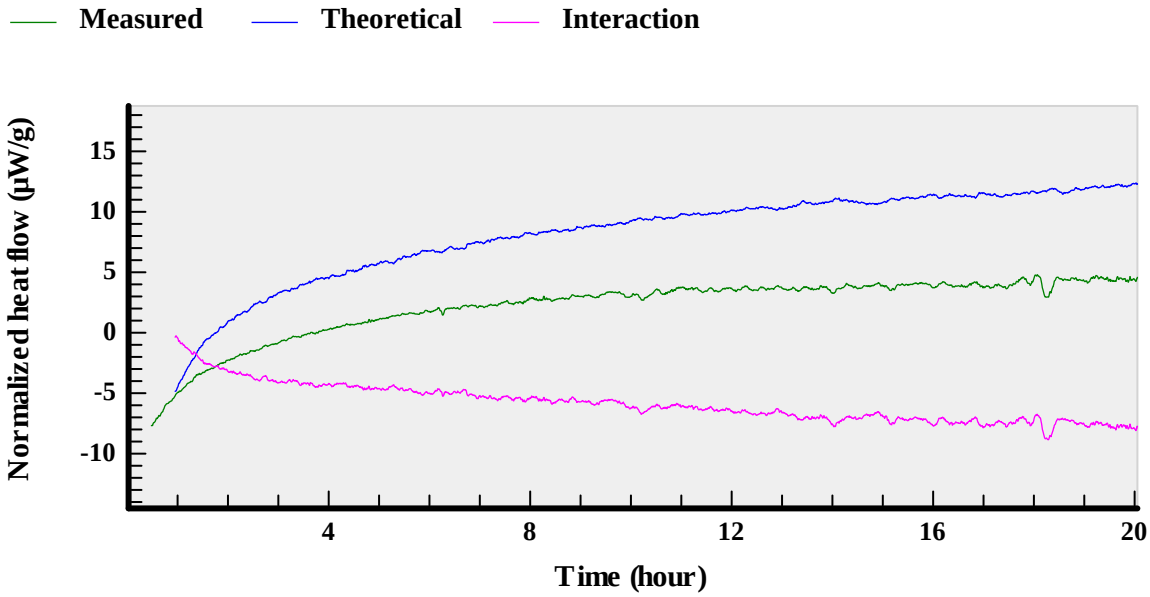

Figure 11: Heat flow data obtained for artemisone and Tween® 20.

3.12 Clofazimine and Tween® 20

The combination of clofazimine and Tween® 20 was investigated for compatibility. The combination contained 334.2 mg Tween® 20 and 20.6 mg clofazimine. The interaction average heat flow was calculated to be -11.56 µW/g and the interaction error was calculated to be 12.03 µW/g. From the heat flow graph (Figure 12) one can see the deviation between the measured heat flow and the theoretical heat flow is significant and therefore the two compounds are not compatible with one another.

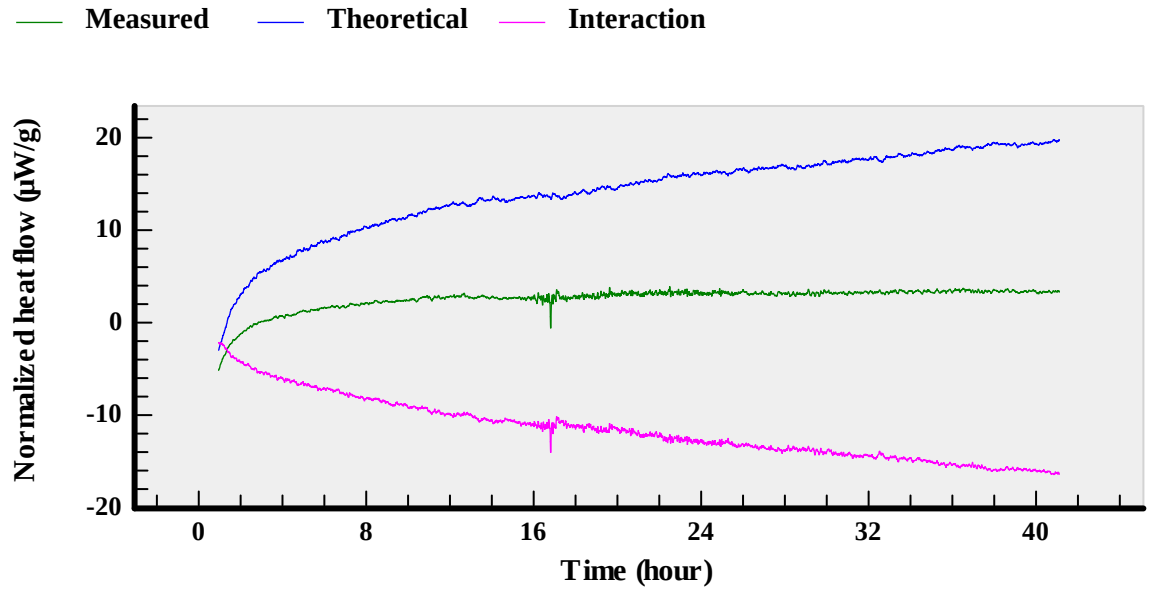

Figure 12: Heat flow data obtained for clofazimine and Tween® 20.

3.13 Decoquinate and Tween® 20

The combination of decoquinate and Tween® 20 was tested with a combination of 19.9 mg decoquinate and 338.3 mg Tween® 20. From Figure 13 it is evident that an incompatibility exists between the two compounds. This can be seen from the difference between the measured heat flow and the theoretically calculated heat flow. An interaction average heat flow with interaction error of -85.15 ± 86.80 µW/g was measured.

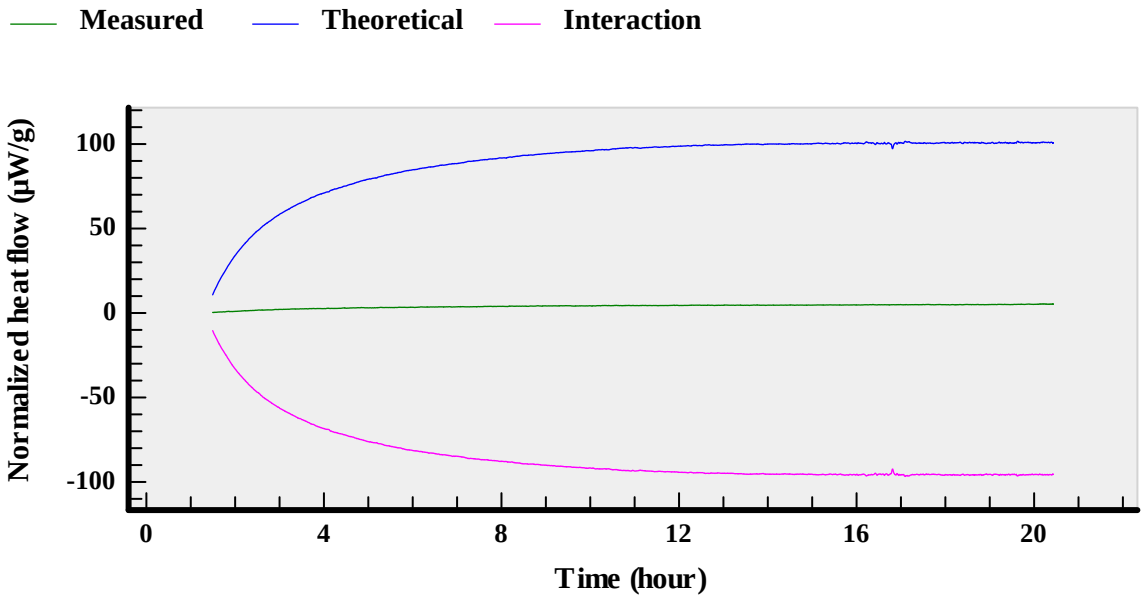

Figure 13: Heat flow data obtained for decoquinate and Tween® 20.

3.14 Phosphatidylcholine and Tween® 20

The compatibility of phosphatidylcholine and Tween® 20 was tested. The combination contained 316 mg phosphatidylcholine and 336.8 mg Tween® 20. A very small difference between the two compounds was identified (-1.49 µW/g) with an interaction error of 4.14 µW/g. The heat flow graph is depicted in Figure 14. From the data it can be concluded that little to no incompatibility exist between phosphatidylcholine and Tween® 20.

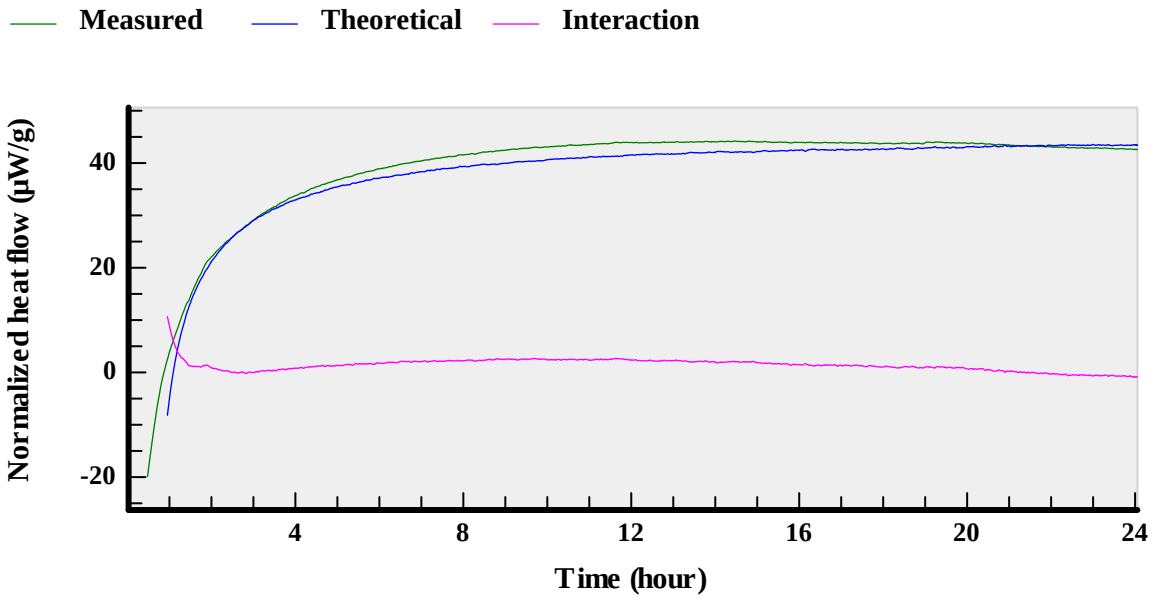

Figure 14: Heat flow data obtained for phosphatidylcholine and Tween® 20.

3.15 Combination of Tween®20 and cholesterol

The combination of Tween®20 and cholesterol was tested for compatibility. An interaction average heat flow of 379.97 nW/g and an interaction error of 3.43 µW/g were calculated. From the interaction graph presented in Figure 15 it is also clear that no incompatibility exist between the two compounds.

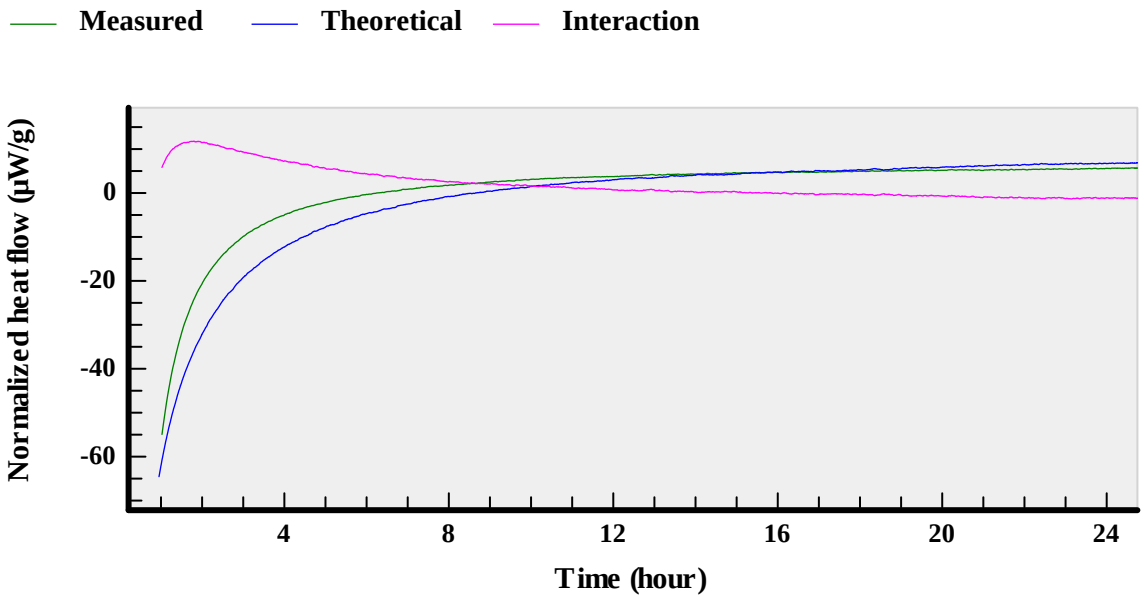

Figure 15: Heat flow data obtained for Tween®20 and cholesterol.

3.16 Decoquinate, artemisone and Tween®20

A combination of decoquinate, artemisone and Tween®20 has been tested for compatibility. The combination contained 20.4 mg decoquinate, 19.5 mg artemisone and 336.7 mg Tween®20. The interaction average heat flow was calculated to be -5.89 ± 6.26 µW/g. Although the difference between the measured and theoretically calculated heat flow is not that significant, the difference still signifies incompatibility between the three components of the mixture (Figure 16). This correlates with the compatibility data obtained with decoquinate in combination with Tween®20.

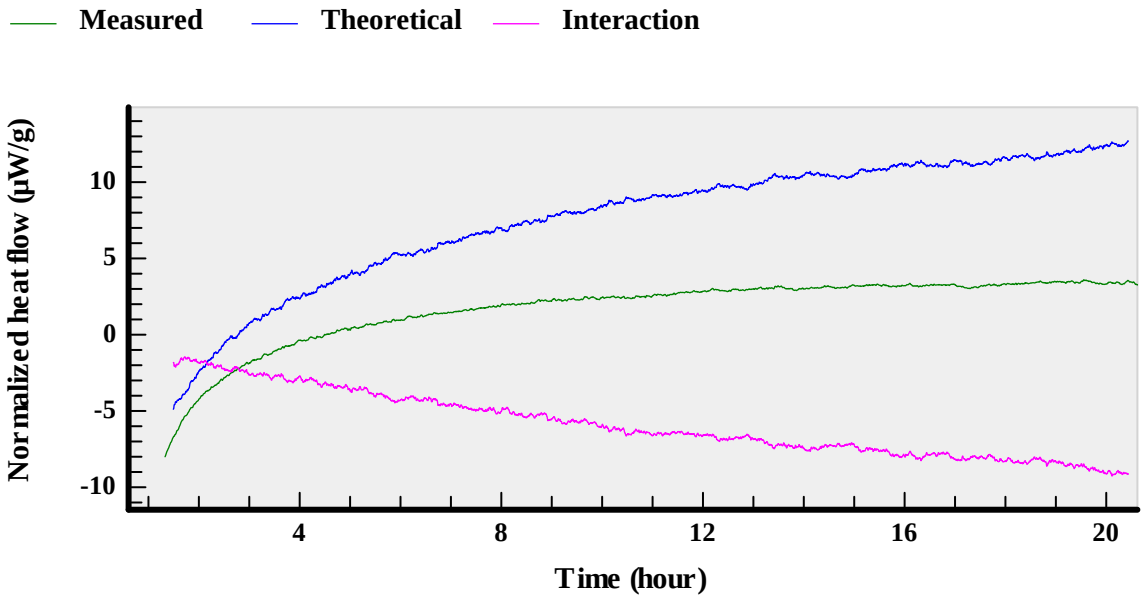

Figure 16: Heat flow data obtained for decoquinate, artemisone and Tween®20.

3.17 Decoquinate, clofazimine and Tween®20

The combination of decoquinate, clofazimine and Tween®20 was investigated for compatibility. The combination contained 19.8 mg decoquinate, 22.9 mg clofazimine and 340.4 mg Tween®20. The interaction average heat flow was calculated to be -7.03 ± 7.85 µW/g. The difference between the measured and theoretically calculated heat flow curve shows that some form of incompatibility exists (Figure 17).

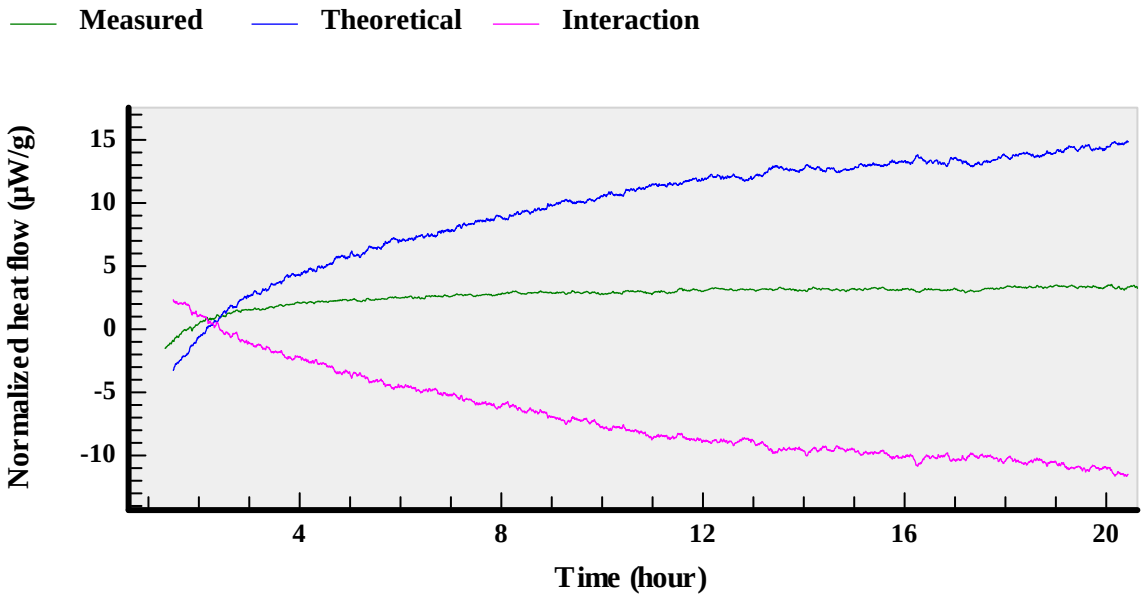

Figure 17: Heat flow data obtained for decoquinate, clofazimine and Tween®20.

3.18 Artemisone, clofazimine and Tween®20

The compatibility of artemisone, clofazimine and Tween®20 was tested. The combination contained 20.7 mg artemisone, 19.5 mg clofazimine and 333.6 mg Tween®20. The interaction average heat flow was calculated to be -10.82 ± 11.17 µW/g. The difference between the physically measured and theoretically determined heat flow shows that some incompatibility exists between the three components.

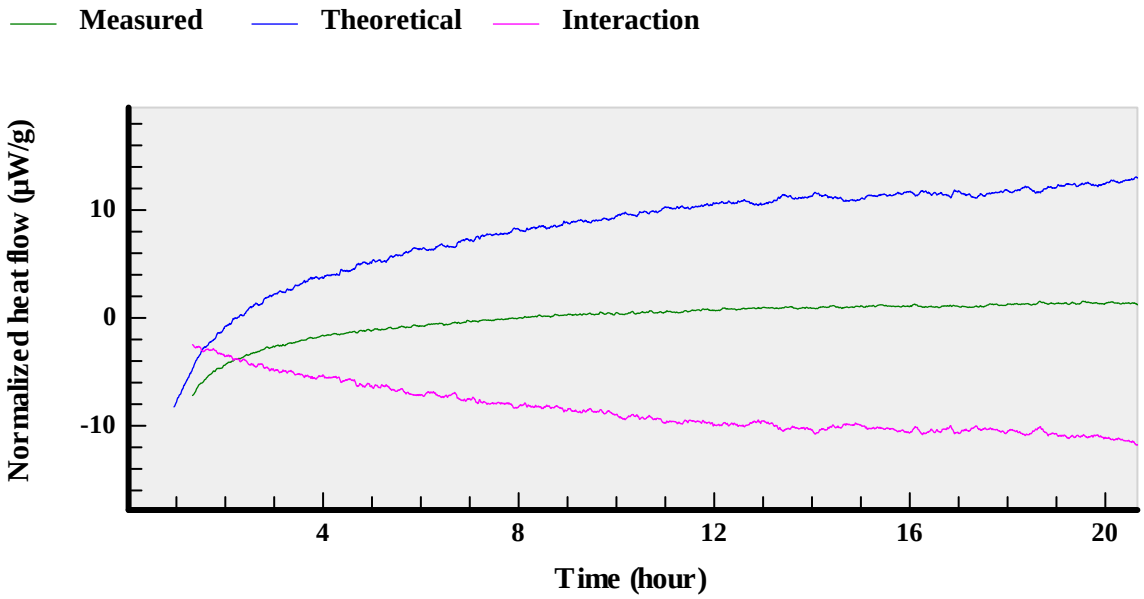

Figure 18: Heat flow data obtained for artemisone, clofazimine and Tween®20.

3.19 Artemisone, clofazimine, decoquinate and Tween®20

The compatibility of artemisone, clofazimine, decoquinate and Tween®20 was tested. For the experiment 22 mg artemisone, 20 mg decoquinate, 19.7 mg clofazimine and 330.9 mg Tween®20 was used. The interaction average heat flow was determined to be -5.47 ± 6.03 µW/g. The difference between the measured and theoretically determined heat flow showed that an incompatibility exist between the four components (Figure 19).

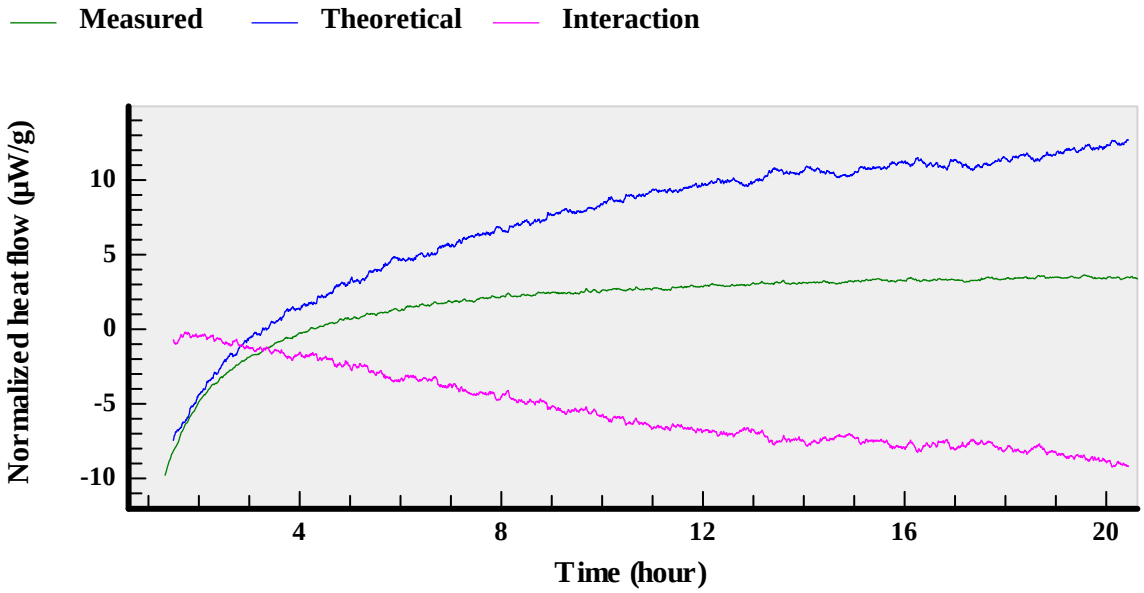

Figure 19: Heat flow data obtained for artemisone, clofazimine, decoquinate and Tween®20.

3.20 Liposomes containing artemisone

Figure 20 shows the interaction heat flow data obtained for a combination ratio of artemisone, phosphatidylcholine and cholesterol. An average heat flow of 1.87 ± 2.51 µW/g was calculated. The difference between the measured and theoretical heat flow curves is minimal and therefore it can be concluded that the difference that do exist between the heat flow curves is due to the incompatibilities identified with the combinations of artemisone and phosphatidylcholine and artemisone and cholesterol. It does however look like the fact that the artemisone is combined with phosphatidylcholine and cholesterol in liposomes, minimises the interaction between the compounds.

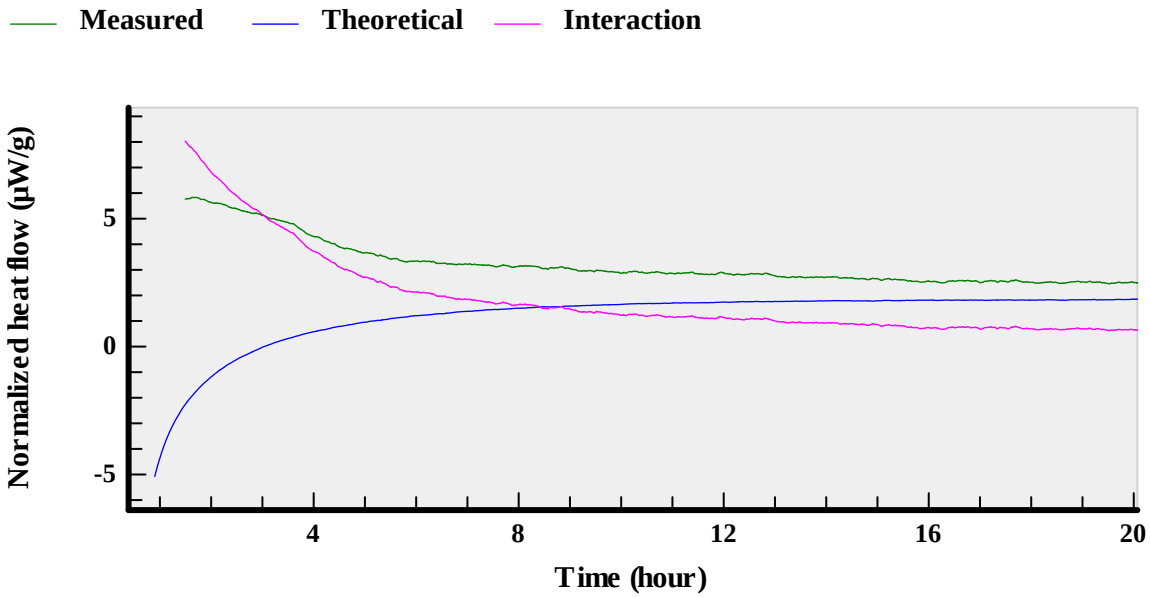

Figure 20: Heat flow *versus* time graph obtained for a combination of artemisone, phosphatidylcholine and cholesterol.

3.21 Liposomes containing clofazimine

The compatibility of clofazimine with phosphatidylcholine and cholesterol when formulated as liposomes was tested. The liposomes contained 4.56 mg clofazimine, 40.12 mg phosphatidylcholine and 59.58 mg cholesterol. The interaction average heat flow was determined to be 21.06 µW/g and the interaction error was determined to be 26.45 µW/g. From the heat flow graph (Figure 21) it can be seen that a deviation do exist between the measured heat flow and the theoretically determined heat flow, however this deviation is not as significant. Therefore it can be concluded that the combination of clofazimine with phosphatidylcholine and cholesterol minimises the incompatibility of clofazimine and phosphatidylcholine. Therefore cholesterol might have a stabilising effect on the combination of clofazimine and phosphatidylcholine.

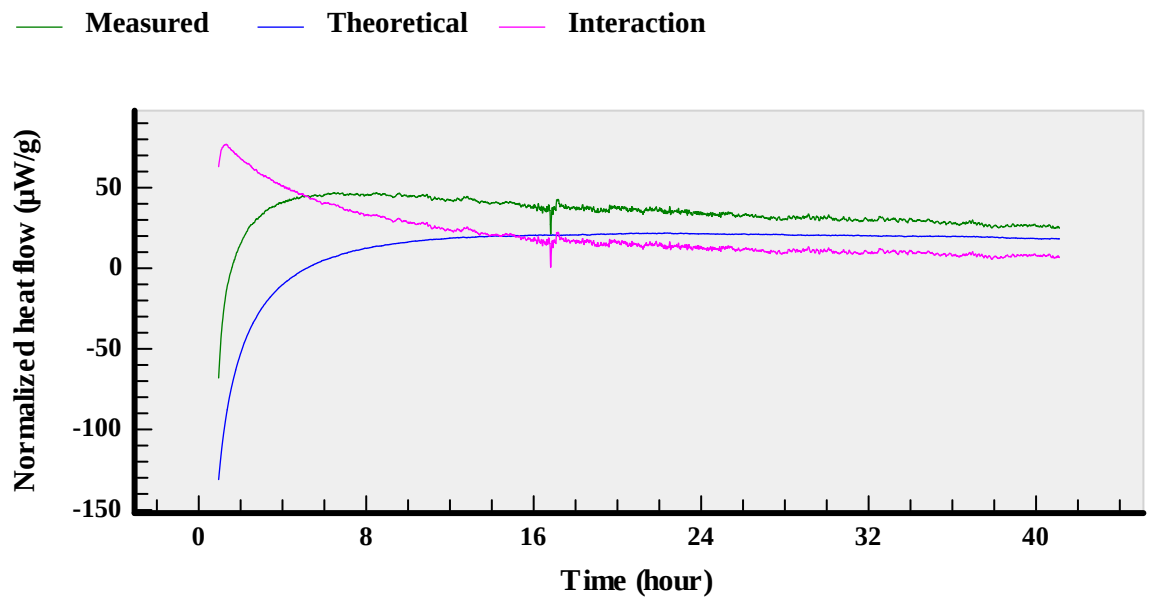

Figure 21: Heat flow *versus* time graph obtained for a combination of clofazimine, phosphatidylcholine and cholesterol.

3.22 Liposomes containing decoquinate

The combination of decoquinate when formulated with phosphatidylcholine and cholesterol as liposomes was tested in terms of compatibility. The formulation consisted of 59.32 mg phosphatidylcholine, 39.40 mg cholesterol and 4.20 mg decoquinate. The interaction average heat flow was calculated to be 250.35 nW/g and the interaction error 1.15 µW/g. From Figure 22 one can deduce that the difference between the two curves (measured and theoretical) is not significant and therefore it can be concluded that the liposomes containing decoquinate do not show incompatibility issues.

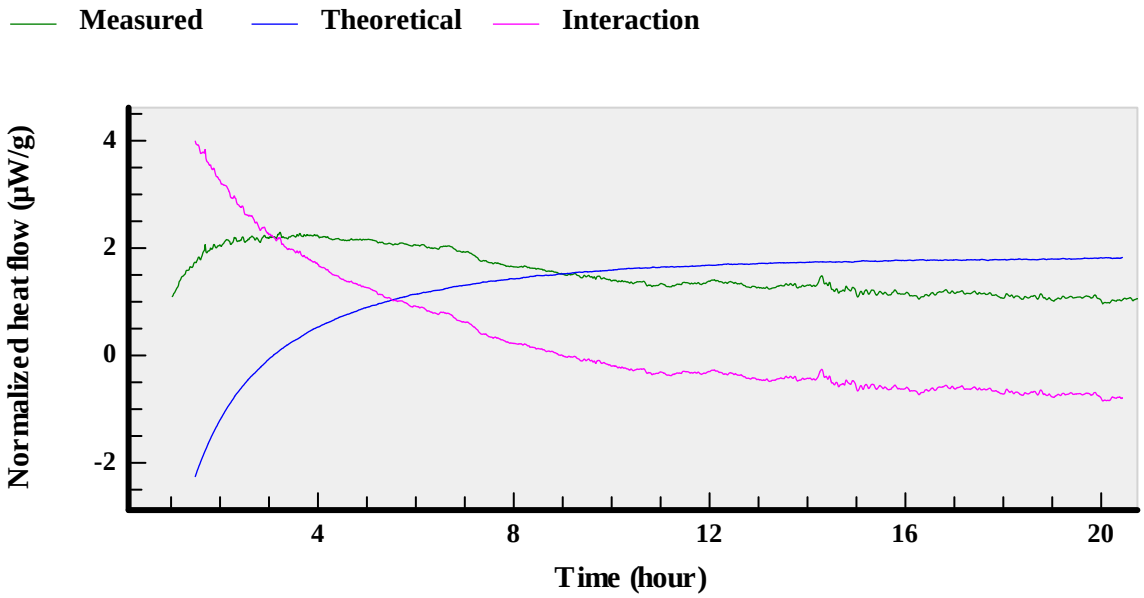

Figure 22: Heat flow *versus* time graph obtained for a combination of decoquinate, phosphatidylcholine and cholesterol.

3.23 Liposomes containing artemisone, decoquinate and clofazimine

A formulation of liposomes containing artemisone, decoquinate and clofazimine were tested for compatibility. The formulation contained 60.18 mg phosphatidylcholine, 39.28 mg cholesterol and 4.1 mg artemisone. From the interaction graph some deviation between the measured and theoretical calculated curves can be identified. However, it is considered to be insignificant and therefore no incompatibility in this formulation was identified (Figure 23). The interaction average heat flow was determined to be 2.28 µW/g and the interaction error 2.86 µW/g.

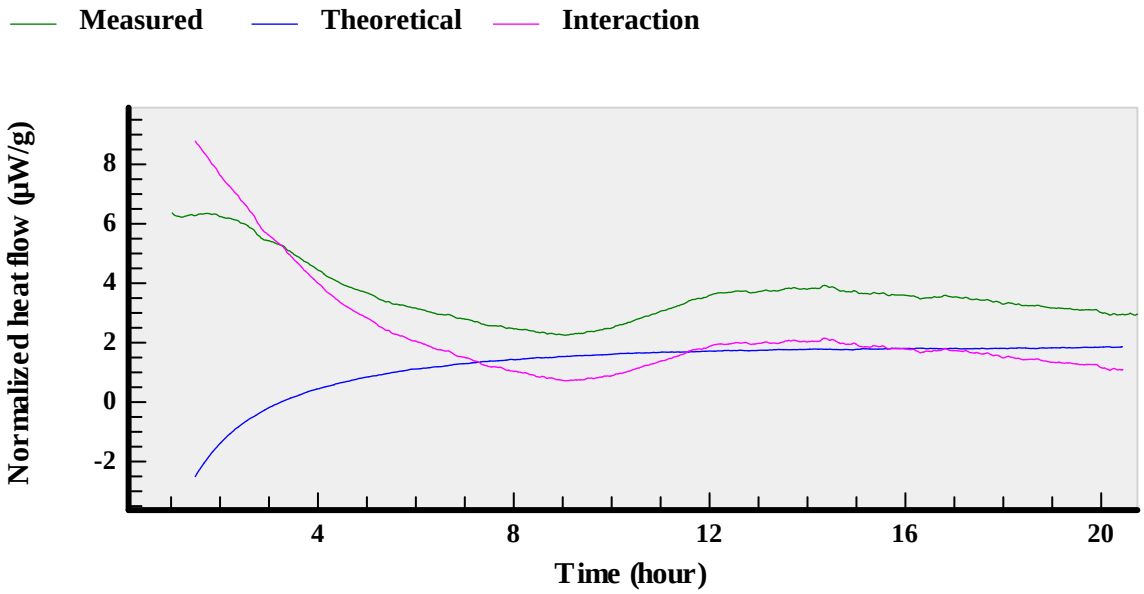

Figure 23: Heat flow *versus* time graph obtained for a combination of decoquinate, phosphatidylcholine and cholesterol when formulated as liposomes.

3.24 Transferosomes containing artemisone

The compatibility of artemisone with phosphatidylcholine when formulated as transferosomes was determined. The combination contained 4.64 mg artemisone and 79.32 mg phosphatidylcholine formulated as transferosomes. The average interaction heat flow was determined to be 653.98 nW/g ± 1.28 µW/g. Although there exists a difference between the measured and theoretical heat flow it is too small to be identified as an incompatibility (Figure 24).

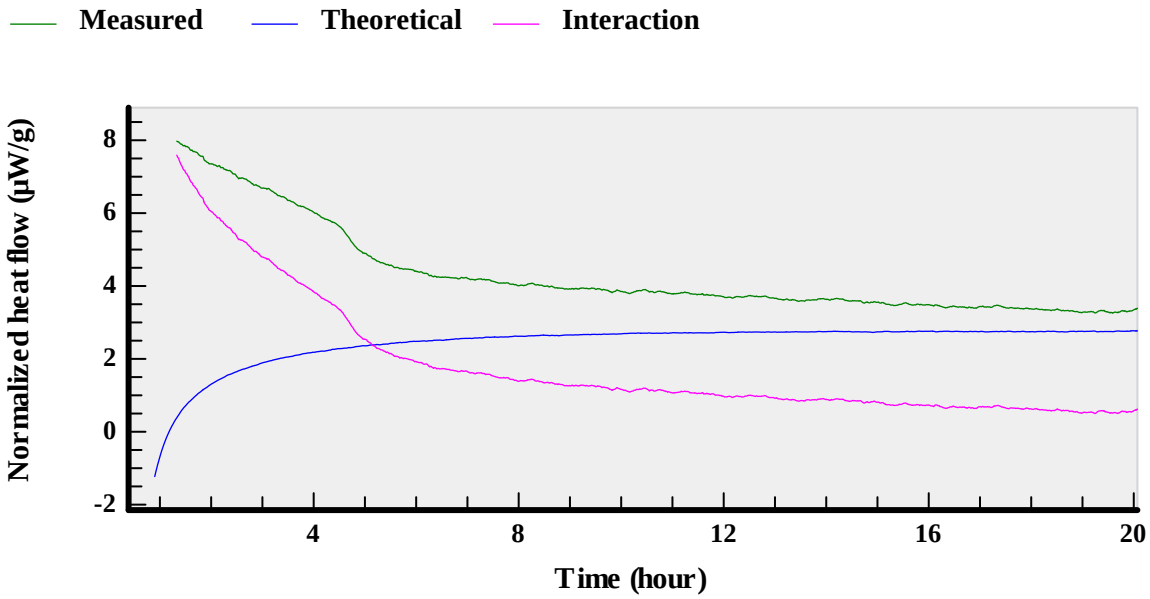

Figure 24: Heat flow data obtained for transferosomes containing artemisone and phosphatidylcholine.

3.25 Transferosomes containing clofazimine

The combination of clofazimine and phosphatidylcholine when combined into transferosomes has been tested. The combination contained 4.26 mg clofazimine and 80.4 mg phosphatidylcholine. The interaction average heat flow was determined to be 724.23 nW/g and the interaction error was calculated to be 2.88 µW/g. The same applies here as for the tranferosomes containing artemisone. There exist a difference in the physically measured and theoretically calculated heat flow, however it is too small to signify an incompatibility (Figure 25).

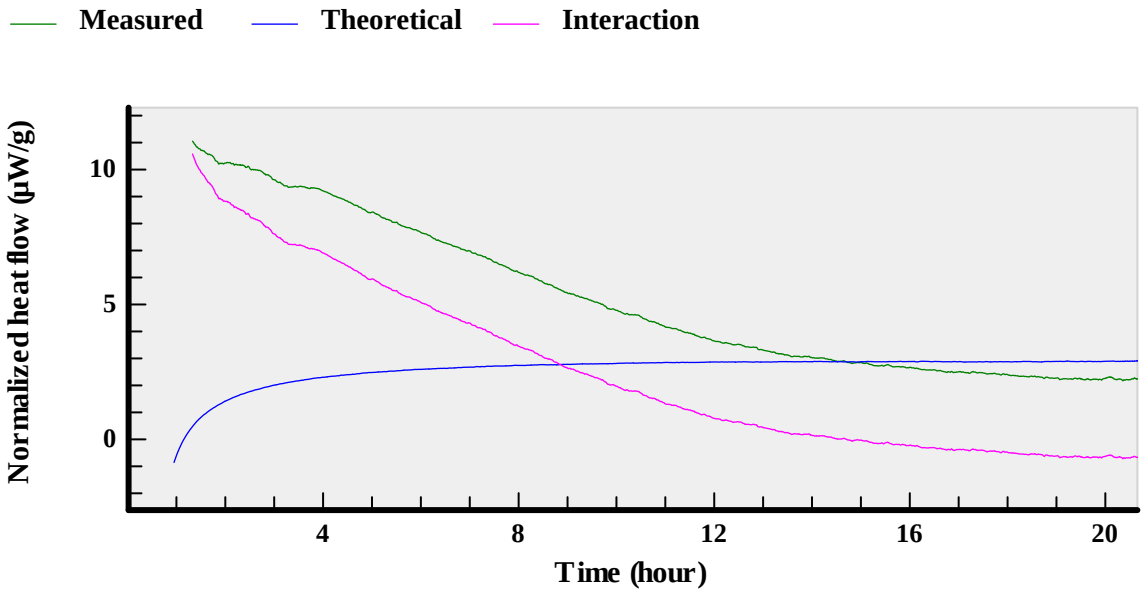

Figure 25: Heat flow data obtained for transferosomes containing clofazimine and phosphatidylcholine.

3.26 Transferosomes containing decoquinate

The combination of decoquinate with phosphatidylcholine formulated as transferosomes have been tested for incompatibility. The interaction average heat flow was calculated to be -42.98 nW/g ± 1.27 µW/g. The measured heat flow correlated very well with the theoretically calculated heat flow and the very small interaction heat flow in the nW-range proves that no incompatibility between decoquinate and phosphatidylcholine when prepared as transferosomes exist (Figure 26). This also correlates very well with the data obtained for decoquinate and phosphatidylcholine when combined within the solid-state.

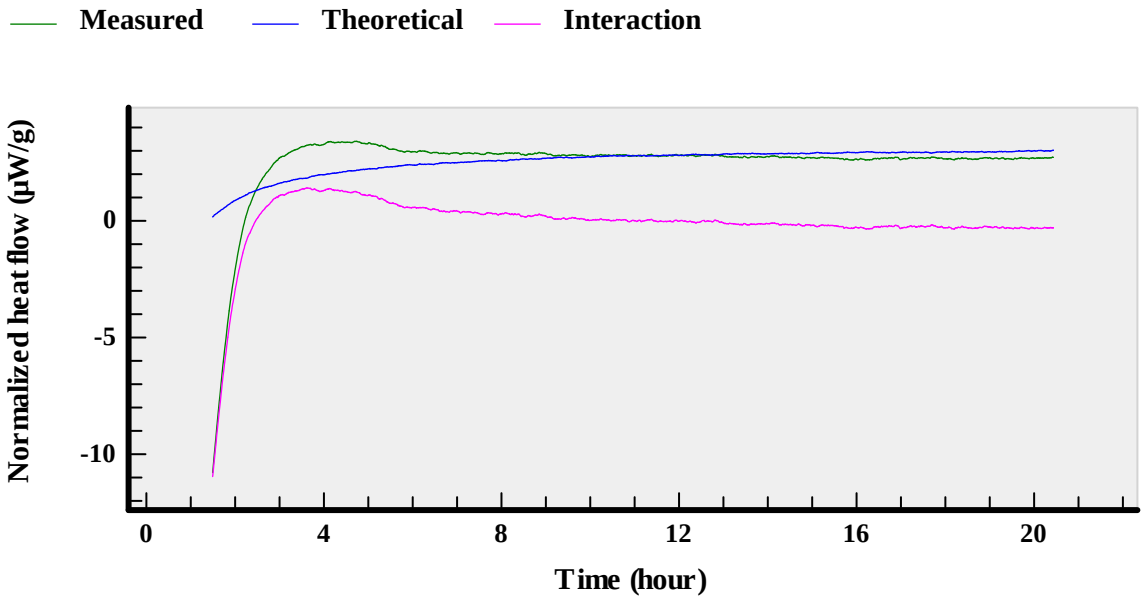

Figure 26: Heat flow data obtained for transferosomes containing decoquinate and phosphatidylcholine.

3.27 Transferosomes containing artemisone, decoquinate and clofazimine

Transferosomes containing artemisone, decoquinate and clofazimine were tested for compatibility. The formulation contained 79.4 mg phosphatidylcholine, 4.6 mg artemisone, 4.58 mg decoquinate and 4.42 mg clofazimine. Figure 27 shows the heat flow graphs obtained for this combination. From all the transferosome combinations this combination showed the greatest difference between the measured and theoretically calculated heat flow curves. The interaction heat flow was calculated to be 4.0 µW/g and the interaction error was determined to be 4.5 µW/g. Although a difference was observed between the measured and theoretical calculated heat flow curves, it was too insignificant to account for an incompatibility. It was therefore concluded that within the transferosome formulation containing all three drugs no incompatibility was observed.

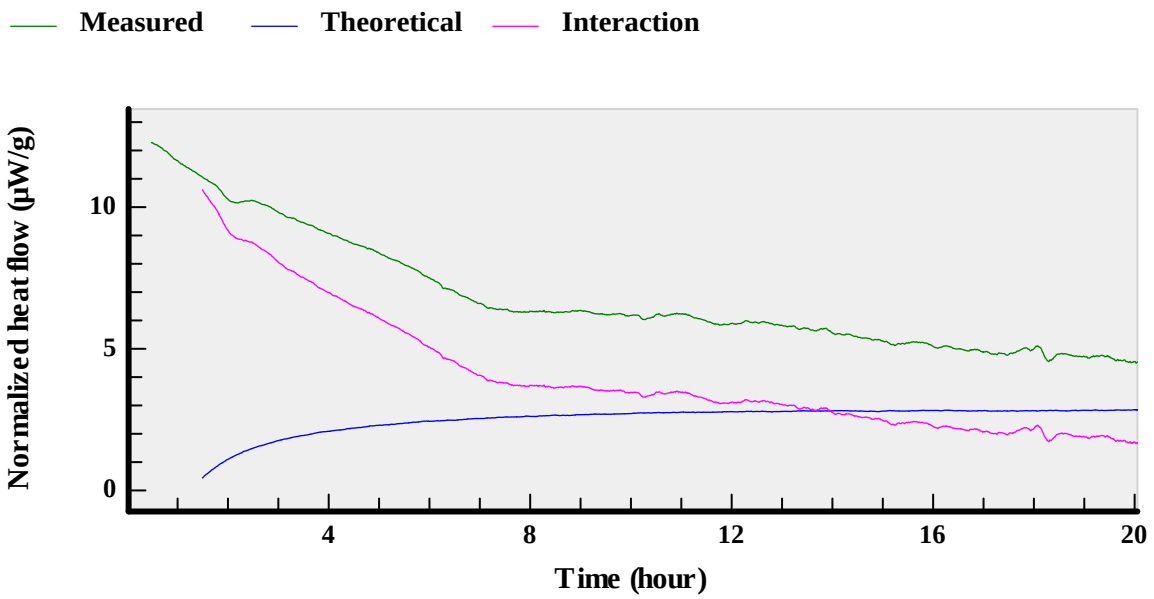

Figure 27: Heat flow data obtained for transferosomes containing artemisone, clofazimine, decoquinate and phosphatidylcholine.

3.28 Niosomes containing artemisone

Niosomes containing artemisone was tested for compatibility. The niosomes tested, contained 66.88 mg Tween®20, 32.78 mg cholesterol and 4.16 mg artemisone. The niosomes were tested against the heat flow data of all the mentioned individual compounds. The calculated heat flow data showed an interaction average heat flow of 748.91 nW/g and an interaction error of 1.25 µW/g. From the curve depicted in Figure 28 it is also clear that the deviation between the measured and theoretically calculated heat flow curves is minimal.

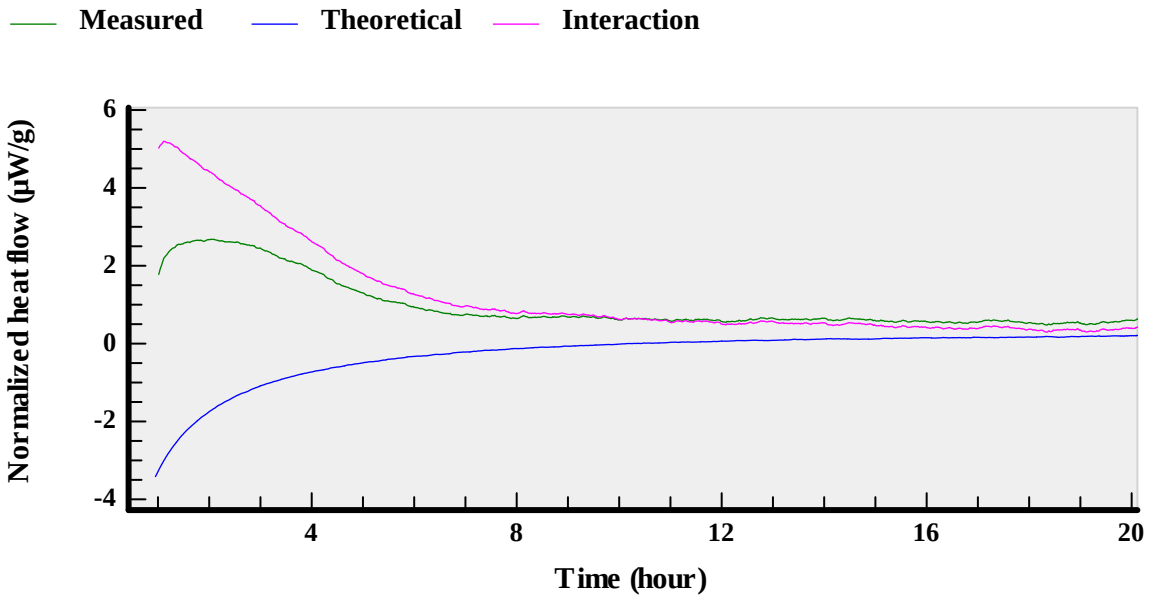

Figure 28: Heat flow data obtained for niosomes containing artemisone.

3.29 Niosomes containing clofazimine

The compatibility of clofazimine when formulated with cholesterol and Tween®20 as niosomes were tested. The formulation was tested against the heat flow of all the individual components. The heat flow data showed that the interaction average heat flow was 140.54 nW/g and the interaction error was 862.53 nW/g. This proved that the formulation of clofazimine in niosomes is stable with no incompatibility issues. Figure 29 also depicts the heat flow data obtained and it is clear that the deviation between the measured and theoretically calculated heat flow curves is insignificant.

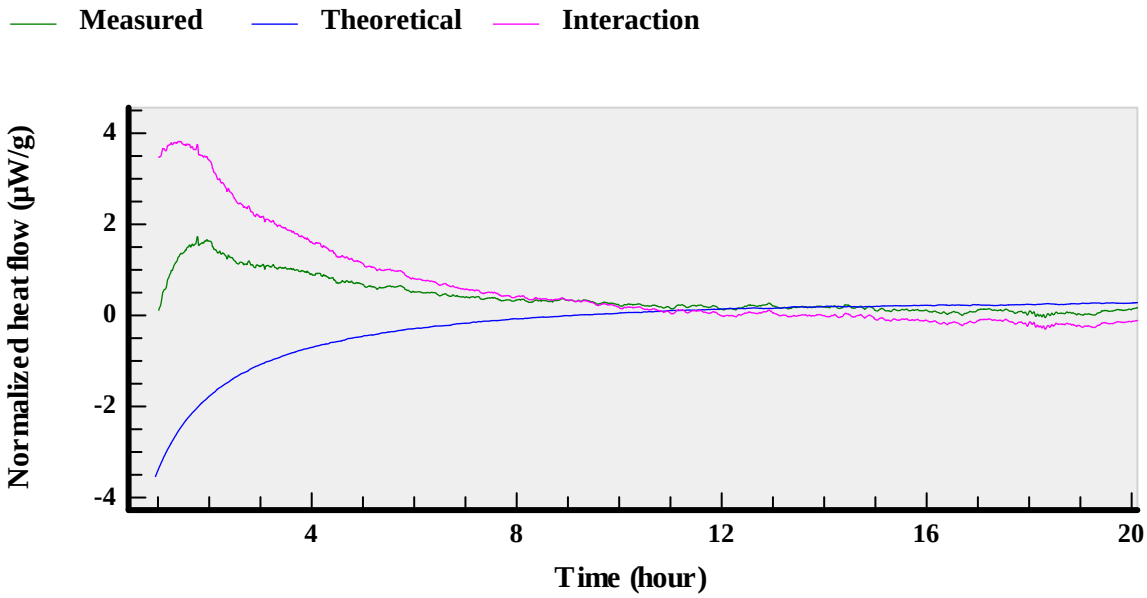

Figure 29: Heat flow data obtained for niosomes containing clofazimine.

3.30 Niosomes containing decoquinate

The combination of Tween®20, cholesterol and decoquinate when formulated as niosomes were tested for compatibility. The formulation contained 75.61 mg Tween®20, 32.9 mg cholesterol and 4.32 mg decoquinate. The interaction average heat flow obtained was 610.13 nW/g and the interaction error 946.94 nW/g. From the data, including the interaction graph, Figure 30, it is apparent that no incompatibility exists between the different components of the niosome formulation.

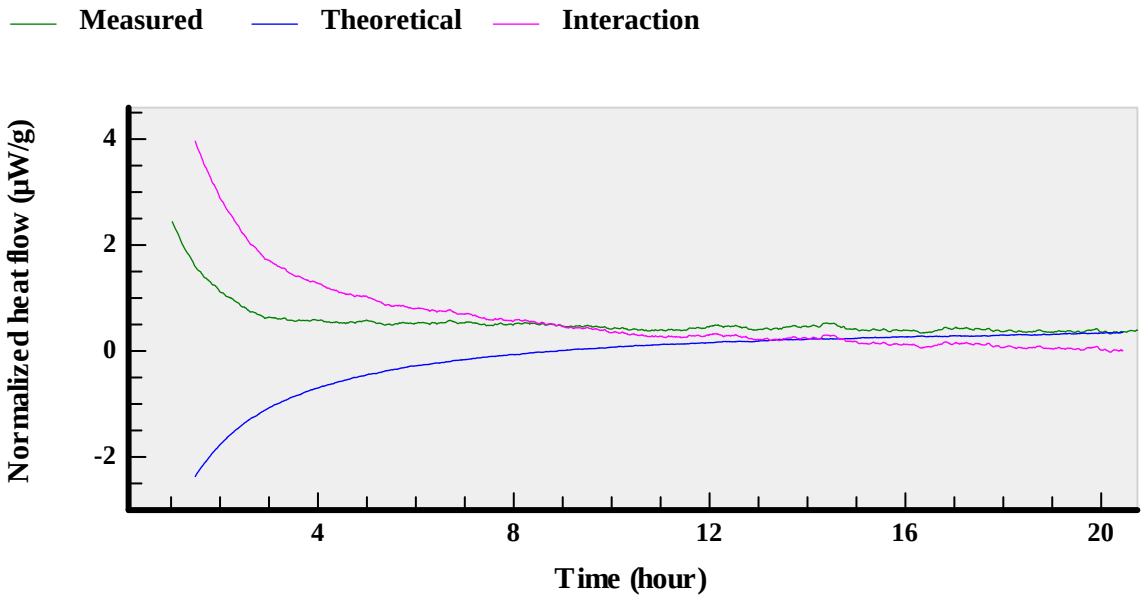

Figure 30: Heat flow data obtained for niosomes containing decoquinate.

3.31 Niosomes containing artemisone, clofazimine and decoquinate

The compatibility of artemisone, clofazimine and decoquinate with Tween®20 and cholesterol when formulated as niosomes were investigated. The heat flow of the niosome formulation was tested against the individual components to establish compatibility. Figure 31 shows the heat flow graph obtained during this experiment.

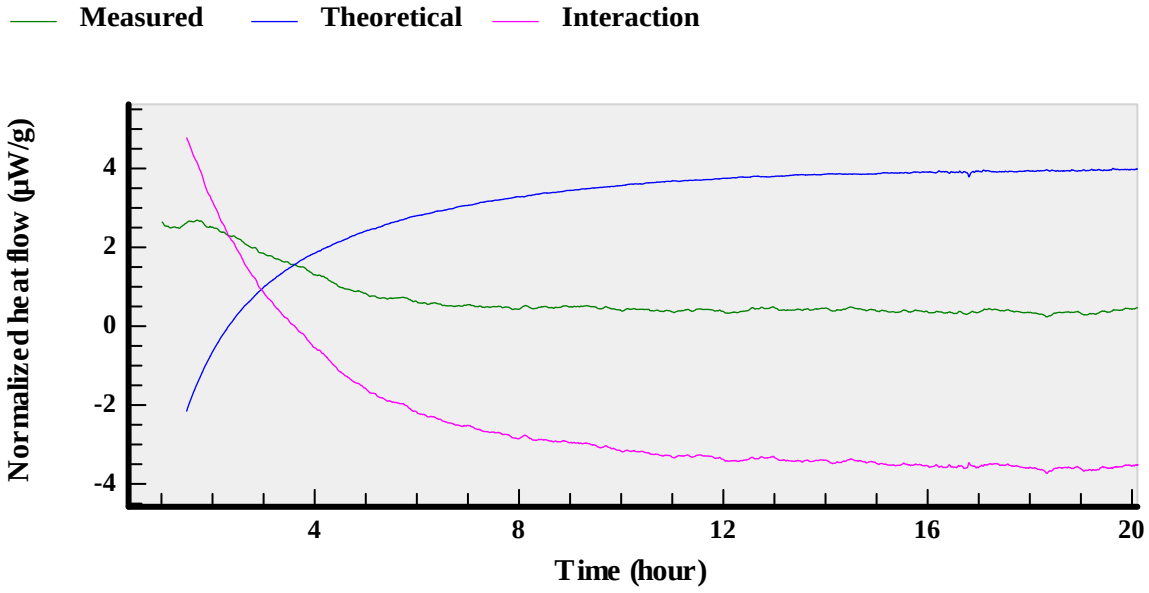

Figure 31: Heat flow data obtained for niosomes containing artemisone, clofazimine and decoquinate.

3.32 CONCLUSION

From this study it became apparent that the three drugs, namely artemisone, clofazimine and decoquinate are compatible with one another within the solid-state form. The combination of the three drugs with phosphatidylcholine showed that artemisone in combination with phosphatidylcholine is incompatible. The compatibility of clofazimine with phosphatidylcholine is somewhat questionable. Decoquinate and phosphatidylcholine were found to be compatible. The combination of all three actives with phosphatidylcholine also showed incompatibility. This isn't really surprising given the fact that phosphatidylcholine and two of the other compounds showed incompatibility.

The combination of artemisone with cholesterol didn't show a definitive incompatibility. However caution must be applied through knowing that a more severe incompatibility might be triggered between the two compounds, either through addition of one or more compound, other formulation excipients or even energy from formulation processes. Compatibility was established between clofazimine and cholesterol, decoquinate and cholesterol, phosphatidylcholine and cholesterol as well as the combination of all three drugs with cholesterol.

It was evident that the compatibility of all three drugs in combination with Tween® 20 will be problematic. Interestingly, the combination of Tween® 20 and phosphatidylcholine didn't show any incompatibility. Cholesterol and Tween® 20 also showed to be compatible. A combination of artemisone, decoquinate and Tween® 20 showed incompatibility, as well as a combination of artemisone, clofazimine and Tween® 20. A combination of clofazimine, decoquinate and Tween® 20 also showed incompatibility. A combination of all three drugs with Tween® 20 also showed incompatibility.

All the liposome formulations proved to be compatible, despite incompatibility of some of the individual combinations. All the transferosome formulations showed compatibility. With the niosomes showing the best compatibility of all the tested formulations.

PROJECT INVESTIGATOR: **REPORT DATE:** 22 March 2017

Annexure C

LIPOSOMES, NIOSOMES AND TRANSFEROSOMES UTILISED FOR TOPICAL DRUG DELIVERY

C.1. INTRODUCTION

During the past few decades the skin as delivery route has become increasingly prevalent, mainly due to the circumvention of the hepatic fist pass effect. Active pharmaceutical ingredients (APIs) can be delivered either topically and/or transdermally via the skin, depending on the treatment strategy. There are, however, also a few obstacles to overcome when formulating for topical application since the skin's primary function is to create a boundary between the body and the surrounding environment, being highly selective as to what will pass through it (Dragicevic-Curic & Maibach, 2015; Tavano *et al.*, 2013). Since skin inhibits the penetration of most compounds, vesicles have been investigated in more detail to increase delivery of APIs to the skin (El Maghraby *et al.*, 2000).

C.2. BACKGROUND

The skin is a complex organ which can mainly be divided into the epidermis, dermis and hypodermis. When focussing on topical delivery of an API, the epidermis is of utmost importance. The stratum corneum (SC) is the outermost layer of the epidermis and is responsible for the barrier function of the skin (Gillet *et al.*, 2011; Tavano *et al.*, 2013). The SC consists of layers of corneocytes, which are non-living cells that have a cornified envelope instead of a plasma membrane. Corneocytes are filled with keratin filaments and are scattered in a lipid-enriched extracellular matrix also containing protein/peptide components. This extracellular matrix, ordered into lamellae parallel to the flat surface of the corneocytes, is the only continuous section in the SC and is therefore likely to be central to the skin's barrier function (Dragicevic-Curic & Maibach, 2015).

For delivery to the skin, an API must adhere to two key rules, namely it must have a molecular mass of less than 500 g/mol and a partition coefficient (log P) of 1–3, though other factors such as solubility, melting point and molecular volume may also influence skin permeation. Tavano *et al.* (2013) established that with vesicles, skin permeation will also be influenced by composition, API solubility in the matrix and viscosity. This confirms that it will be quite a challenge to deliver specific compounds into, and through the skin. For this reason the focus has been placed on the use of delivery systems to promote skin permeation. Delivery systems, such as vesicles (Figure C.1), were chosen for this study, since they are capable of encapsulating both hydrophilic

and lipophilic APIs. Vesicles are colloidal particles with an aqueous compartment surrounded by lipid bilayers (Dragicevic-Curic & Maibach, 2015; Mahale *et al.*, 2012). Vesicles mostly create a "depot-effect" due to their lipophilicity and significantly higher API concentrations have been reached at target organs (Jain *et al.*, 2014; Kaur & Singh, 2014). There are currently a variety of vesicles being studied, but for this study focus will be placed on liposomes, niosomes and transferosomes.

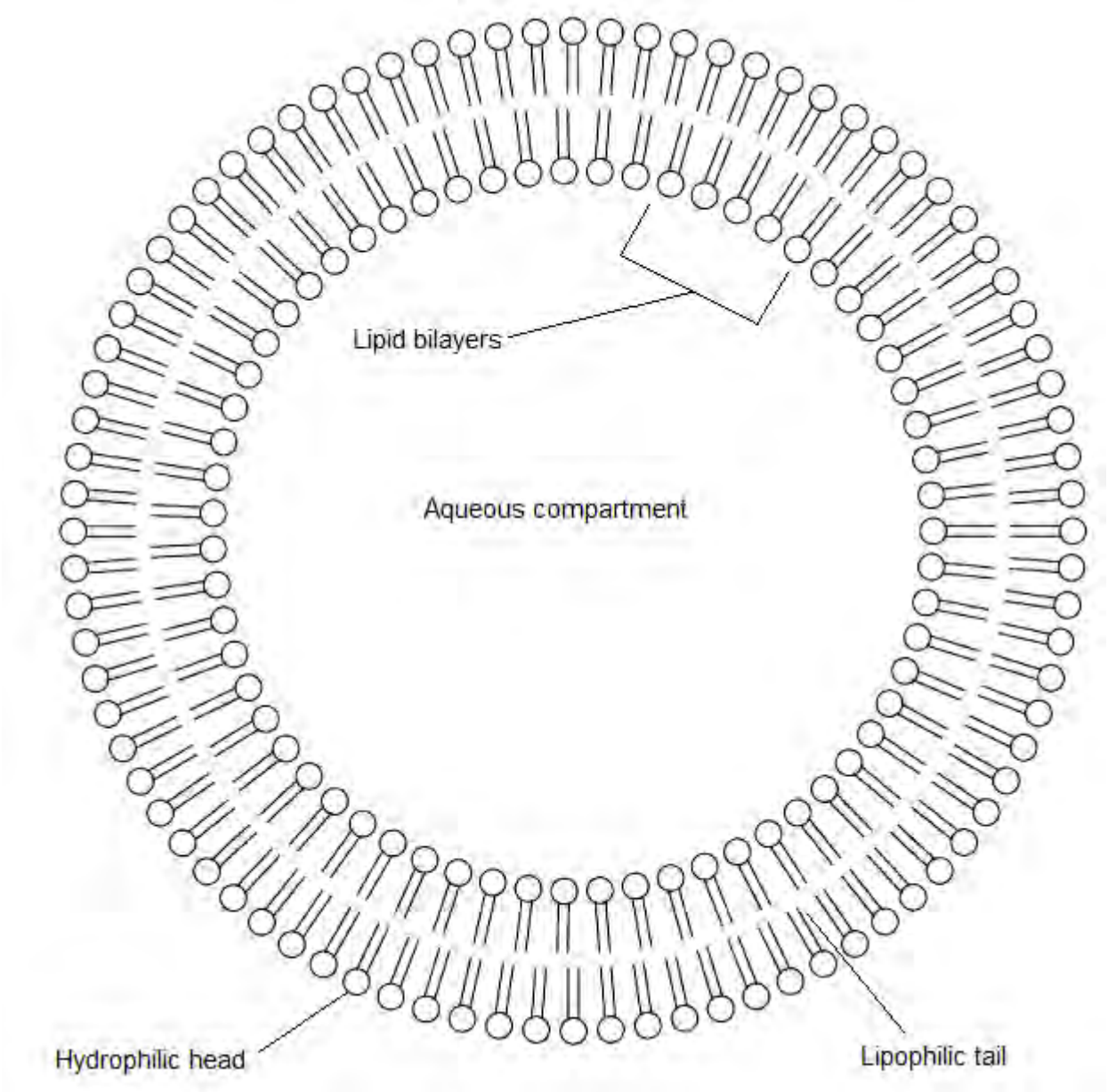

Figure C.1: Diagrammatical presentation of a unilamellar vesicle (Wiechers, 2008)

C.2.1. LIPOSOMES

Liposomes were initially reported in 1980 as a skin penetration enhancer for the primary use of delivering compounds topically (Zhao *et al.*, 2013). Liposomes typically consist of an aqueous centre enclosed by a lipid membrane consisting of cholesterol and phospholipids (Barry, 2001;

Kaur & Singh, 2014; New, 1990). They can entrap compounds both within the aqueous compartment, as well as in the lipid membrane, improving the efficacy and lowering toxicity of some APIs, including clofazimine (Isacchi *et al.*, 2012; Kaur & Singh, 2014; Vanniasinghe *et al.*, 2009). The liposome membrane is similar to the lipid portion of natural cell membranes and can be constructed of natural components, rendering liposomes a safe and effective vehicle for medical applications (New, 1990).

The main components of liposomes are cholesterol and phospholipids. Phospholipids are the major components of biological membranes. Phosphatidyl choline (PC) molecules, also called lecithin, are the most common phospholipids, which are amphiphatic molecules with a pair of hydrophobic hydrocarbon chains linked with a hydrophilic polar head-group (phosphocholine) through a glycerol bridge. In an aqueous medium the PC molecules align themselves in planar bilayer sheets to minimise adverse interactions, which are entirely eliminated when these sheets fold onto themselves to form closed sealed vesicles. PC can be derived from natural and synthetic sources, though they are more readily extracted from egg yolk and soybeans. It is used as the main phospholipid in liposomes since it is chemically inert and has lower cost implications relative to other phospholipids (New, 1990).

Cholesterol, on the other hand, does not form bilayer structures by itself; nonetheless, it can be incorporated into phospholipid membranes in high concentrations. Cholesterol to PC ratios can be 1:1 or 2:1, yet ratios in natural membranes vary from 0.1–1. It is an amphiphatic molecule which attaches to the membrane with its hydroxyl group towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. Cholesterol has a minimal effect on the position of the main transition temperature; however, with increasing concentrations a phase transition is eliminated altogether, thus, reducing the enthalpy of phase change to zero at a 1:1 ratio. This alters the fluidity of the membrane both above and below the phase transition temperature. Below this temperature the membrane packing is weakened and fluidity is increased, whereas above this temperature the membranes are condensed through reduction in freedom of the acyl chains and fluidity is decreased. In short, higher concentrations of cholesterol decrease membrane permeability at temperatures higher than the main transition temperature. Egg yolk lecithin membranes (used in this study) have a transition temperature of -15°C to -7°C, compared to membranes from mammalian sources that range from 0–40°C (New, 1990).

Liposome membranes are semi-permeable, thus, the rate of diffusion of molecules and ions across the membrane differs substantially. Molecules, which are highly soluble in organic and aqueous, media will pass practically unrestricted through the membrane, whereas polar solutes (e.g. glucose) and compounds with a higher molecular weight will only pass through the membrane very slowly. As a result of the semi-permeable nature of the phospholipid membrane,

a concentration difference of the solute between both sides of the membrane can cause osmotic pressure which will lead to accumulation of water molecules on one side of the membrane. This can cause the liposome to swell if higher concentrations of solute are entrapped in the vesicle surrounded by lower concentrations in the aqueous phase. Swelling of the liposome will increase the area of the membrane, which in turn will significantly increase the spacing between adjacent phospholipid molecules, and accelerated leakage through the membrane can subsequently occur. Furthermore, complete rupturing of the vesicle may sometimes occur, which will cause total content loss of the liposome into the aqueous phase before the liposome re-seals again (New, 1990).

Liposomes are classified according to size and shape since they range in size from 25 nm to even 1000 nm or larger (Isacchi *et al.*, 2012; Manconi *et al.*, 2011; Vanniasinghe *et al.*, 2009; Zhao *et al.*, 2013), and they can be bound by a single bilayer membrane or multiple concentric membrane lamellae stacked on one another. Multilamellar vesicles (MLVs) range from 100–1000 nm in diameter and typically comprise five or more concentric lamellae. Small unilamellar vesicles (SUVs) are defined as liposomes at the lowest limit of size for phospholipid vesicles and are 15–25 nm in diameter, whereas large unilamellar vesicles (LUVs) have a diameter of approximately 1 000 nm (New, 1990; Vanniasinghe *et al.*, 2009).

Liposomes utilised in the topical/transdermal delivery of APIs have gained significant interest, since their membrane structure is similar to natural membranes as obtained in the skin, thus minimising obstruction from the skin. Liposomes are expected however to remain at the surface of the skin, and dehydrate themselves where after they fuse with the skin lipids and release their entrapped content (Barry, 2001). Topical delivery is therefore more likely than transdermal delivery, though some cases of transport processes have been reported depending on the formulation (El Maghraby *et al.*, 2000).

C.2.2. NIOSOMES

Niosomes, similar to liposomes, have attracted immense interest for API delivery, since they are biodegradable, non-toxic, amphiphilic and are penetration enhancers effective in modulating API release properties (Agarwal *et al.*, 2001). Niosomes have been investigated for their ability to enhance API transport across the SC and improving the retention time of APIs in skin tissues, thus reducing systemic absorption. They were first introduced as an element of cosmetic applications developed by L'Oreal (Mahale *et al.*, 2012; Mali *et al.*, 2013). Their size ranges in diameter from 50–15 000 nm, depending on the production method and composition. Niosomes can also be classified as unilamellar or multilamellar vesicles (Agarwal *et al.*, 2001; Jain *et al.*, 2014; Kaur & Singh, 2014; Mali *et al.*, 2013; Sharma *et al.*, 2009; Tavano *et al.*, 2013).

Typically, niosomes consist of non-ionic surfactants and may or may not contain cholesterol, while adding cholesterol results in less leakage from the vesicles and providing rigidity to the bilayers (Barry, 2001; Kumar & Rajeshwarrao, 2011; Mahale *et al.*, 2012; Varun et al., 2012). Surfactants that can be used include Pluronic L64 (non-ionic), Aerosol OT (anionic), Spans (non-ionic) and Tweens (non-ionic), although uncharged molecules are preferred for dermal applications, as neutral compounds will permeate the skin barrier more readily. It was also found that the charge of the surfactant influences the size of the vesicles (Tavano *et al.*, 2013). Nanometer sized niosomes were mainly documented when Span 60, Span 20 and Tween 20 were used as surfactants (Mali *et al.*, 2013). The non-ionic surfactants contribute most of the desirable properties of niosomes, which include the ability to maintain pH up to physiological pH as well as improving the bioavailability of many APIs through inhibiting p-glycoprotein (Mahale *et al.*, 2012). They can encapsulate lipophilic as well as hydrophilic APIs and protect these APIs against enzymatic and acidic degradation (Sharma *et al.*, 2009).

Niosomes have a few advantages over liposomes which include the fact that they are structurally and chemically more stable, have lower production costs and they do not require special conditions for protection and storage (Kaur & Singh, 2014; Verun *et al.*, 2012). They are osmotically active and are more flexible in structure (Jain *et al*, 2014). They also significantly enhance cutaneous API retention when compared to the commercial formulation RetinA® and phosphatidylcholine liposomes (Manconi *et al.*, 2011).

C.2.3. TRANSFEROSOMES

Liposomes and niosomes are not suitable for transdermal delivery of APIs due to their poor skin permeability, as well as their aggregation and fusion tendency in skin tissue. This led to the production of transferosomes (meaning to carry across), which can reversibly alter their membrane structure to enable them to penetrate through narrow skin pores (Gillet *et al.*, 2011; Jain *et al.*, 2014). The first generation transferosomes were introduced in 1992, which can also be termed highly deformable, elastic liposomes, consisting of phospholipids and a single chain co-surfactant. The co-surfactant acts as an “edge activator” by destabilising the lipid bilayer and thus increasing the deformability (Gillet *et al.*, 2011; Kumar & Rajeshwarrao, 2011; Manconi *et al.*, 2012). Co-surfactants that can be used include sodium cholate, sodium deoxycholate, Tween 80 and Span 80 (El Maghraby *et al.*, 2000; Jain *et al.*, 2014).

Barry (2001) mentioned that vesicle sizes of 200–300 nm can pass through intact skin due to their ultra-deformability. The diameter of transferosomes is considerably smaller than that of conventional liposomes and can vary from 90–340 nm (Gillet *et al.*, 2011; Jain *et al.*, 2014). These vesicles can be used for topical and transdermal API delivery and can efficiently entrap APIs of both low and high molecular weight. Encapsulated APIs can be released in a controlled manner, but transferosomes are chemically unstable and expensive to manufacture (Jain *et al.*, 2014).

C.3. PREPARATION OF VESICLES

C.3.1. MATERIALS

Clofazimine (CLF) was kindly donated by Cipla Inc. (Mumbai, India), whereas artemisone (ART) and decoquinate (DQ) were supplied by the MALTBRedox MRC South African University Flagship Projects from the Pharmaceutical Chemistry Department of the North-West University (Potchefstroom, South Africa). The vesicles were manufactured using L-α-Phosphatidylcholine from egg yolk ~60% (Sigma-Aldrich, South Africa), cell culture suitable cholesterol (Sigma-Aldrich, South Africa), cell culture tested Tween® 20 (Sigma-Aldrich, South Africa) and absolute ethanol from Associated Chemical Enterprises (ACE) in South Africa. The water used was purified by a Milli-Q® water purification system from Millipore® (United States of America). Methanol (ACE, South Africa) and acetone (ACE, South Africa) were used as lipid solvents and glass beads with a diameter of 2 mm (Merch, Germany) were used during the thin film manufacturing method. All chemicals used were of analytical grade.

C.3.2. METHOD OF PREPARATION

The equipment utilised in the preparation of the vesicles are:

- Hot plate/stirrer (Figure C.2)
- Ultrasonicator bath (Figure C.3)
- Hielscher ultrasonic processor UP200St (200 W, 26 kHz, Figure 3.4).

Figure C.2: Labcon® hotplate and stirrer

Figure C.3: Transsonic® TS540 ultrasonicator bath

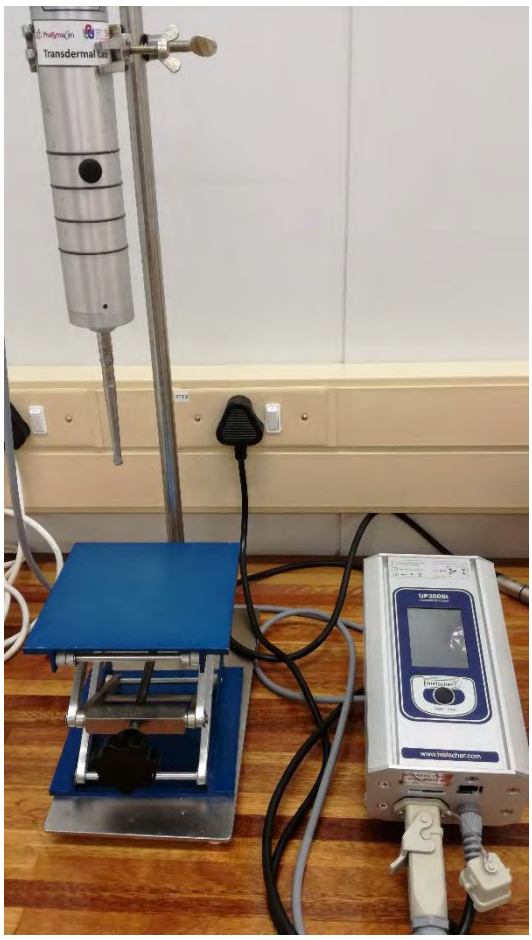

Figure C.4: Hielscher® ultrasonic processor UP200St at 200 W and 26 kHz

A modified version of the thin-film hydration method was used to prepare the vesicles (Agarwal *et al.*, 2001; Shaji & Lal, 2014). The lipophilic phase (Part A) was accurately weighed in a glass beaker and dissolved in a 3 ml acetone:methanol (2:1) mixture. The API(s) were also added to Part A since they are mostly lipophilic, and to promote accumulation in the vesicles. The Part A mixture was placed onto a hot plate, with a Teflon® coated magnetic stirrer set to a medium speed and low heat, and left until Part A was completely dissolved in the organic solvent. After complete dissolution, the magnetic stirrer was removed and the mixture was carefully heated to 45°C while swirling the mixture in the beaker to ensure all the organic solvent evaporated and only an even, thin film of lipids was left behind in the beaker (Figure C.5).

Figure C.5: Lipid film containing clofazimine in a beaker

An aqueous phase (Part B) phosphate buffer solution (pH 5.5) was added (10 ml) to Part A together with 5 glass beads and left in the sonicator bath for 15 min. The glass beads were removed and the vesicles were probe sonicated at 200 W for 2 min in order to ensure smaller, evenly sized vesicles. While using the probe sonicator, the sample was placed in another beaker filled with ice to ensure that the temperature did not increase above 45°C. The vesicle dispersion was placed in a refrigerator at 4°C for at least 2 h to enable vesicles to form and settle properly. The dispersions of the different vesicles types can be seen in Tables C.1-C.3.

Table C.1: Liposome vesicles (5%)

Ingredients		Amount
Part A	Phosphatidyl choline	300 mg
	Cholesterol	200 mg
	API(s)	1% (100 mg each)
Part B	Phosphate buffer solution	10 ml

Table C.2: Transferosome vesicles (5%)

Ingredients		Amount
Part A	Phosphatidyl choline	400 mg
	Ethanol	100 mg
	API(s)	1% (100 mg each)
Part B	Phosphate buffer solution	10 ml

Table C.3: Niosome vesicles (5%)

Ingredients		Amount
Part A	Tween 20	335 mg
	Cholesterol	168 mg
	API(s)	1% (100 mg each)
Part B	Phosphate buffer solution	10 ml

Originally an API concentration of 0.2% (m/v) was used, though after noticing very low peaks with HPLC analysis, it was decided to use a 1% (m/v) concentration API for skin diffusion studies. It was, however, discovered that a 1% API concentration displayed unstable colloidal dispersions, and thus physical tests were done on dispersions ranging from 0.2–1% API concentrations at 0.2% intervals to determine at what specific API concentration the vesicles were considered unstable. Preliminary studies indicated that the vesicles were considered unstable at 0.6% API (m/v), and some dispersions at 0.4% API. Therefore, characterisation studies were not conducted on the 0.6% and 0.8% API dispersions.

C.4. PRE-FORMULATION OF VESICLES

C.4.1. DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is a technique used to characterise the stability of a biomolecule directly in its natural form. This is achieved by measuring the heat change associated with the molecule's thermal denaturation when heated at a constant rate (Malvern instruments Ltd., 2016). The DSC is used for measuring, among others, melting temperature, latent heat of melting, reaction energy and temperature, denaturisation temperatures, and precipitation energy and temperatures (Anderson materials Inc., 2016).

The APIs were mixed with individual excipients in a 1:1 (w/w) ratio in a mortar and pestle for compatibility studies. The mixtures and single compounds were analysed by DSC, and the resulting thermograms compared to determine whether any incompatibilities occurred. Possible incompatibilities were indicated by a peak shift, appearance of new peaks or disappearance of peaks in the DSC thermograms. The DSC-60 Shimadzu® instrument (Shimadzu®, Japan) was

used to record the DSC thermograms. All samples weighed approximately 3–5 mg and were placed in aluminium crimped cells. The cells were heated to the desired temperature, starting at 25°C and up to a maximum temperature of 300°C, with nitrogen gas flow set at 35 ml/min and a heating rate set to 10°C/min. The thermograms of the three APIs, as well as their combinations can be observed in Figure C.6.

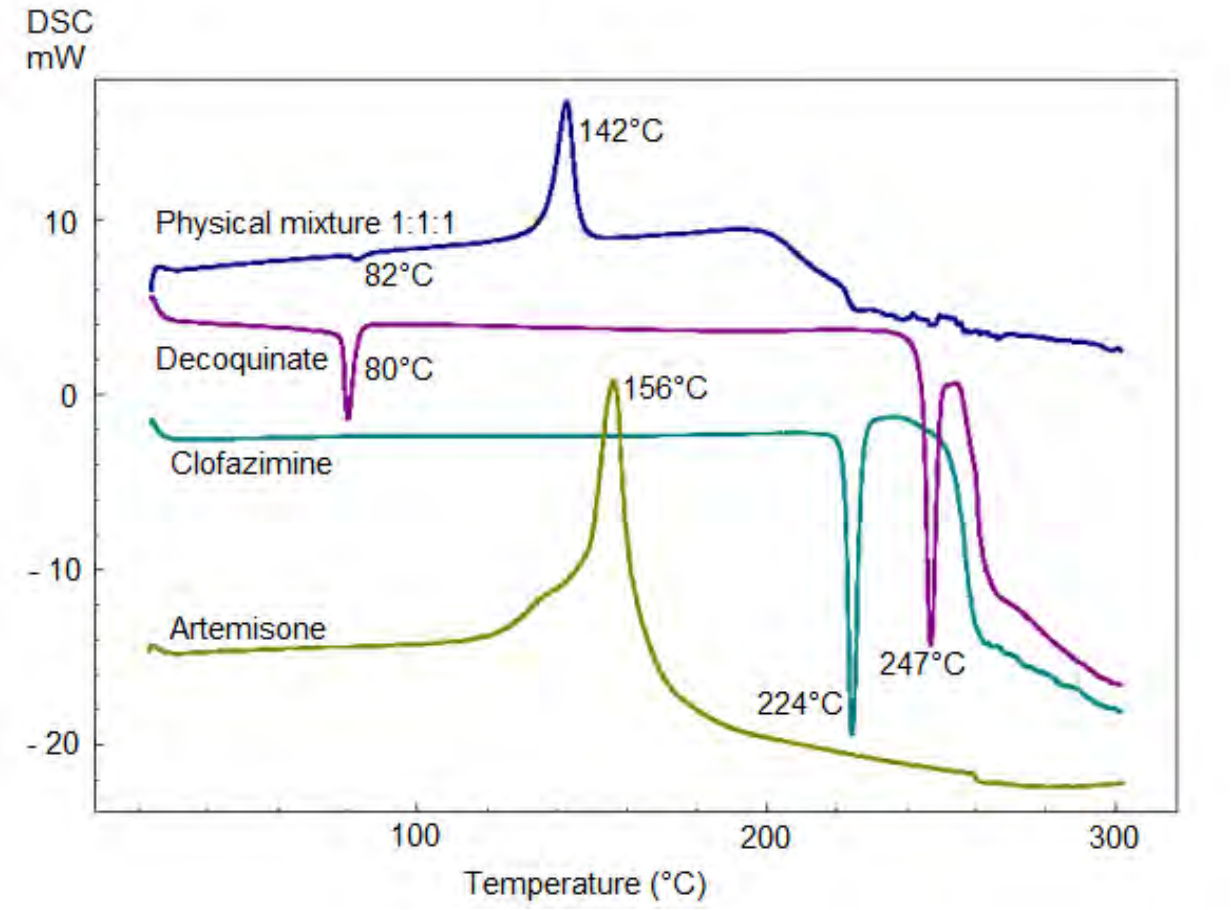

Figure C.6: DSC thermogram of the three APIs and their combination

Figure C.6 illustrates a melting point of 156°C for artemisone and 224°C for clofazimine. Decoquinate showed melting points of 80°C and 247°C, though the first melting point obtained was possibly due to the presence of an impurity or moisture. In order to determine what caused the first melting point, a thermal activity monitoring experiment was subsequently performed. The combination of the three compounds (physical mixture) presented a lower melting point, which could be explained by the artemisone that started to melt first, followed by the decoquinate and clofazimine dissolving in the artemisone.

C.4.2. ISOTHERMAL CALORIMETRY

The thermal activity monitor (TAM) is an isothermal microcalorimeter designed to monitor a wide range of chemical and biological reactions. Both exothermic and endothermic processes can be

quantified. The TAM is often used for its sensitivity and to study reactions that traditional calorimeters such as the DSC cannot facilitate (Fauske & Associates, 2016).

Compatibility testing was conducted using a 2277 Thermal Activity Monitor (TAMIII) (TA Instruments, United States of America), equipped with an oil bath with a stability of ± 100 µK over 24 h. The calorimeters' temperature was maintained at 40°C. During the compatibility studies the heat flow was measured for the single components, as well as for the mixtures. Calorimetric outputs observed for the individual samples were summed to provide a theoretical response. This calculated hypothetical response represents a calorimetric output that would be expected if the two materials do not interact with each other. If the materials interact, the measured calorimetric response will differ from the calculated theoretical response (TA Instruments, 2017).

Compatibility of different combinations of clofazimine (CLF), artemisone (ART), decoquinate (DQ), Tween® 20, cholesterol, phosphatidylcholine (PC), niosomes, transferosomes and liposomes were analysed. The results can be seen in Table.C.4 below.

Table C.4: Compatibility report of different ingredients used for vesicle preparation

No	Ingredients	Compatibility	Average interaction heat flow (µW/g)
1	ART + CLF + DQ	No incompatibility	14.57 ± 17.97
2	ART + PC	Incompatibility exist; may be due to oxidative PC	18.19 ± 22.68
3	CLF + PC	Incompatibility exist, but less than combination 2	32.71 ± 33.66
4	DQ + PC	No incompatibility	79.89 ± 80.20
5	ART + CLF + DQ + PC	Measured and theoretical curves differ more than 100 nW/g showing a degree of incompatibility, though difficult to clearly identify	34.36 ± 36.25
6	ART + cholesterol	Some degree of interaction, though not significant	-15.71 ± 19.06
7	CLF + cholesterol	No incompatibility	6.25 ± 6.73
8	DQ + cholesterol	No incompatibility	13.81 ± 18.73
9	ART + CLF + DQ + cholesterol	No incompatibility	11.44 ±12.76
10	PC + cholesterol	No incompatibility	3.68 ± 26.20
11	ART + Tween® 20	Incompatibility exist	-7.99 ± 8.34
12	CLF + Tween® 20	Incompatibility exist	-11.56 ±12.03
13	DQ + Tween® 20	Incompatibility exist	-85.15 ± 86.80
14	PC + Tween® 20	No incompatibility	-1.49 ± 4.14
15	Tween® 20 + cholesterol	No incompatibility	0.38 ± 3.43
16	DQ + ART + Tween® 20	Incompatibility exist, though no significant difference	-5.89 ± 6.26
17	DQ + CLF + Tween® 20	Incompatibility exist	-7.03 ± 7.85
18	ART + CLF + Tween® 20	Incompatibility exist	-10.82 ± 11.17
19	ART + CLF + DQ + Tween® 20	Incompatibility exist	-5.47 ± 6.03
20	Liposomes + ART	Incompatibility is minimised through this combination	1.87 ± 2.51
21	Liposomes + CLF	Incompatibility is minimised through this combination, cholesterol might have a stabilising effect	21.06 ± 26.45
22	Liposomes + DQ	No incompatibility	0.25 ± 1.15
23	Liposomes + ART + CLF + DQ	No incompatibility	2.28 ± 2.86
24	Transferosomes + ART	No incompatibility	0.65 ± 1.28
25	Transferosomes + CLF	No incompatibility	0.72 ± 2.88
26	Transferosomes + DQ	No incompatibility	-0.04 ± 1.27
27	Transferosomes + ART + CLF + DQ	No incompatibility	4.00 ± 4.50
28	Niosomes + ART	Incompatibility is minimal	0.75 ± 1.25
29	Niosomes + CLF	No incompatibility	0.14 ± 0.86
30	Niosomes + DQ	No incompatibility	0.61 ± 0.95
31	Niosomes + ART + CLF + DQ	Incompatibility is probable	-2.43 ± 3.02

From this study it became apparent that the three APIs, artemisone, clofazimine and decoquinate are compatible with one another within the solid-state form. The combinations of the three APIs with PC showed artemisone in combination with PC to be incompatible. The incompatibility of clofazimine with PC is slightly less than artemisone and PC, whereas decoquinate and PC were found to be compatible. The combination of all three actives with PC also showed incompatibility. This was not surprising given the fact that PC and two of the other compounds showed incompatibility.

The artemisone/cholesterol combination did not show a definitive incompatibility. However, caution must be exercised, as it is known that a more severe incompatibility might be triggered between the two compounds, either through addition of one or more compounds, other dispersion excipients or even energy from production processes. Compatibility was established between clofazimine and cholesterol, decoquinate and cholesterol, PC and cholesterol, as well as the combination of all three APIs together with cholesterol.

It was evident that the incompatibility of all three APIs in combination with Tween® 20 would be problematic. Interestingly, the combination of Tween® 20 and PC did not show any incompatibility, as was the case with cholesterol and Tween® 20. The artemisone/decoquinate/Tween® 20 combination, the artemisone/clofazimine/Tween® 20 combination, as well as clofazimine/decoquinate/Tween® 20 exhibited clear incompatibilities. A combination of all three APIs with Tween® 20 also showed incompatibility.

All the liposome dispersions proved to be compatible, despite incompatibility of some of the individual combinations. All the transferosome dispersions displayed compatibility, with the niosomes showing the best compatibility of all the dispersions tested.

C.4.3. HOT-STAGE MICROSCOPY

Hot-stage microscopy (HSM) combines thermal analysis and microscopy to characterise the physical properties of materials as a function of temperature. HSM is used in the pharmaceutical industry in various ways to confirm transitions observed using other techniques (Vitez *et al.*, 1998).

A small amount of API was placed on a microscope slide with either a cover slide only, or the API first covered with silicon oil (Fluka Chemica, Switzerland) and then a cover slide (in the case of solvates). A Nikon® Eclipse E400 thermo-microscope (Tokyo, Japan), equipped with a Leitz 350 heating unit (Wetzlar, Germany) and a Metratherm 1200d thermostat were used for the HSM experiments. A Nikon® Simple Polarising Attachment (Tokyo, Japan) was fixed on the same microscope for the polarising optical microscopy. Images were taken using a Nikon® Coolpix

5400 digital camera (Tokyo, Japan) attached to the microscope. Images taken can be seen in Figures C.7-C.10.

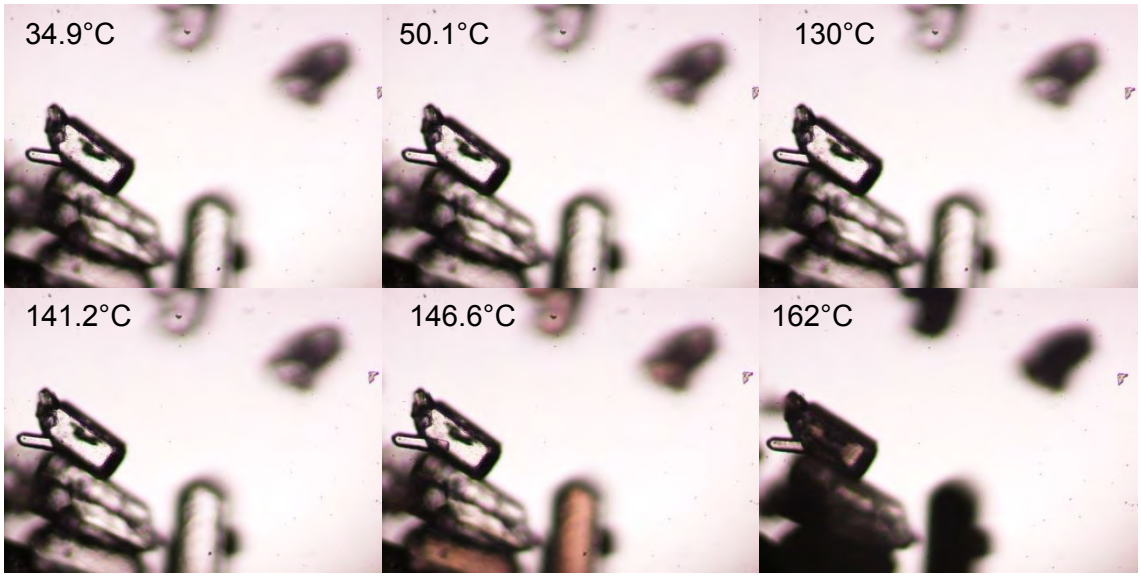

Figure C.7: Hot stage microscopy micrographs of artemisone during continuous heating

Artemisone has rectangular crystals, which start to melt at 162°C as seen in the last image in Figure C.7.

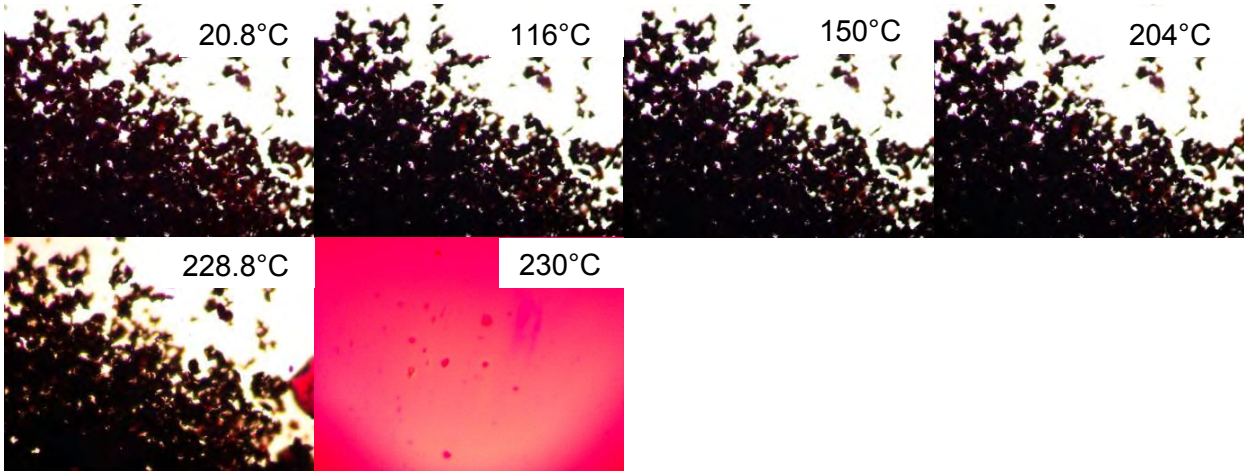

Figure C.8: Hot stage microscopy micrograph of clofazimine during continuous heating

Clofazimine is a brick-red powder (Figure C.8), which did not break down before melting at 228.8°C when the powder started to change colour. At 230°C the clofazimine was completely melted.

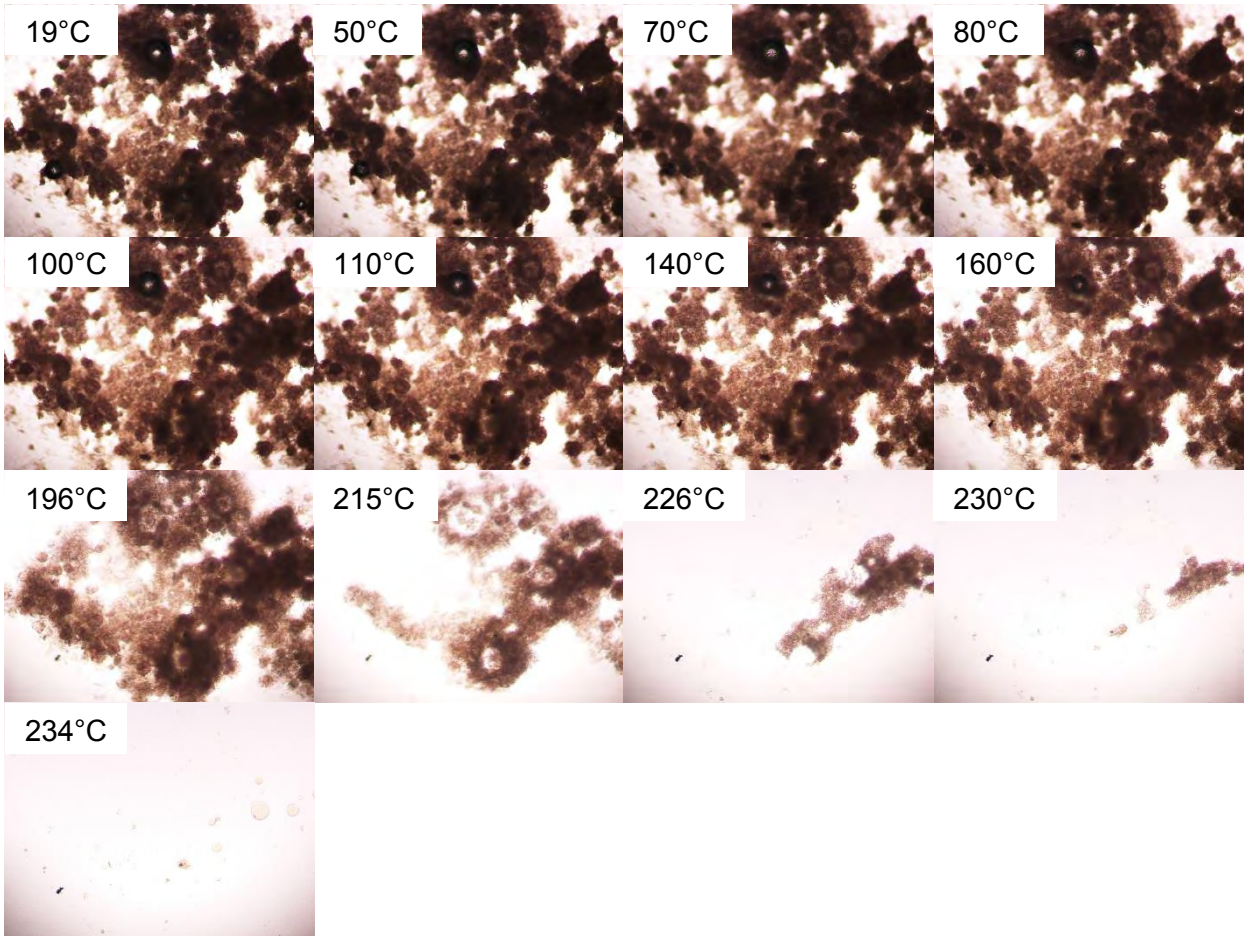

Figure C.9: Hot stage microscopy micrograph of decoquinate during continuous heating

Decoquinate is a very dense, cream-coloured powder, which displays no disruption prior to melting, since there is no discolouration before it melts. The melting point is at 196°C, as observed in the image differences between 160°C and 196°C. During DSC experiments a melting point peak at 80°C was revealed that led to the belief that decoquinate might start to break down at that temperature or that impurities might be included in the powder. Silicon oil was thus added to the decoquinate and images were taken at 80°C. Results indicated that no impurities were present and no breakdown of the compound commenced at 80°C.

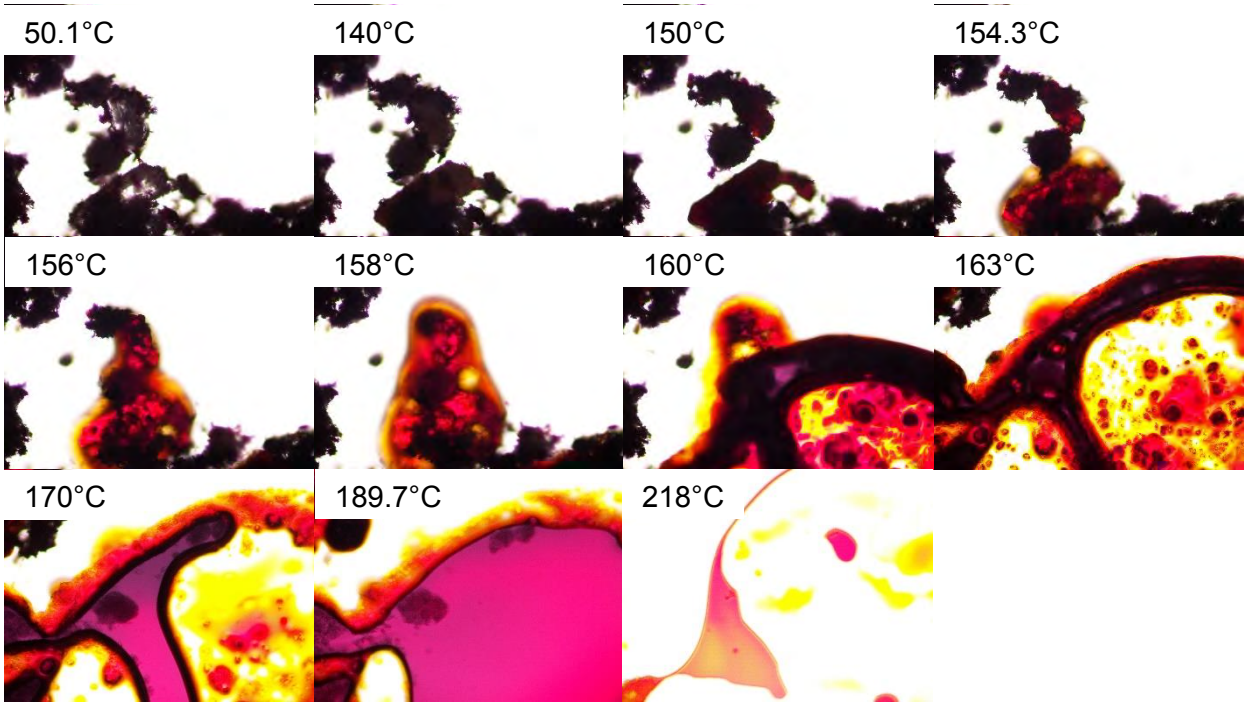

Figure C.10: Hot stage microscopy micrograph of artemisone, clofazimine and decoquinate during continuous heating

Next, the three APIs were added together and heated continuously. At 154.3°C artemisone started to melt, and clofazimine and decoquinate dissolved in the artemisone. At 189.7°C all the compounds were completely melted.

C.5. CHARACTERISATION

C.5.1. TRANSMISSION ELECTRON MICROSCOPY

Vesicle formation and size were first determined by transmission electron microscopy (TEM). Liposomes, niosomes and transferosomes were prepared in the phosphate buffer solution and also in pure water with no API(s) added as they cause crystallisation that obscure the vesicles. The vesicle sample was diluted with water until a clear dispersion was visible. A drop of the diluted sample was placed on a carbon coated formvar grid. The grids were placed in a closed petri-dish together with a drop of 4% aqueous osmium tetroxide and the vesicles were allowed to vapour fix for 10 min. The osmium vapour fixes lipids and ensure that the lipids are more electron-dense for enhanced imaging with the TEM.

After 10 min at room temperature (i.e. 25°C) excess fluid was carefully removed from the grid with Whatman® filter paper, taking care not to rub the grid and possibly remove/damage the vesicles. Thereafter, a drop of 4% aqueous uranyl acetate was placed on the grid for 40 s and washed in three changes of ultrapure water. It was dried using filter paper and left at room temperature for 20 min. The grid was placed in the single tilt holder and examined using an FEI Tecnai G2 20S-

Twin TEM at 120 kV. Images were digitally captured with a Gatan® bottom mount camera using DigitalMicrograph® software, which was also used to measure the vesicle size. Images obtained with the TEM can be seen in Figures C.11-C.12.

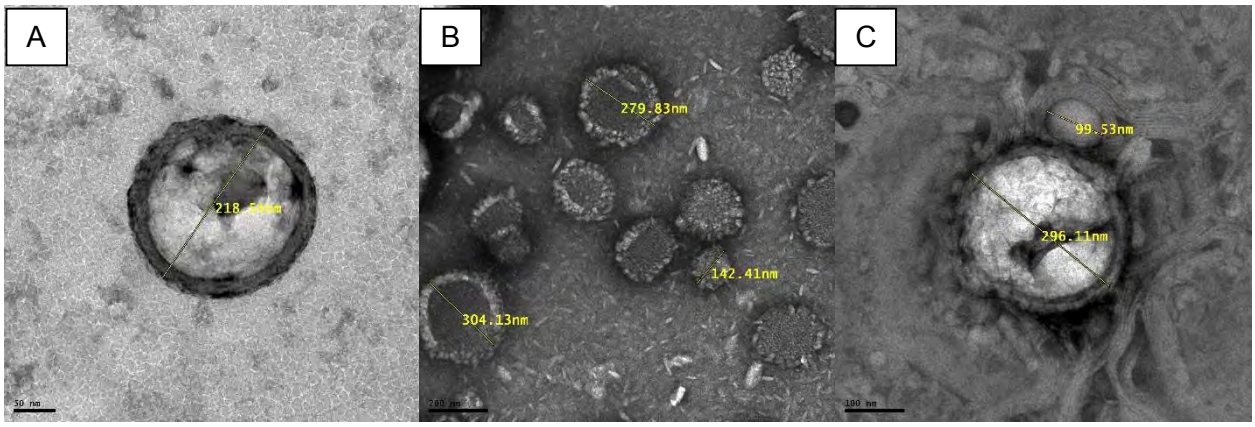

Figure C.11: TEM imaging illustrating: A. Liposomes, B. Niosomes and C. Transferosomes prepared with PBS as the aqueous phase

The phosphate buffer solution (PBS) formed crystals during the drying/preparation process for TEM imaging as can clearly be seen in Figure C.11B. Figure C.11C depicts membranes that were formed but never attached to form vesicles. New samples were prepared that contained no buffer, but only pure water as the aqueous phase (Figure C.12).

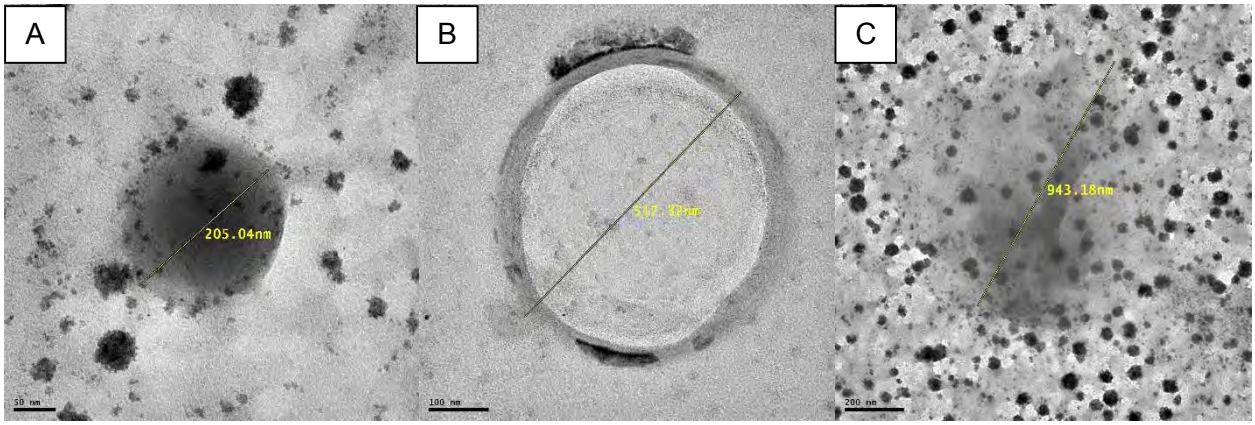

Figure C.12: TEM imaging illustrating: A. Liposomes, B. Niosomes and C. Transferosomes prepared with pure water

Most of the vesicles displayed an approximate size of 200 nm (section C.5.3) and the images above are to show the form of the vesicles. The transferosome in Figure C.12C is approximately 943.18 nm in size, though the small dark spots are also formed vesicles with approximate sizes of 50 nm if compared to the scale on the bottom left of the image.

C.5.2. ENCAPSULATION EFFICIENCY

Encapsulation efficacy is determined by removing unentrapped API(s) from the entrapped API(s) and is calculated using the following equation (Eq. C.1):

$$\%EE = \frac{A - B}{A} X100 \qquad \text{Eq. C.1}$$

Where: A = amount of API added to dispersion
B = amount of API unentrapped

The (A – B) at the top of the fraction may also be substituted with C, which is the amount of API entrapped in the vesicles (Gillet *et al.*, 2011; New, 1990). This will depend on what method of unentrapped-entrapped separation will be used. For this study, the centrifugation separation method was used. Since SUV(s) were prepared, it is stated that the vesicles should be centrifuged at 2 000 *g* (or 3335 rpm) for 20 min to determine encapsulation (New, 1990). The centrifuge used, was the Eppendorf® 5804 R centrifuge equipped with an A-4-44 rotor. The density of the liposomes was higher than that of the unentrapped API, thus the pellet that formed (Figure C.13), were the liposomes, and the supernatant the unentrapped API. An amount of the supernatant was diluted with ethanol until a transparent solution was formed, the sample was filtered to remove vesicle residue and analysed in triplicate by means of HPLC. The encapsulation efficiency of the different dispersions at different API concentrations can be seen in Table C.5.

Figure C.13: Dispersions after centrifugation showing the formation of pellets

Table C.5: Encapsulation efficiency (%EE) of the vesicles in the different dispersions

	Compound and Concentration		Liposomes	Niosomes	Transferosomes
Single API dispersions	**ART**	0.2%	87.94	74.84	12.89
	CLF		86.22	97.86	45.18
	DQ		55.01	98.25	93.98
Combination	**ART**		79.02	71.11	32.35
	CLF		87.01	97.20	44.68
	DQ		41.78	97.20	95.86
Combination	**ART**	0.4%	89.95	84.21	68.15
	CLF		90.81	85.59	71.53
	DQ		73.06	91.15	97.88
Single API dispersions	**ART**	1%	96.54	94.34	85.39
	CLF		94.04	97.94	87.33
	DQ		96.39	93.12	97.24
Combination	**ART**		96.87	93.77	87.24
	CLF		91.23	95.54	87.89
	DQ		94.32	92.71	98.50

Though the entrapment efficacy seems relatively higher for the lower API concentration dispersions, this is not an accurate representation of the amount of API that can be entrapped in the vesicles. The vesicle concentration in the aqueous medium remained at 5% in 10 ml PBS. Only the concentration of API increased. For this reason the amount (mg) of API entrapped for each dispersion (calculated from the exact amount of API added to each dispersion) is presented in Table C.6.

Table C.6: Amount (mg) of API entrapped in the vesicles for the different dispersions with the initial amount added to dispersion in brackets

Formulations	Compound	Liposomes	Niosomes	Transferosomes
Single API dispersions 0.2%	**ART**	16.88 (19.2)	15.04 (20.1)	2.63 (20.4)
	CLF	16.90 (19.6)	20.45 (20.9)	9.22 (20.4)
	DQ	10.12 (18.4)	19.75 (20.1)	18.80 (20.0)
Combination 0.2%	**ART**	15.01 (19.0)	13.44 (18.9)	6.11 (18.9)
	CLF	16.62 (19.1)	18.37 (18.9)	8.53 (19.1)
	DQ	8.02 (19.2)	18.37 (18.9)	17.83 (18.6)
Combination 0.4%	**ART**	35.98 (40.0)	33.94 (40.3)	28.01 (41.1)
	CLF	37.32 (41.1)	34.84 (40.7)	28.76 (40.2)
	DQ	30.03 (41.1)	37.64 (41.3)	38.76 (39.6)
Single API dispersions 1%	**ART**	99.05 (102.6)	95.38 (101.1)	84.96 (99.5)
	CLF	97.33 (103.5)	97.45 (99.5)	88.90 (101.8)
	DQ	96.39 (100.0)	94.14 (101.1)	97.34 (100.1)
Combination 1%	**ART**	97.06 (100.2)	93.77 (100.0)	87.15 (99.9)
	CLF	91.14 (99.9)	95.44 (99.9)	87.71 (99.8)
	DQ	95.36 (101.1)	94.19 (101.6)	98.60 (100.1)

Higher amounts of APIs are entrapped in the vesicles with higher concentrations of APIs added. This will lead to larger and more unstable vesicles, which can be confirmed by the zeta-potentials and sizes of the dispersions.

C.5.3. ZETA-POTENTIAL, SIZE AND SIZE DISTRIBUTION

The zeta-potential, as well as vesicle size and distribution of the vesicles was determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern® Instruments Ltd., Worcestershire, UK) at 25°C. The zeta-potential is the electrostatic potential at interfaces and indicates the electrostatic repulsion between particles in a system. The higher the zeta-potential, the stronger the repulsion and the more stable the colloidal system becomes (DT, 2013; Zhao *et al.*, 2014). Zeta-potential is measured in mV (milli-volts) and cannot exceed 100 mV. If the electric charge alone is considered to keep the dispersion stable, the common dividing line between stable and unstable dispersions is usually taken at 30 mV (positive or negative), with higher values considered to be more stable. The zeta-potential is acknowledged as a brilliant index of the interaction between colloidal particles, and can be used to assess the stability of colloidal systems. Viscosity is also usually lower the higher the zeta-potential (Colloidal Dynamics, 1999; Malvern Instruments Ltd., 2017). To prepare the samples for the Zetasizer, one drop of each

dispersion was placed in a separate beaker and 10 ml ultrapure water was added. The sample was lightly swirled to ensure even distribution of the particles. A “size and zeta-potential” folded capillary cell (DTS1060) was properly filled with the diluted sample for analysis. The zeta-potentials for the dispersions are shown in Table C.7.

Table C.7: The average zeta-potentials for the different dispersions

Vesicle	Dispersion and Concentration		Zeta-potential (mV)
Liposomes	Blank	0%	-53.3 ± 5.9
	ART	0.2%	-44.4 ± 6.7
	CLF		**-28.6 ± 3.98**
	DQ		-39.7 ± 5.7
	Combination		-32.7 ± 4.9
	Combination	0.4%	**-22.5 ± 3.98**
	ART	1%	-55.1 ± 4.5
	CLF		-31.5 ± 4.8
	DQ		-57.5 ± 5.3
	Combination		**-21.6 ± 4.6**
Transferosomes	Blank	0%	-35.7 ± 5.3
	ART	0.2%	-49.96 ± 7.8
	CLF		**-9.4 ± 3.8**
	DQ		-47.7 ± 5.5
	Combination		**-25.5 ± 3.9**
	Combination	0.4%	**-17.0 ± 3.9**
	ART	1%	-43.9 ± 8.9
	CLF		**-5.5 ± 3.3**
	DQ		-44.7 ± 5.9
	Combination		**-15.5 ± 3.8**
Niosomes	Blank	0%	-43.3 ± 5.5
	ART	0.2%	**-26.4 ± 4.7**
	CLF		**-25.8 ± 5.2**
	DQ		**-27.9 ± 4.7**
	Combination		**-19.8 ± 5.0**
	Combination	0.4%	**-4.3 ± 4.8**
	ART	1%	**-17.0 ± 3.8**
	CLF		**-8.5 ± 3.5**
	DQ		**-11.5 ± 4.3**
	Combination		**-6.6 ± 4.8**

Values presented in bold are the dispersions where the zeta-potential indicates that the colloidal dispersions might not be stable and aggregation, flocculation or vesicle rupture may occur (Malvern Instruments Ltd., 2017). The size and size distribution were furthermore determined with the Zetasizer Nano ZS and the data are shown in Table C.8-C.9. The size range for this instrument is 0.6 nm-6 µm. Niosome samples presented a high polydispersity index (PDI, approximately ≥ 0.6), indicating that the size distribution of the particles was too wide-ranging for obtaining quality data (Malvern instruments Ltd., 2004).

Table C.8: Average sizes (n=3) of the niosomes with 0.2% API and 4 min sonication

Vesicle	Dispersions	Size (d.nm)	PDI
Niosomes	ART	416.95	0.55
	CLF	1008.8	1.00
	DQ	1284.5	0.91
	Combination	418.4	0.66

The data acquired did not meet quality criteria of the instrument and the size distribution did not deliver a single peak with an even distribution pattern (Figure C.14). The polydispersity index was too high and large sedimenting particles were present in the samples. This indicated that the niosomes needed to be sonicated for a longer time period to ensure smaller sizes and a narrower size distribution.

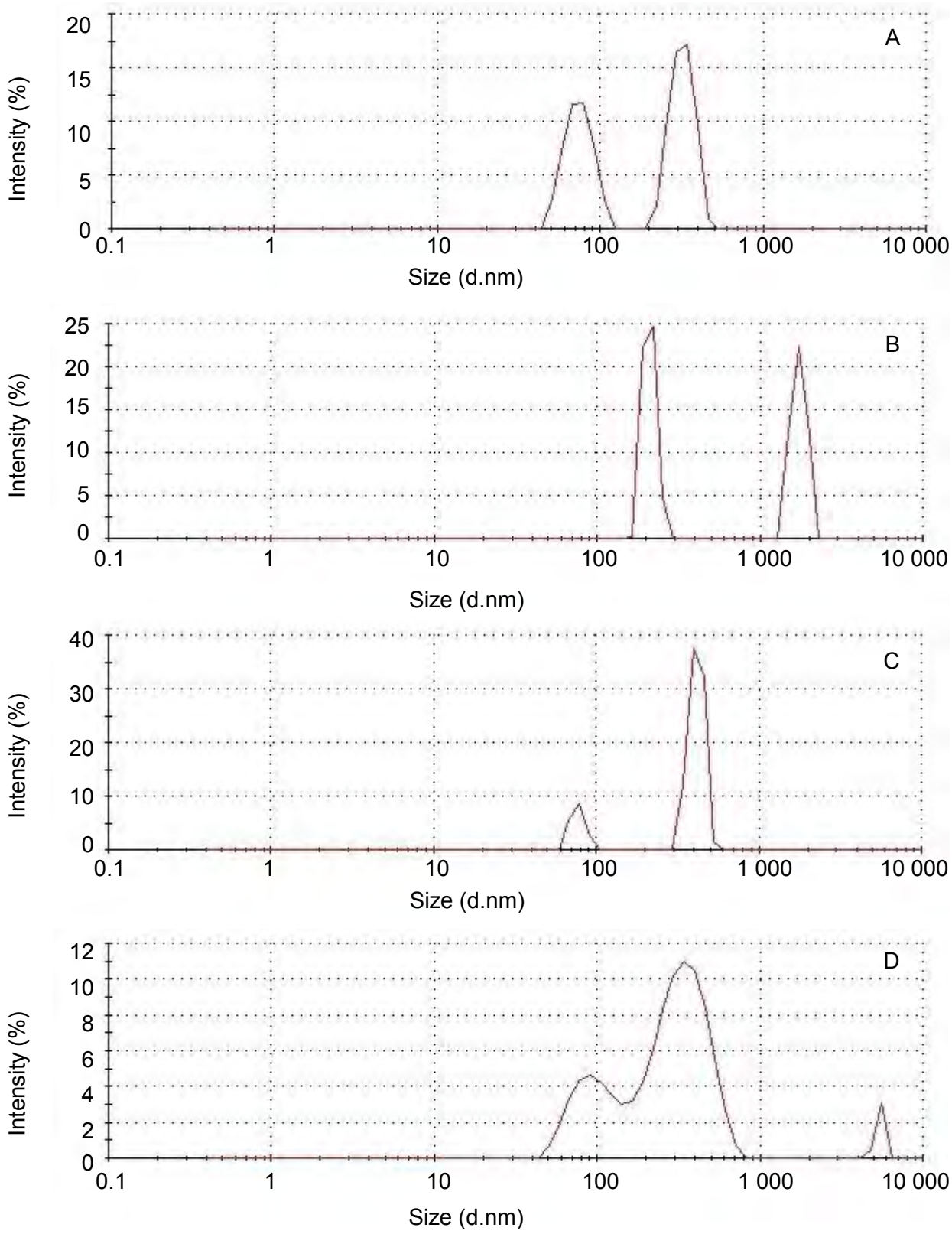

Figure C.14: Size distribution of niosomes containing 0.2% artemisone (A), clofazimine (B), decoquinate (C), and a combination of all three APIs (D), sonicated 4 min

The niosome dispersion needed to be sonicated longer for a more optimal size distribution and to lower the PDI. After careful evaluation it was determined that the niosomes needed to be sonicated for 8 min at 2 min intervals, taking care not to overheat the niosomes.

Table C.9: Average sizes (n=3) of the vesicles of different dispersions

Vesicle	Dispersion and Concentration		Size (d.nm)	PDI
Liposomes	Blank	0%	154.0	0.306
	ART	0.2%	170.2	0.427
	CLF		153.3	0.366
	DQ		208.97	0.526
	Combination		167.4	0.471
	Combination	0.4%	**606.0**	**0.632**
	ART	1%	146.6	0.239
	CLF		**481.7**	**0.618**
	DQ		**252.7**	**0.452**
	Combination		**824.1**	**0.673**
Transferosomes	Blank	0%	106.3	0.257
	ART	0.2%	95.4	0.232
	CLF		89.8	0.257
	DQ		117.2	0.425
	Combination		99.3	0.269
	Combination	0.4%	**205.0**	**0.461**
	ART	1%	123.7	0.394
	CLF		**1222.0**	**0.892**
	DQ		**367.5**	**0.482**
	Combination		**524.7**	**0.696**
Niosomes	Blank	0%	167.5	0.525
	ART	0.2%	333.9	0.593
	CLF		284.6	0.523
	DQ		208.9	0.465
	Combination		430.2	0.565
	Combination	0.4%	**546.9**	**0.621**
	ART	1%	**437.4**	**0.481**
	CLF		**2461.0**	**0.939**
	DQ		**670.1**	**0.628**
	Combination		**984.9**	**0.925**

The data indicated in bold did not meet the quality criteria, since either a high PDI or a size distribution that was too broad, was displayed. It was found that all the dispersions' vesicle sizes increased with higher concentrations APIs and the size distribution at 0.4% API delivered

inadequate data. This can, together with zeta-potential data, indicate unstable vesicle dispersions. Vesicle sizes between measurements are irregular. The increase of API concentration from 0.2% to 0.4% and then from 0.4% to 1% clearly shows a decrease in vesicle stability. This evidently indicates the influence of an API on vesicle formation. Focussing on the 1% API dispersions, it can likewise be seen that clofazimine had a more dominant effect on the vesicle sizes and size distributions. This can more clearly be observed in Figures C.15-C.26.

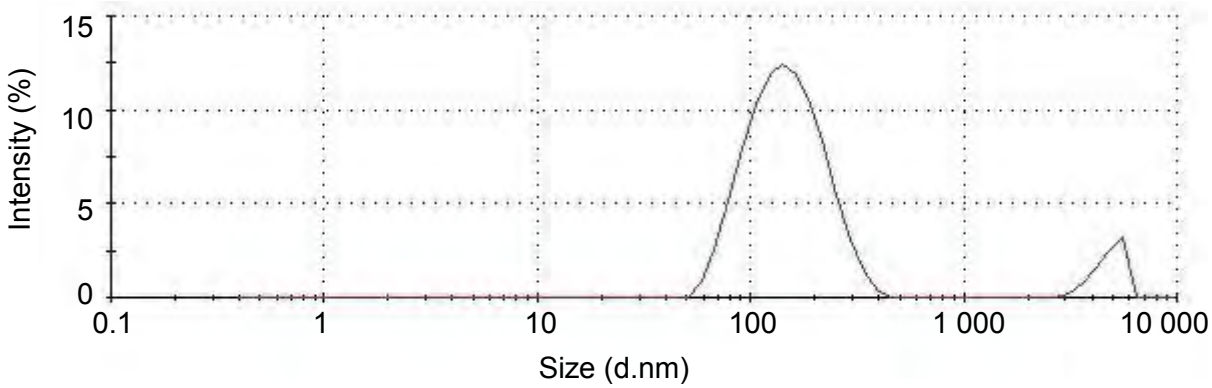

Figure C.15: Size distribution of liposomes with no APIs

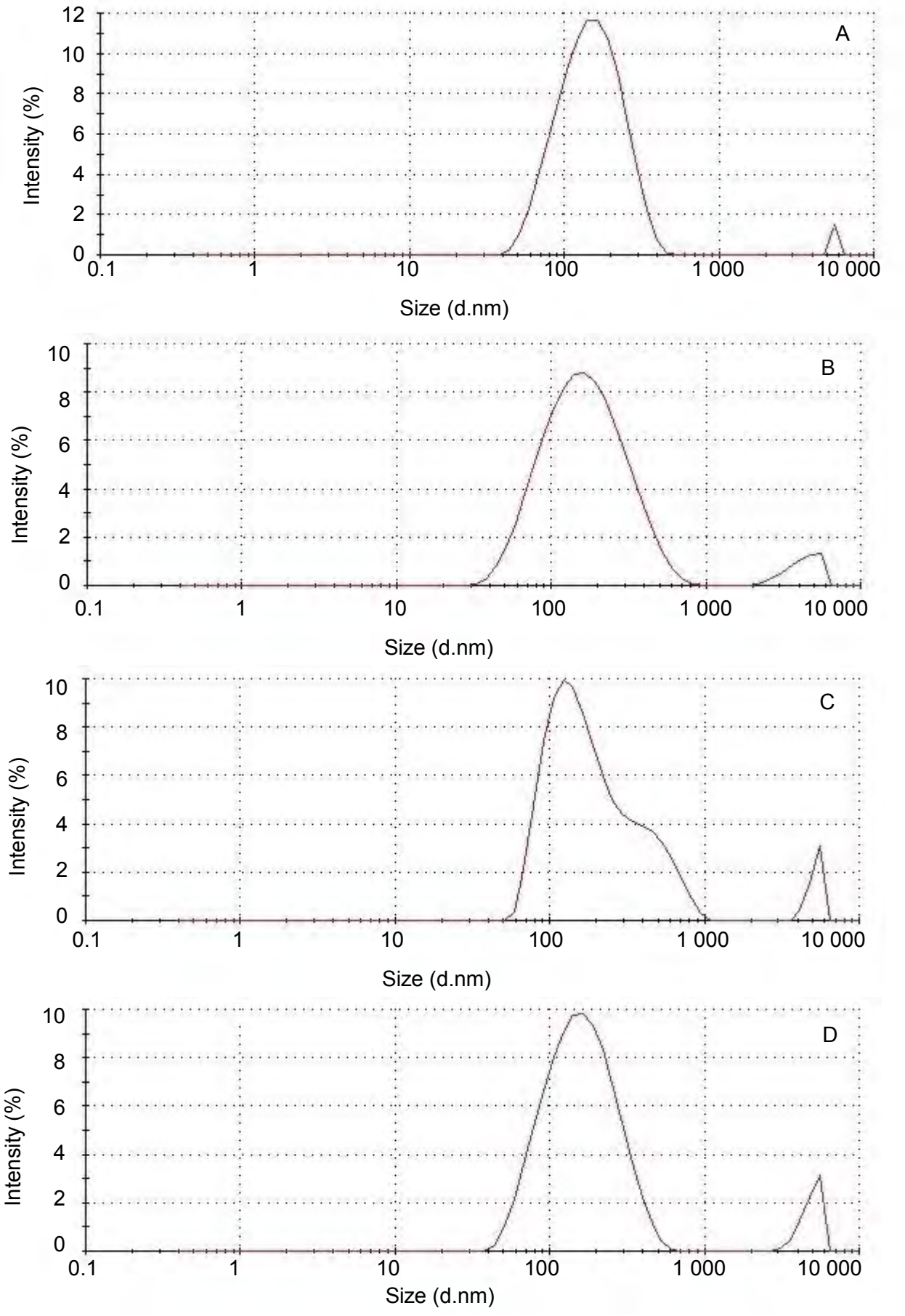

Figure C.16: Size distribution of the liposomes containing 0.2% artemisone (A), clofazimine (B), decoquinate (C), and all three APIs (D)

In Figure C.16 A–D it can be seen that a 0.2% API concentration yields a more uniform size distribution in liposomes, with a single, mostly symmetrical peak.

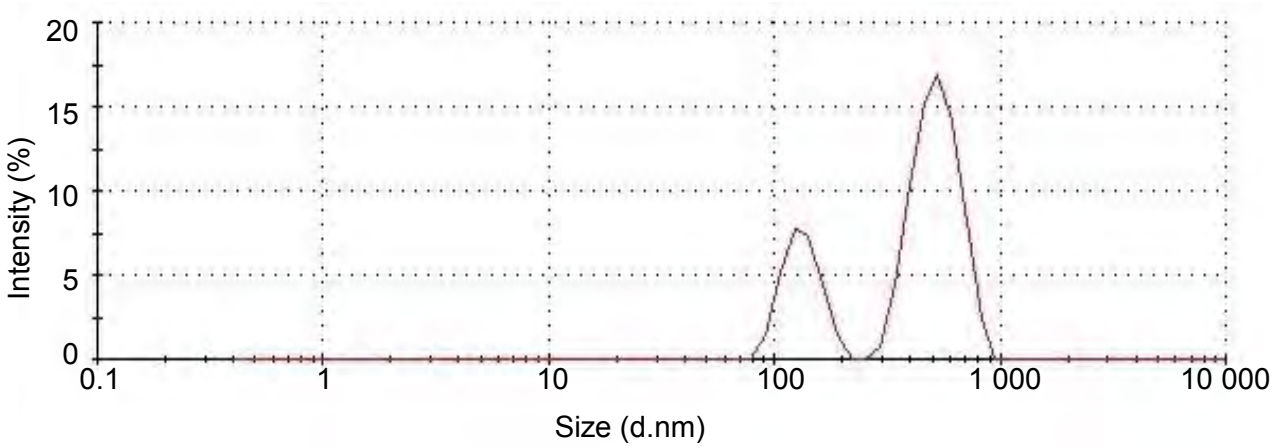

Figure C.17: Size distribution of the liposomes in the dispersion containing 0.4% of all three APIs

The liposomes with a 0.4% API concentration yielded two peaks (Figure C.17), indicating a non-uniform size distribution of the liposomes. It was confirmed with a PDI of 0.632, as can be seen in Table C.9.

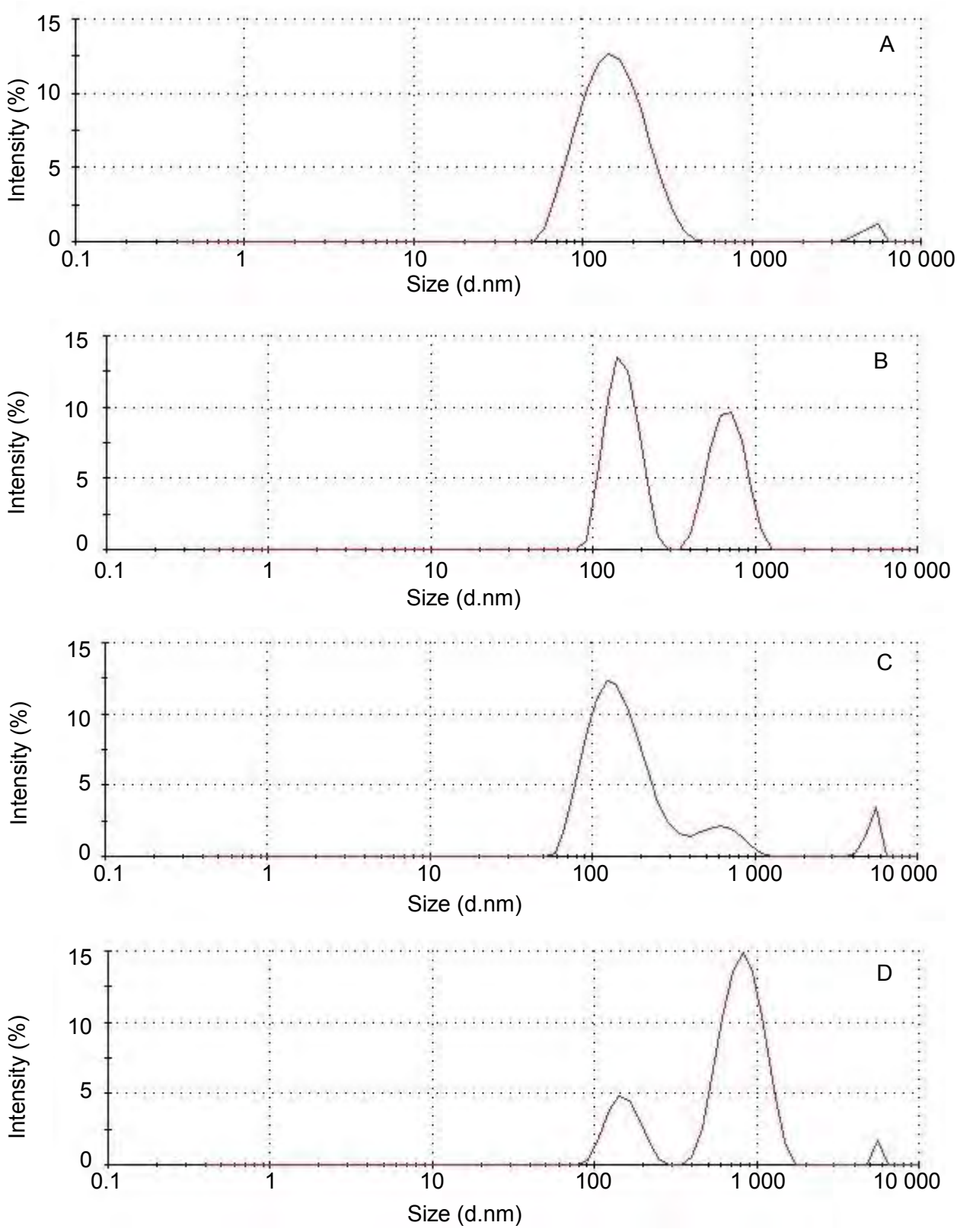

Figure C.18: Size distribution of the liposomes containing 1% artemisone (A), clofazimine (B), decoquinate (C), and all three APIs (D)

Observing the liposome dispersions containing 1% APIs, it was evident that only the dispersion containing artemisone delivered a uniform size distribution. The combination and clofazimine

dispersions, both displayed two peaks, which also portrayed the least favourable size distribution. PDI values in Table C.9 verified these results.

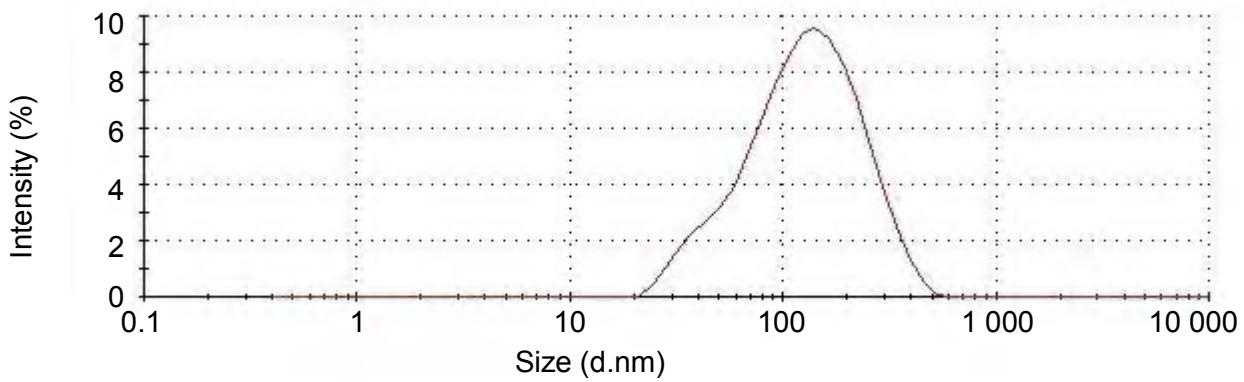

Figure C.19: Size distribution of transferosomes in the blank dispersion

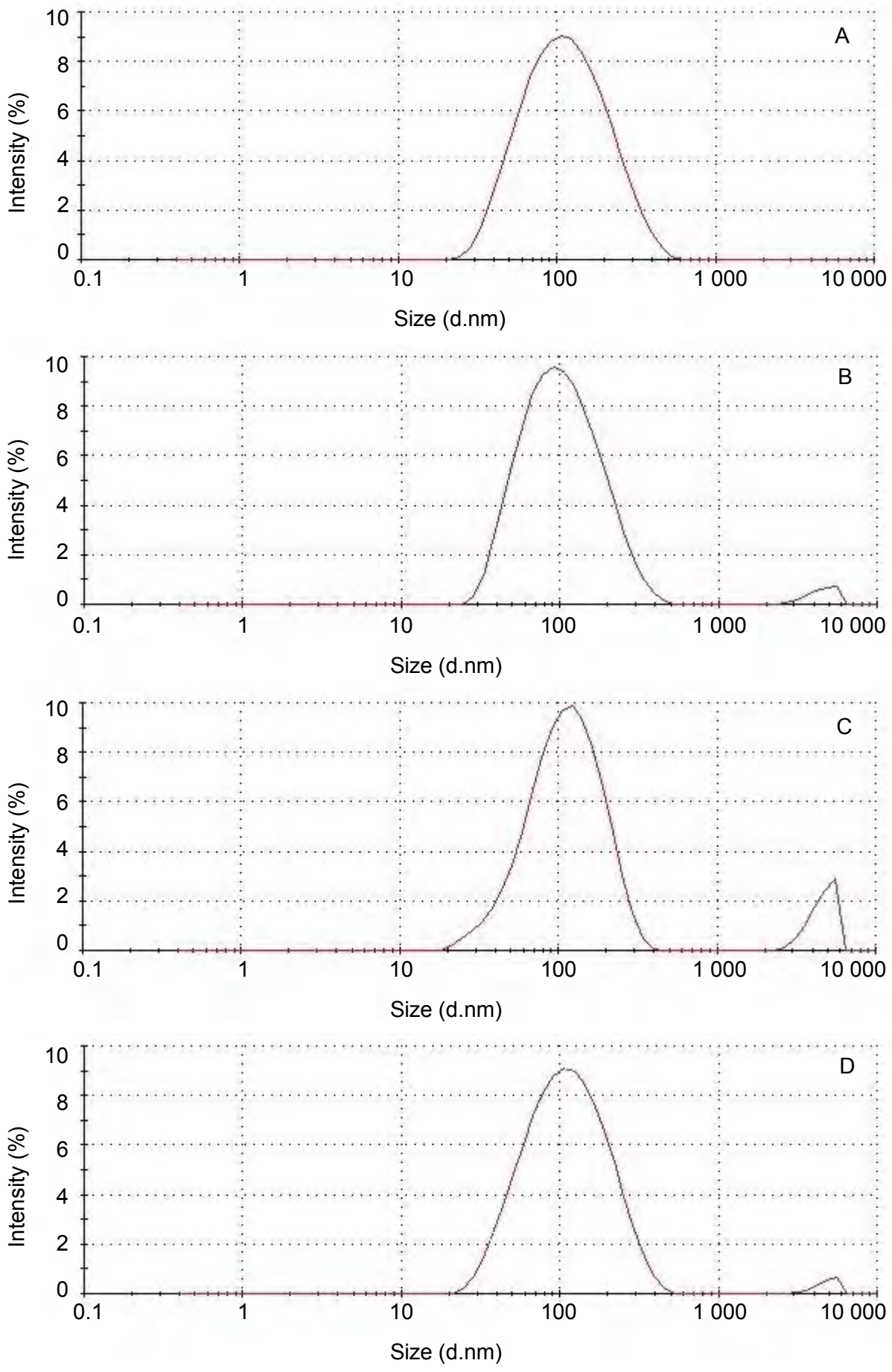

Figure C.20: Size distribution of the transferosomes containing 0.2% artemisone (A), clofazimine (B), decoquinate (C), and a combination of all three APIs (D)

All the dispersions (Figures C.20 A–D) containing 0.2% APIs in transferosomes delivered a good size distribution. The transferosomes were, on average, smaller compared to the liposomes.

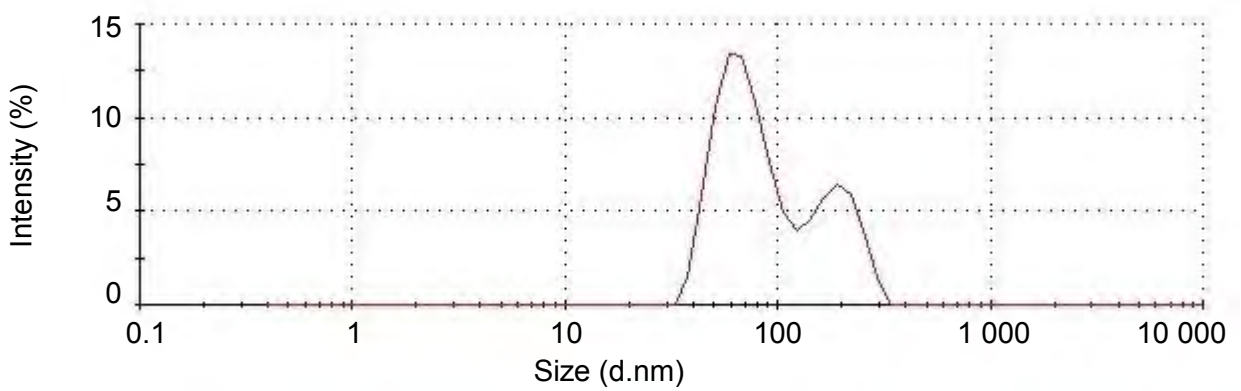

Figure C.21: Size distribution of the transferosomes in the dispersion containing 0.4% of all three APIs

The size distribution for the combination dispersion of 0.4% APIs was not ideal, with a double peak and a PDI of 0.461. Though this PDI on its own does not signify inadequate size distribution, the double peak obtained indicated that data requirements were not met.

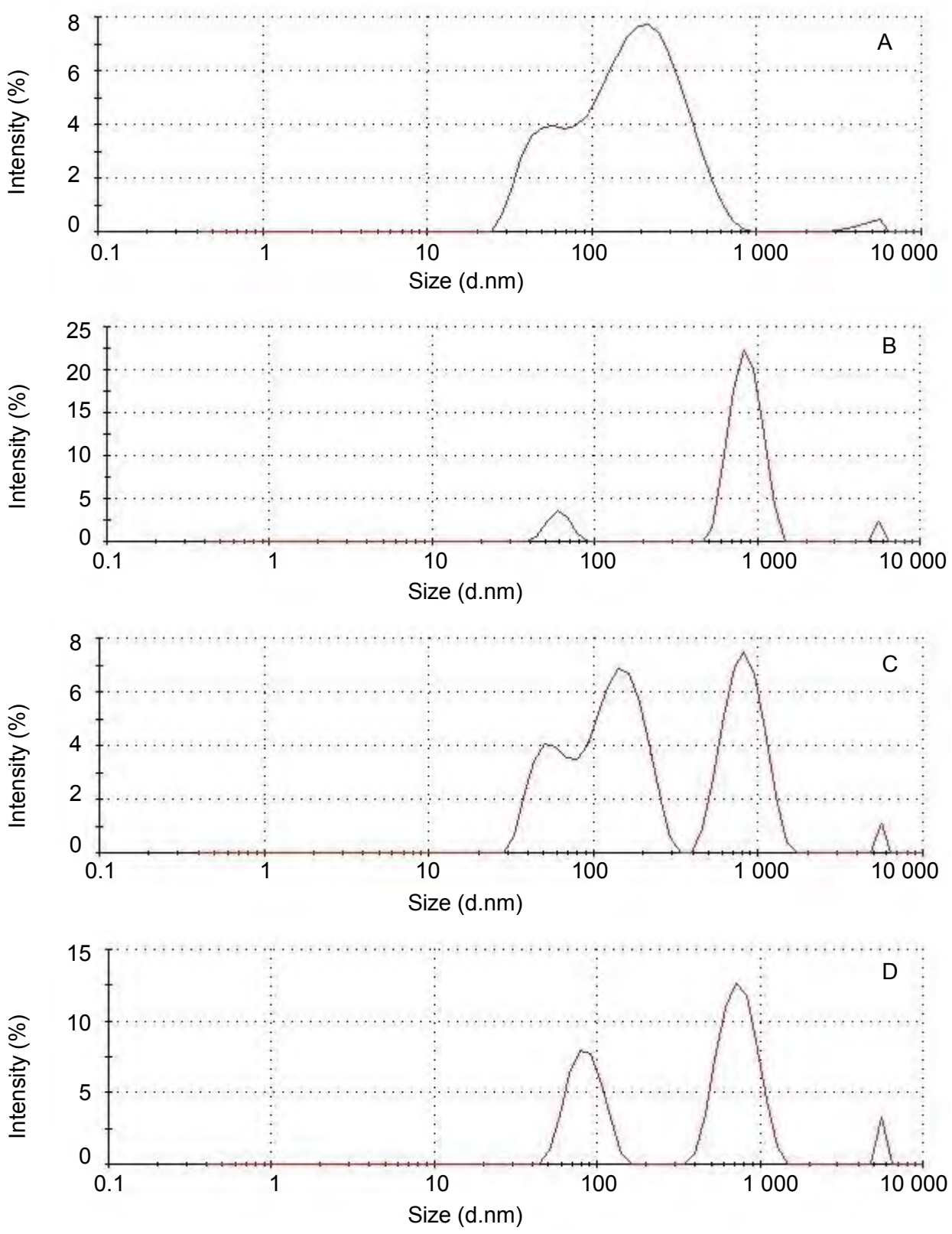

Figure C.22: Size distribution of the transferosomes containing 1% artemisone (A), clofazimine (B), decoquinate (C), and a combination of all three APIs (D)

Only the 1% artemisone (Figure C.22 A) in the transferosome dispersion delivered an acceptable size distribution with a PDI of 0.394. The other transferosome dispersions containing 1% API all displayed undesired size distribution, with the 1% clofazimine dispersion again being the least

acceptable dispersion with a PDI of 0.892. These results are confirmed with the varying amount and shapes of the peaks obtained.

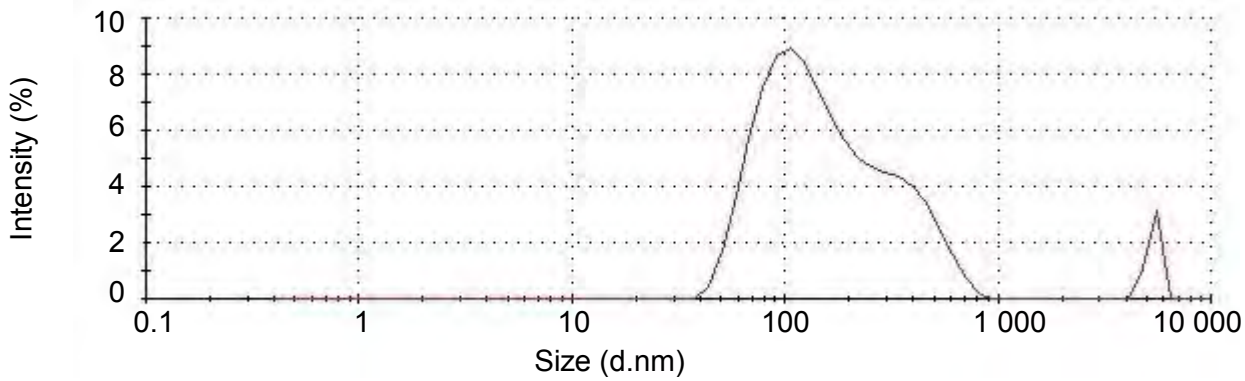

Figure C.23: Size distribution of niosomes in the blank dispersion

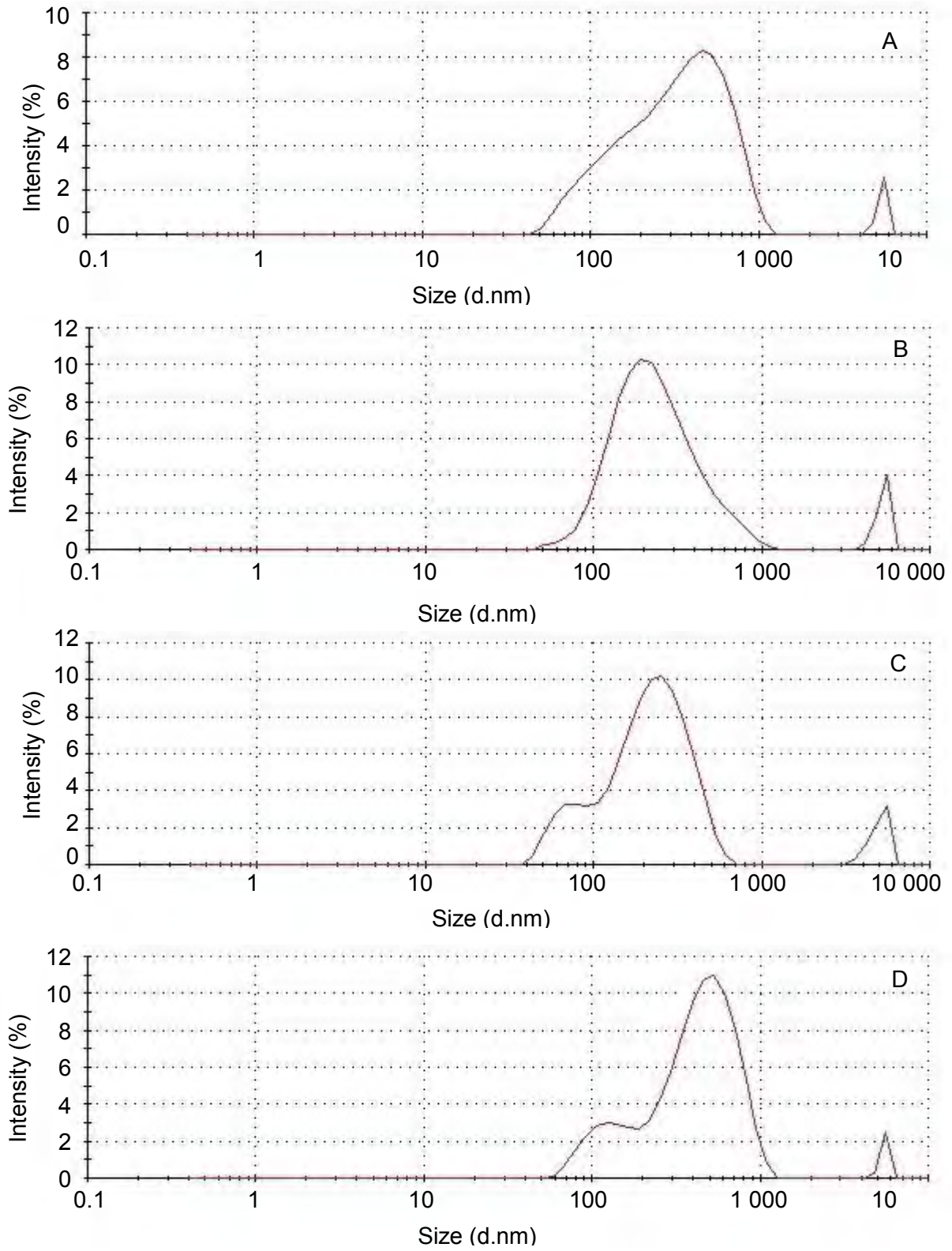

Figure C.24: Size distribution of the niosomes containing 0.2% artemisone (A), clofazimine (B), decoquinate (C), and a combination of all three APIs (D)

All the 0.2% API concentration dispersions met the quality criteria, although they did not deliver symmetrical peaks, but rather peaks with tails or a slight bump on one side. This can be affirmed by all the PDIs being more than 0.5, except for the 0.2% decoquinate in niosomes, which presented a PDI value of 0.465.

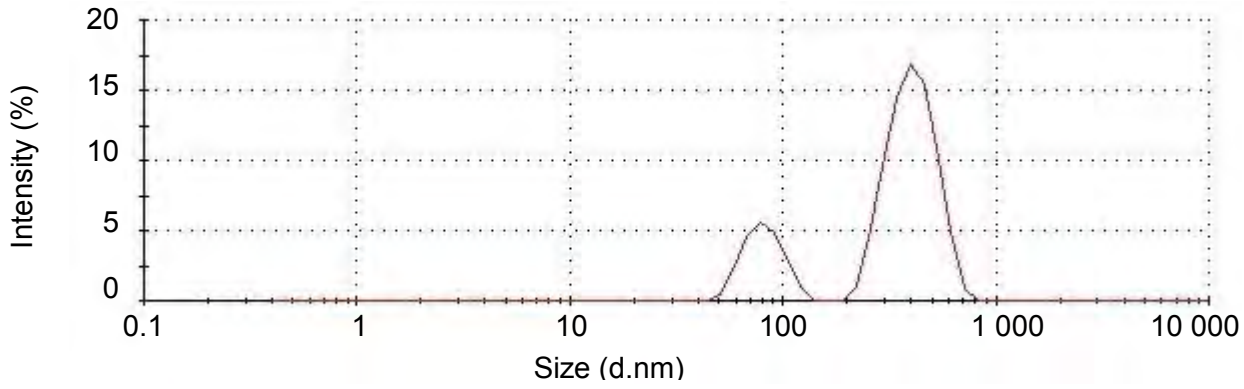

Figure C.25 Size distribution of the niosomes in the dispersion containing 0.4% of all three APIs

The size distribution for the combination dispersion containing 0.4% APIs, showed two peaks, indicating an unfavourable size distribution as was confirmed by a PDI of 0.621.

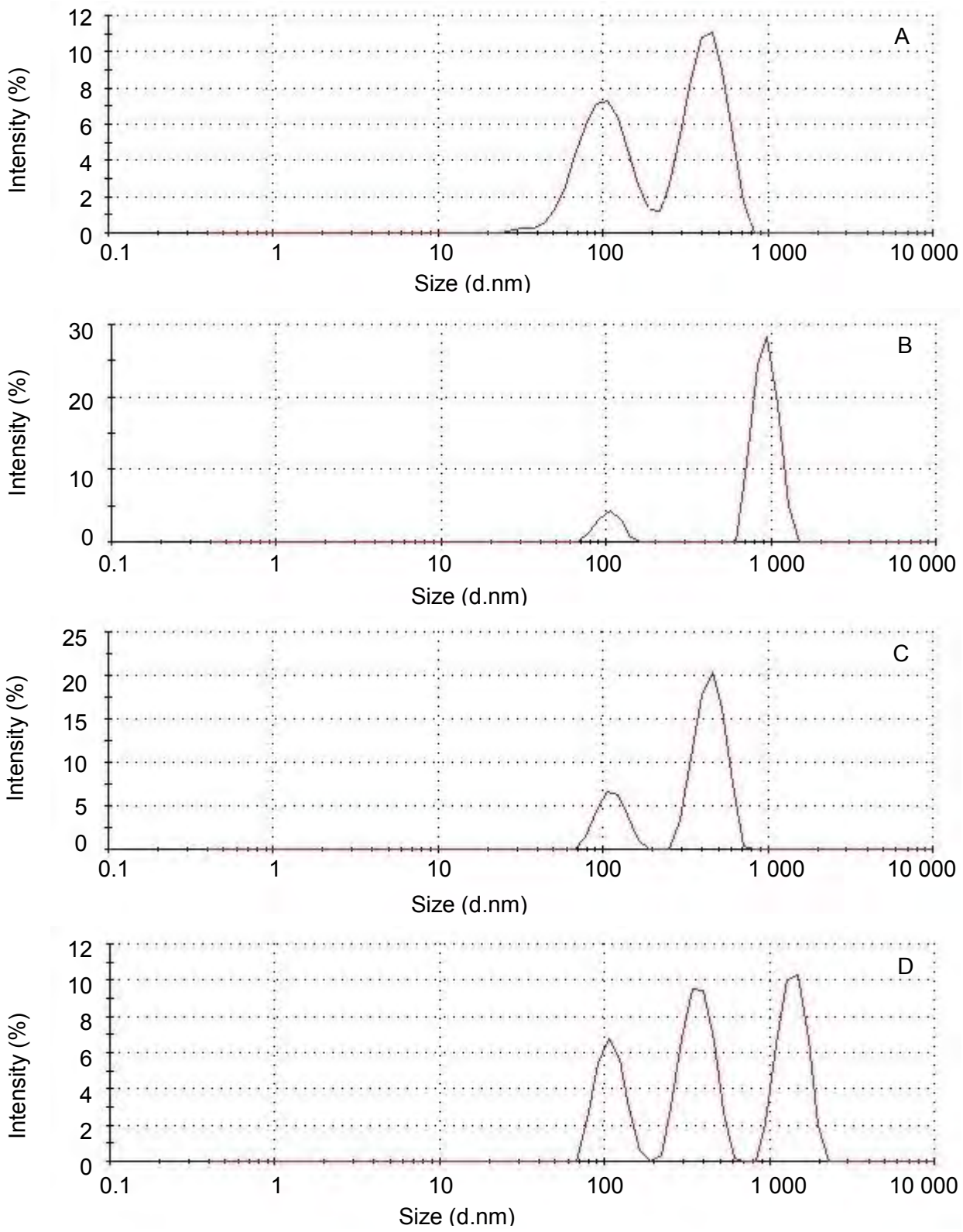

Figure C.26: Size distribution of the niosomes containing 1% artemisone (A), clofazimine (B), decoquinate (C), and a combination of all three APIs (D)

None of the 1% API concentration dispersions showed desirable size distributions. Sharp, double, and even triple peaks could be observed with PDI values as high as 0.939 and an average diameter of 2461 nm for the 1% clofazimine dispersion.

From the zeta-potential, size and size distribution data it is clear that of the three vesicles used, the niosomes delivered the least desirable data when the three selected APIs were incorporated into these dispersions. The liposomes, on the other hand, were more robust when the API concentration was increased.

C.5.4. pH

The pH was determined for each of the dispersions using a Mettler® Toledo pH meter (Mettler® Toledo International Inc., USA) with a Mettler® Toledo Inlab® 410 NTC electrode 9823 (Figure C.27). The pH of the skin's surface ranges from 5.4–5.9 and has a high buffering capacity against large pH changes. When applying a formulation with a pH higher than 9 or lower than 4, skin irritation occurs and the barrier function of the skin is compromised. Therefore, topical formulations should be buffered as close to the skin's surface pH as possible. Physiological pH of human skin can be influenced by the dissociation constant (pKa) of an API and cause dermal irritation when exposed to the skin for more than 24 h. A pKa of between 4–8 has shown minimal irritation (Paudel *et al.*, 2010). The pH values of the different dispersions were measured in triplicate and the averages calculated (Table C.10).

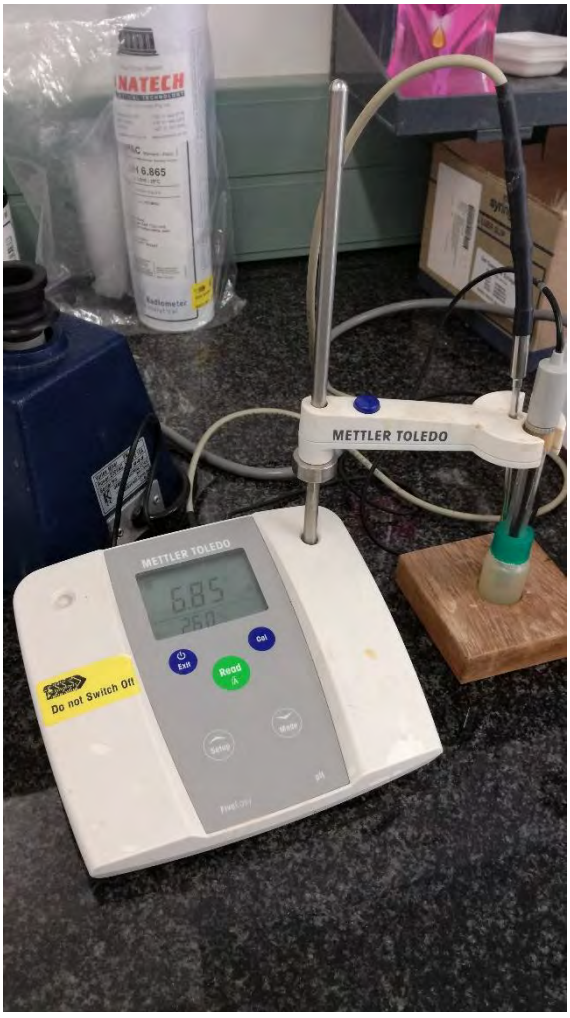

Figure C.27: Mettler® Toledo pH meter

Table C.10: Average pH measurements (n=3) of the different dispersions at room temperature (25±1.0°C)

Vesicle	Dispersions and concentration API		pH
Liposomes	Blank	0%	5.50
	ART	0.2%	5.67
	CLF		5.37
	DQ		5.72
	Combination		5.22
	Combination	0.4%	5.32
	ART	1%	5.51
	CLF		5.49
	DQ		5.51
	Combination		5.44
Transferosomes	Blank	0%	5.37
	ART	0.2%	5.58
	CLF		5.26
	DQ		5.60
	Combination		5.06
	Combination	0.4%	5.44
	ART	1%	5.36
	CLF		5.53
	DQ		5.34
	Combination		5.43
Niosomes	Blank	0%	5.19
	ART	0.2%	4.95
	CLF		5.17
	DQ		4.99
	Combination		5.53
	Combination	0.4%	5.29
	ART	1%	5.11
	CLF		5.24
	DQ		5.06
	Combination		5.75
No vesicles	ART	0.2%	5.63
	CLF		5.69
	DQ		5.69
	Combination		5.78
	Combination	0.4%	5.89
	ART	1%	5.68
	CLF		5.70
	DQ		5.67
	Combination		5.48

All the pH values collected were between 4 and 9 as preferred for topical application. Most of the values acquired were even between 5.4-5.9, the same as the skin pH, which are ideal.

C.5.5. VISCOSITY

The viscosity of the dispersions was measured using a Brookfield® Viscometer model DV2T™ (Stoughton, USA, Figure C.28) connected to a circulating water bath with a Brookfield® temperature controller to maintain the temperature in the water jacket at 25±1.0°C. A SC4-18 spindle was used for this study and set to 100 rpm, where the torque is approximately 20%. The spindle was immersed in the dispersion without causing a disturbance. Viscosity readings were taken every 10 s for 1 min. The experiment was performed in triplicate and the average viscosity for each dispersion was determined (Table C.11). Generally, it is assumed that more viscous formulations will inhibit or eliminate the skin partitioning and absorption of molecules within a formulation, partly due to poor skin distribution. This may not always be the case, since it was also found that thin layers of a more viscous formulation may enhance skin permeation due to the formation of an occlusive layer. Careful analysis of results is thus necessary to avoid false interpretations (Leite-Silva *et al.*, 2012). The viscosities of the dispersions were found to be very close to that of water, which has a viscosity of 1 cP (or mPa.s in SI units) at 20°C and 0.89 mPa.s at 25°C (Crittenden *et al.*, 2012).

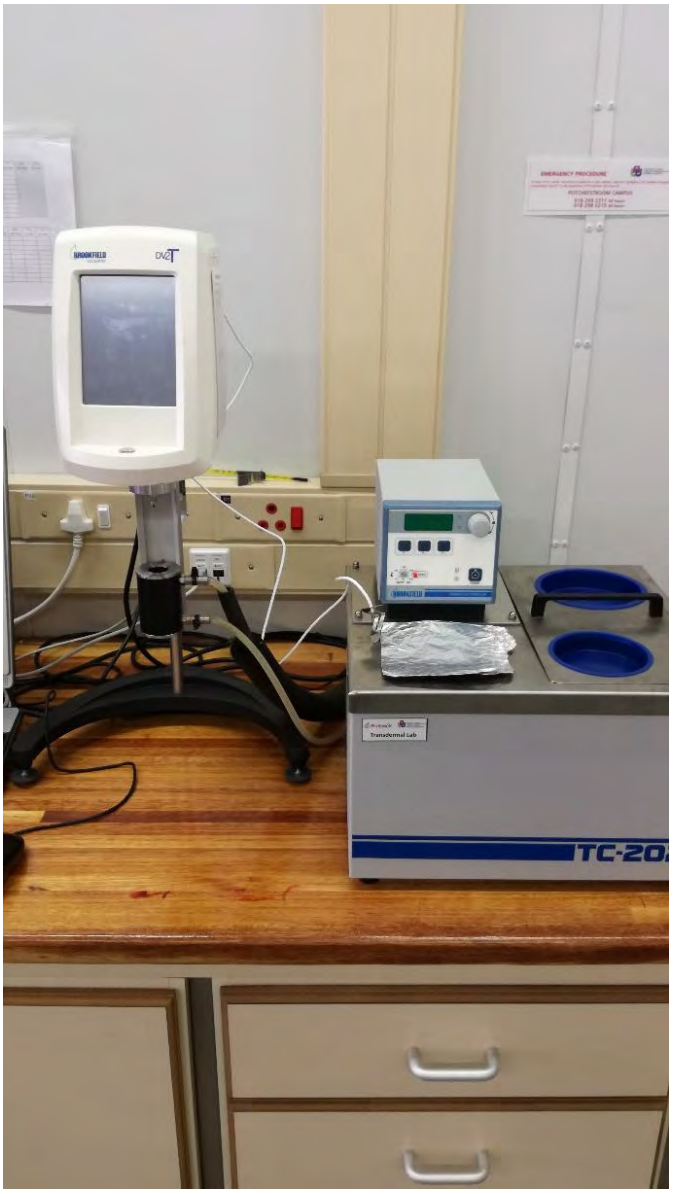

Figure C.28: A Brookfield® Viscometer used for measuring viscosity

Table C.11: The average viscosity (mPa.s, n=12) for the different dispersions at 25±1.0°C

Vesicle	Dispersions and concentration API		Viscosity (mPa.s)
Liposomes	Blank	0%	1.88
	ART	0.2%	2.14
	CLF		1.85
	DQ		1.98
	Combination		2.05
	Combination	0.4%	2.53
	ART	1%	1.89
	CLF		2.44
	DQ		1.87
	Combination		2.89
Transferosomes	Blank	0%	1.41
	ART	0.2%	1.45
	CLF		1.60
	DQ		1.48
	Combination		1.86
	Combination	0.4%	1.60
	ART	1%	1.45
	CLF		1.54
	DQ		1.47
	Combination		1.78
Niosomes	Blank	0%	1.71
	ART	0.2%	1.65
	CLF		1.84
	DQ		2.04
	Combination		2.15
	Combination	0.4%	1.65
	ART	1%	1.71
	CLF		1.66
	DQ		1.69
	Combination		1.99
No vesicles	ART	0.2%	1.32
	CLF		1.32
	DQ		1.43
	Combination		1.49
	Combination	0.4%	1.12
	ART	1%	1.15
	CLF		1.13
	DQ		1.14
	Combination		1.13

C.6. EFFICACY AGAINST TUBERCULOSIS

The dispersions were prepared and sent to the University of Stellenbosch for efficacy studies against *Mycobacterium tuberculosis* (*Mtb*). This was done as part of the Flagship Programme and because the University is equipped with a bio-hazardous level 3 laboratory where the specific methods for analysis were already established and validated. The studies were conducted by, and under supervision of Dr. Andile H Ngwane. Results obtained (Table C.13) from samples sent (Table C.12) to Stellenbosch are displayed below.

Table C.12: Dispersions prepared for efficacy against tuberculosis

API	Amount (mg) API per 10 ml dispersion	Liposomes (sample no)	Niosome (Sample no)	Transferosomes (Sample no)
Artemisone	100	L1	N6	T11
Clofazimine	100	L2	N7	T12
Decoquinate	100	L3	N8	T13
All 3 APIs	300	L4	N9	T14
Blank	0	L5	N10	T15

As indicated in Table C.12, each of the 10 ml samples contained 100 mg of a selected API and the combination dispersions contained a total amount of 300 mg API (i.e. 100 mg of each API). The dispersions were supplied in liquid form with instructions that if a little sediment formed on the bottom of the test tube, gentle shaking of the tube would be necessary prior to administration onto the cell cultures. These samples were stored at 4°C for no longer than 7 days prior to testing.

The minimum inhibitory concentrations (MICs) against H37Rv of clofazimine, artemisone and decoquinate ranged from 1.2, > 80 and > 10 µM, respectively, and therefore the dispersions containing a single API were tested at 1.2, 80 and 10 µM, correspondingly. During the experiment, clofazimine was considered the most active API of the selected APIs and thus its MIC (Lechartier and Cole; 2015) was utilised as a bench mark for the other APIs.

Mtb H37Rv was inoculated for 1:10 dilution from stock cultures in a 25 cm^2 tissue flask for 4 days and the optical density at 600 nm (OD_{600}) reached 0.6. These cultures were sub-cultured at 1:5 dilutions into 75 cm^2 until OD_{600} reached 0.3 for the experiment. Aliquots of 10 ml from this culture were transferred into 25 cm^2 tissue flasks. Each culture flask was treated with the desired amount of API (as mentioned above) for 24 h as well as one control flask with no API.

At the time of treatment, 200 µl of the untreated culture flasks were used to count colony forming units (CFU) per ml. After 24 h of inoculation at 37°C, 200 µl samples from each culture flask were centrifuged, suspended and washed twice in 7H9 broth and CFUs were plated on 7H11 agar.

After 12–15 days the CFUs were counted and the percentage inhibition of the different dispersions calculated as shown in Table C.13. The control culture was used as the positive control at 0% inhibition and INH as the negative control (83% inhibition) since INH is a known API against tuberculosis.

Table C.13: Efficacy against *Mycobacterium tuberculosis* H37Rv

Sample no	Average CFU/ml	Standard deviation	% inhibition (relative to control)
L1	2.87×10^8	± 3.8	31
L2	2.4×10^8	± 5.1	42
L3	2.97×10^8	± 4.6	29
L4	2.42×10^8	± 2.1	42
L5	3.68×10^8	± 1.8	12
N6	3.35×10^8	± 4.1	20
N7	1.98×10^8	± 2.5	52
N8	3.83×10^8	± 2.6	8
N9	2.6×10^8	± 2.0	38
N10	2.68×10^8	± 4.9	36
T11	2.6×10^8	± 4.7	38
T12	2.57×10^8	± 2.1	38
T13	2.7×10^8	± 1.8	35
T14	3.67×10^8	± 2.3	12
T15	2.4×10^8	± 3.1	42
L4 (0.5 MIC)	3.13×10^8	± 2.9	25
N9 (0.5 MIC)	3.25×10^8	± 2.1	22
T14 (0.5 MIC)	3.17×10^8	± 3.7	24
Isoniazid (INH)	7.0×10^7	± 2.8	83
Artemisone	3.0×10^8	± 2.3	28
Decoquinate	3.0×10^8	± 2.3	28
Clofazimine	2.4×10^8	± 3.3	42
Clofazimine (0.5 MIC)	3.57×10^8	± 3.0	14
Control culture	4.17×10^8	± 4.6	0

Inhibition data for artemisone, decoquinate and clofazimine in the different dispersions as well as the clean API can be seen in Table C.14.

Table C.14: Percentage inhibition of the three APIs, combination of the APIs and also when encapsulated into selected vesicles

	API	Liposomes	Niosomes	Transferosomes
Artemisone	28	31	20	38
Clofazimine	42	42	52	38
Decoquinate	28	29	8	35
Combination	-	42	38	12
Blank	-	12	36	42

C.6.1. EFFECT OF EMPTY VESICLES ON TUBERCULOSIS CELLS

The blank dispersions (i.e. vesicles without any API) also displayed some activity, which is a possible indication that the cholesterol added to the liposome and niosome dispersions may be responsible for the activity observed. This is surprising since literature states that *Mtb* uses host cholesterol for growth and energy, and also that a high cholesterol diet can enhance the bacterial load (Ouellet *et al.*, 2011). Transferosomes do not contain cholesterol and consist mainly of phosphatidylcholine (PC). However, transferosomes were found to be most active when the inhibitory effects of the three blank dispersions were compared. Acton (2012) noted that PC was found to have no activity against tuberculosis. PC, however, is very lipophilic and the data might be explained by an occlusion effect the lipids might have on the cells, causing cell death.

C.6.2. EFFECTIVITY OF A COMBINATION OF APIs AGAINST TUBERCULOSIS

From Table C.14 it is clear that the combination of these APIs did not enhance activity against tuberculosis when compared to the single-API dispersions. When we firstly look at artemisone, the activity of the API (28%) was higher than when formulated in niosomes (20%), though the transferosome dispersion showed highest activity (38%). Clofazimine showed highest activity in the niosome dispersion (52%), though the clean API's activity (42%) was higher than when formulated in transferosomes (38%). Decoquinate showed the overall lowest activity of 8% inhibition when formulated in niosomes. Decoquinate in transferosomes (35%) was higher than when formulated in liposomes (29%) and clean decoquinate (28%).

C.6.3. EFFECT OF THE TYPE OF VESICLE USED TO ENCAPSULATE THE APIs ON TUBERCULOSIS CELLS

Of the three combination dispersions, liposomes delivered the most significant inhibition (42%), as well as a very narrow inhibition distribution (dispersions L1–L3). The combination dispersion of niosomes delivered an inhibition percentage (38%) not significantly lower than that of the liposome dispersion. The combination dispersion in transferosomes, however, delivered the

lowest percentage inhibition (12%). Though no definite conclusions can be made from this data, further investigation into clofazimine would be beneficial, since clofazimine powder and clofazimine in the different vesicles delivered the highest percentage inhibition. Clofazimine is a documented anti-tuberculous API that was investigated in the 1990s, but which caused skin pigmentation due to ceroid lipofuscinosis (Job *et al.*, 1990; Smith *et al.*, 2016). Further investigation into methods to deliver it locally, while limiting the severe skin pigmentation might be advantageous for CTB. There is a definite difference between data from the three vesicles, and since vesicles are ideal for carrying APIs with non-ideal properties through/into skin, further investigation into vesicles would be beneficial.

C.7. SUMMARY

Liposomes, transferosomes and niosomes were successfully prepared and viewed with TEM. Size and size distribution were conducted with the Zetasizer Nano ZS and results showed that the vesicle sizes were within the limits stated by literature. The transferosome dispersions displayed the smallest vesicles that were, furthermore, confirmed by literature. These results are an indication that transdermal delivery through this dosage form may be further improved. Liposomes delivered good size distribution and sizes only slightly larger than transferosomes. On the other hand, the prepared niosomes showed the largest vesicles, which will possibly improve topical delivery. Niosome sizes, however, were more broadly distributed and presented a high PDI (> 0.6), indicating that the method of production may need some alteration. The time sonicated was subsequently lengthened, which in turn caused a more favourable size distribution. The zeta-potential values for the dispersions were more than -30 mV, with a few exceptions, which may indicate that those dispersions are unstable and vesicles may aggregate or leak API after some time.

The dispersions were manufactured to have a pH-value of no less than five since this is the lowest pH at which dispersions can be topically applied to avoid skin irritation. The pKa values of the APIs were used to calculate at which pH they would be mostly unionised as unionised particles pass more readily across lipid membranes. The proportion of unionised API determines the effective membrane gradient for diffusion, although ionised particles may also diffuse across the stratum corneum to a limited extent (Barry, 2002; Vaidyanathan e*t al.*, 1985). A pH approximately 5.5 was attained by preparing a 1.5 strength phosphate buffer solution. It was determined that a lower pH would produce higher concentrations unionised particles, but the pH had to be kept above 5.

Viscosity was subsequently determined for each dispersion in order to later interpret skin diffusion data, since viscosity influences spreadability of a formulation and the formulation may cause an occlusion effect if the viscosity is high. The viscosity measured for the dispersions was close to that of water, which ensured easy and even spreadability.

When tested against tuberculosis, activity was found for all of the dispersions, with clofazimine in niosomes being the highest and decoquinate in niosomes the lowest. Combination of the APIs did not increase activity compared to the single-API dispersions. Surprisingly, the blank dispersions also showed some activity, which cannot be fully explained in the case of the transferosomes. When omitting the combination dispersions, transferosomes and liposomes delivered narrowly distributed data with the transferosomes being slightly higher.

REFERENCES

ACTON, A. 2012. Tuberculosis: New insights for the Healthcare Professional: 2011 Edition. https://books.google.co.za/books?id=HjIlcl5deFUC&pg=PT115&lpg=PT115&dq=phosphatidylcholine+against+tuberculosis&source=bl&ots=0JoLR8YpCS&sig=OzipfIV7NY4ch0WKTsMs4f8MTeQ&hl=en&sa=X&ved=0ahUKEwiymNK9h4bWAhUDD8AKHVIiDO8Q6AEISjAE#v=onepage&q=phosphatidylcholine%20against%20tuberculosis&f=false Date of access: 2 September 2017.

AGARWAL, R., KATARE, O.P. & VYAS, S.P. 2001. Preparation and in vitro evaluation of liposomal/niosomal delivery systems for antipsoriatic drug dithranol. *International journal of pharmacceutics.*, 288:43-52.

ANDERSON MATERIALS INC. 2016. Differential scanning calorimetry (DSC) thermal analysis. http://www.andersonmaterials.com/dsc.html Date of access: 21 July 2016.

BARRY, B. 2002. Transdermal drug delivery. (*In* Aulton, M. E., *ed*. Pharmaceutics: The science of dosage form design. 2nd ed. New York: Churchill Livingstone. p. 499-533.)

BARRY, B.W. 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. *Eur. J. Pharm. Sci.*, 14:101-114.

COLLOIDAL DYNAMICS: Leaders in colloid measurement. 1999. Electroacoustics tutorials: The zeta potential. 4p.

CRITTENDEN, J.C., TRUSSEL, R.R., HAND, D.W., HOWE, K.J. & TCHOBANOGLOUS, G., eds. 2012. MWH's water treatment: Principles and design. 3rd ed. New Jersey: John Wiley & Sons, Inc. 1901p.

DRAGICEVIC-CURIC, N. & MAIBACH, H.I., eds. 2015. Percutaneous penetration enhancers - Chemical methods in penetration enhancement: Drug manipulation strategies and vehicle effects. 1st ed. New York: Springer. 341p.

DT: DISPERSION TECHNOLOGY INC. 2013. Zeta potential, short tutorial. http://www.dispersion.com/zeta-potential-short-tutorial Date of access: 17 May 2016.

EL MAGHRABY, G.M.M., WILLIAMS, A.C. & BARRY, B.W. 2000. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. *International journal of pharmaceutis*, 196:63-74.

FAUSKE & ASSOCIATES, LLC. 2016. Thermal stability. http://www.fauske.com/chemical-industrial/thermal-stability Date of access: 21 July 2016.

GILLET, A., LECOMTE, F., HUBERT, P., DUCAT, E., EVRARD, B. & PIEL, G. 2011. Skin penetration behaviour of liposomes as a function of their composition. *European journal of pharmaceutics and biopharmaceutics*, 79:43-53.

ISACCHI, B., BERGONZI, M.C., GRAZIOSO, M., RIGHESCHI, C., PIETRETTI, A., SEVERINI, C. & BILIA, A.R. 2012. Artemisinin and artemisinin plus curcumin liposomal formulations: enhanced antimalarial efficacy against *Plasmodium berghei*-infected mice. *European journal of pharmaceutics and biopharmaceutics*, 80:528-534.

JAIN, S., JAIN, V. & MAHAJAN, S.C. 2014. Lipid based vesicular drug delivery systems. *Advances in pharmaceutics*, 2014:1-12.

JOB, C.K., YODER, L., JACKOBSON, R.R. & HASTINGS, R.C. 1990. Skin pigmentation from clofazimine therapy in leprosy patients: A reappraisal. *Journal of the American academy of dermatology*, 23(2):236-241.

KAUR, I.P. & SINGH, H. 2014. Nanostructured drug delivery for better management of tuberculosis. *Journal of controlled release,* 18:36-50.

KUMAR, G.P. & RAJESHWARRAO, P. 2011. Nonionic surfactant vesicular systems for effective drug delivery – an overview. *Acta pharmaceutica sinica B*, 1(4):208-219.

LECHARTIER, B. & COLE, S.T. 2015. Mode of action of clofazimine and combination therapy with benzothiazinones against *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy*, 59(8):4457-4463.

LEITE-SILVA, V.R., DE ALMEIDA, M.M., FRADIN, A., GRICE, J.E. & ROBERTS, M.S. 2012. Delivery of drugs applied topically to the skin: Advanced formulation. http://www.medscape.org/viewarticle/771038_3 Date of access: 18 May 2016.

MAHALE, N.B., THAKKAR, P.D., MALI, R.G., WALUNJ, D.R. & CHAUDHARI, S.R. 2012. Niosomes: novel sustained release nonionic stable vesicular systems – an overview. *Advances in colloid and interface science*, 183-184:46-54.

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*, 3:587-592.

MALVERN INSTRUMENTS LTD. 2004. Zetasizer nano series: User manual (MAN0317; Issue 1.1). Malvern Instruments Ltd.: England. 270p.

MALVERN INSTRUMENTS LTD. 2016. Differential scanning calorimetry: confident characterisation of protein stability. http://www.malvern.com/en/products/technology/differential-scanning-calorimetry/ Date of access: 21 July 2016.

MALVERN INSTRUMENTS LTD. 2017. An introduction to zeta-potential. https://www2.warwick.ac.uk/fac/cross_fac/sciencecity/programmes/internal/themes/am2/booking/particlesize/mrk654-01_an_introduction_to_zeta_potential_v3.pdf Date of access: 23 May 2017.

MANCONI, M., SINICO, C., CADDEO, C., VILA, A.O., VALENTI, D. & FADDA, A.M. 2011. Penetration enhancer containing vesicles as carriers for dermal delivery of tretinoin. *International journal of pharmaceutics*, 412:37-46.

MANCONI, M., CADDEO, C., SINICO, C., VALENTI, D., MOSTALLINO, M.C., LAMPIS, S., MONDUZZI, M. & FADDA, A.M. 2012. Penetration enhancer-containing vesicles: composition dependence of structural features and skin penetration ability. *European journal of pharmaceutics and biopharmaceutics*, 82:352-359.

NEW, R.R.C, *ed*. 1990. Liposomes: a practical approach. 1st ed. New York: Oxford university press. 301p.

OUELLET, H., JOHNSTON, J.B. & ORTIZ DE MONTELLANO, P.R. 2011. Cholesterol catabolism as a therapeutic target in *Mycobacterium tuberculosis*. *Trends in microbiology*, 19(11):530-539.

PAUDEL, K.S., MILEWSKI, M., SWADLEY, C.L., BROGDEN, N.K., GHOSH, P. & STINCHCOMB, A. 2010. Challenges and opportunities in dermal/transdermal delivery. *Therapeutic delivery*, 1(1): 109-131.

SHAJI, J. & LAL, M. 2014. Preparation, optimization and evaluation of transferosomal formulation for enhanced transdermal delivery of a COX-2 inhibitor. *International journal of pharmaceutics and pharmaceutical sciences*, 6(1):467-477.

SHARMA, S.K., CHAUHAN, M. & ANILKUMAR, N. 2009. Span-60 niosomal oral suspension of fluconazole formulation and *in vitro* evaluation. *Journal of pharmaceutical research and health care*, 1(2):142-156.

SMITH, M., ACCINELLI, A., TEJADA, F.R. & KHAREL, M.K. 2016. Chapter 28 – Drugs used in Tuberculosis and leprosy. *Side effects of drugs annual,* 38:283-293.

TA INSTRUMENTS. 2017. Microcalorimetry. http://www.tainstruments.com/pdf/brochure/MicrocalorimetryBrochure.pdf Date of access: 29 August 2017.

TAVANO, L., GENTILE, L., ROSSI, C.O. & MUZZALUPO, R. 2013. Novel gel-niosomes formulations as multicomponent systems for transdermal drug delivery. *Colloids and surfaces B Biointerfaces.*, 110:281-288.

VAIDYANATHAN, R., CHAUBAL, M.G. & VASAVADA, R.C. 1985. Effect of pH and solubility on in vitro skin penetration of methotrexate from a 50% v/v propylene glycol-water vehicle. *International journal of pharmaceutics*, 25(1):85-93.

VANNIASINGHE, A.S., BENDER, V. & MANOLIOS, N. 2009. The potential of liposomal drug delivery for the treatment of inflammatory arthritis. *Seminars in arthritis and rheumatism*, 39:182-196.

VARUN, T., SONIA, A. & BHARAT, P. 2012. Niosomes and liposomes – Vesicular approach towards transdermal drug delivery. *International journal of pharmaceutical and chemical sciences*, 1(2):632-644.

VITEZ, I.M., NEWMAN, A.W., DAVIDOVICH, M. & KIESNOWSKI, C. 1998. The evolution of hot-stage microscopy to aid solid-state characterizations of pharmaceutical solids. *Thermochimica acta*, 324:187-196.

WIECHERS, J.W. 2008. Science and Applications of Skin Delivery Systems. 1st ed. Carol Stream: Allured Publishing Corporation. 511p.

ZHAO, C., TONG, K., TAN, J., LIU, Q., WU, T. & SUN, D. 2014. Colloidal properties of montmorillonite suspensions modified with polyetheramine. *Colloids and surfaces A: Physicochemical and engineering aspects,* 457:8-15.

ZHAO, Y-Z., LU, C-T., ZHANG, Y., XIAO, J., ZHAO, Y-P., TIAN, J-L., XU, Y-Y., FENG, Z-G. & XU C-Y. 2013. Selection of high efficient transdermal lipid vesicle for curcumin skin delivery. *International journal of pharmaceutics*, 454:302-309.

Annexure D

TRANSDERMAL DIFFUSION STUDIES OF DIFFERENT VESICLE DISPERSIONS

D.1. INTRODUCTION

Transdermal delivery of APIs across the skin into the systemic circulation, or topical delivery into the skin has been found a convenient route of administration (Dwivedi *et al.* 2016; Luo & Lane, 2015). This route has many advantages, such as enhanced patient compliance, reduced dosing and avoiding the hepatic first-pass effect, to name only a few. This route, however, comes with many challenges as well. For example, only a limited amount of APIs can be delivered through this route due to the formidable barrier function of the stratum corneum (Ahad *et al.*, 2017; Haine *et al.*, 2017). APIs have to be both lipophilic and hydrophilic to pass into/through the skin, they have to be potent enough to pass this layer in therapeutic amounts, and the molecules should be small in size and have no charge (Malinovskaja-Gomez *et al.*, 2017).

To improve the permeability of non-ideal APIs, many penetration enhancers have been investigated (Ashtikar *et al.*, 2016). For this study the use of vesicles has been applied for the topical delivery of artemisone, clofazimine and decoquinate. Vertical Franz diffusion cells (Figure B.1) were used in studying *in vitro* transdermal delivery, which was done to determine permeation of the APIs through dermatomed black female skin over a period of 12 h. A Franz cell consists of a donor compartment (top) and a receptor compartment (bottom). The receptor compartment is filled with phosphate buffer solution (pH 7.4) which represents human blood and the donor compartment is filled with a specific formulation to be tested. The skin, on which the formulations are tested, is subsequently clamped between the two compartments.

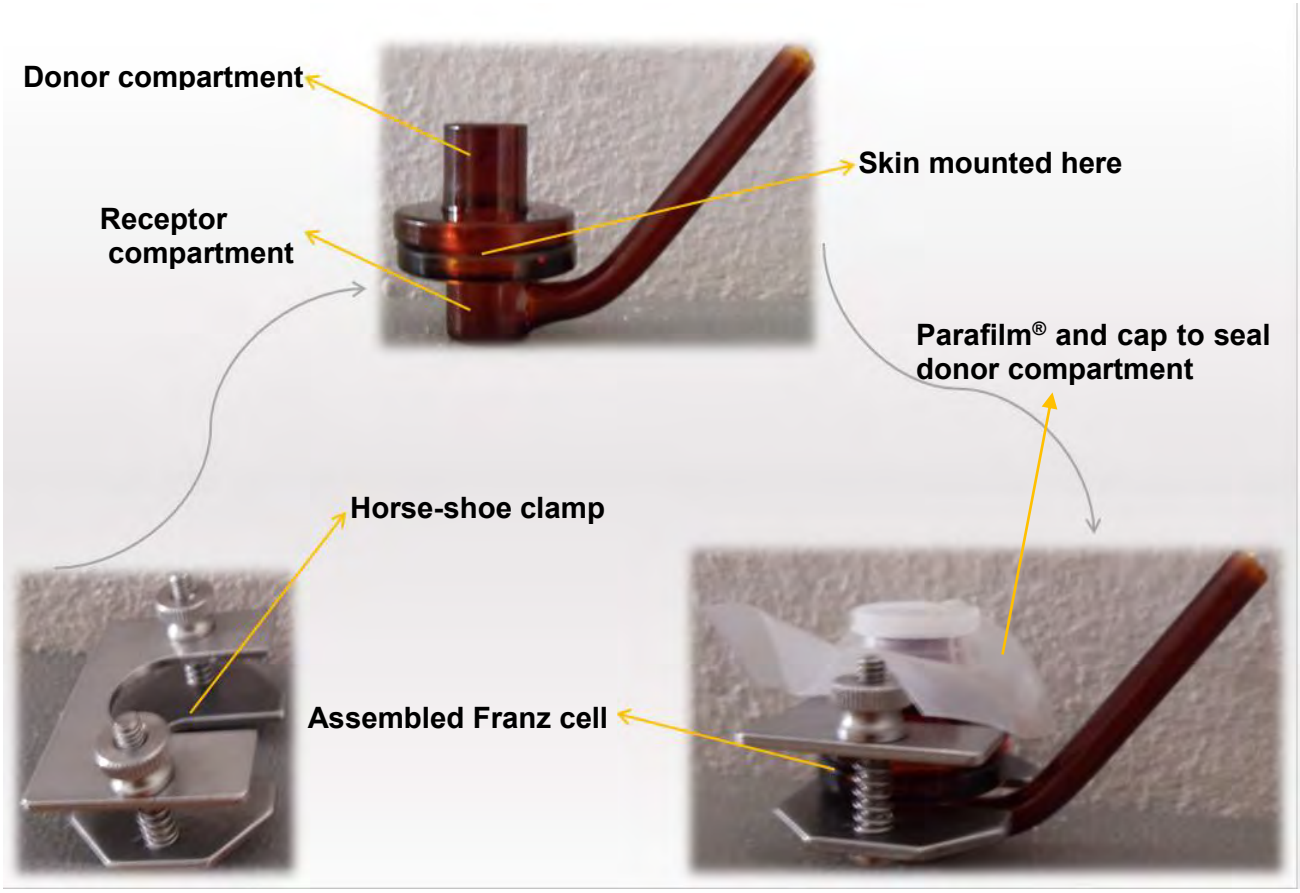

Figure D.1: Vertical Franz diffusion cell components and assembly

D.2. METHODS AND MATERIALS

D.2.1. PREPARATION OF PHOSPHATE BUFFER SOLUTION

Phosphate buffer solution was prepared by dissolving 1.571 g sodium hydroxide (NaOH) in 393.4 ml deionised water and adding this solution slowly to a solution of 6.81 g potassium dihydrogen phosphate (KH_2PO_4) in 250 ml deionised water. The pH of the buffer solution was adjusted to 7.4 with either 2 M H_3PO_4 (to lower pH) or 2 M NaOH (to increase the pH). The phosphate buffer solution was then stored in a refrigerator at 2–4°C for no longer than three months.

D.2.2. SKIN PREPARATION

Skin obtained to conduct permeation studies was donated by anonymous female donors who had undergone abdominoplastic surgery. Caucasian skin is normally used, but for this study, black skin was procured due to the scarcity of skin. The thickness of the stratum corneum in Caucasian and black skin is generally the same, though black skin has more layers and is thus more compact (Richards *et al.*, 2003). This might suggest that results from this study may result in lower values than which would have been obtained from Caucasian skin. Ethical approval for the procurement

and preparation of the skin was issued by the Research Ethics committee of the North-West University with the reference number NWU-00114-11-A5.

Full thickness skin was removed from the donors (Figure D.2) and frozen at -20°C for no longer than 6 months. Prior to conducting the studies, the skin was left to thaw and thereafter visually inspected for defects such as stretch marks and/or holes/large hair follicles. The skin was cut into pieces of approximately 2 cm x 4 cm and 400 µm thick with a Zimmer® electric dermatome model 8821 (Figure D.3). The skin pieces were placed on Whatman® filter paper, covered in aluminium foil and stored in a freezer at -20°C until the studies were conducted (within 24 h).

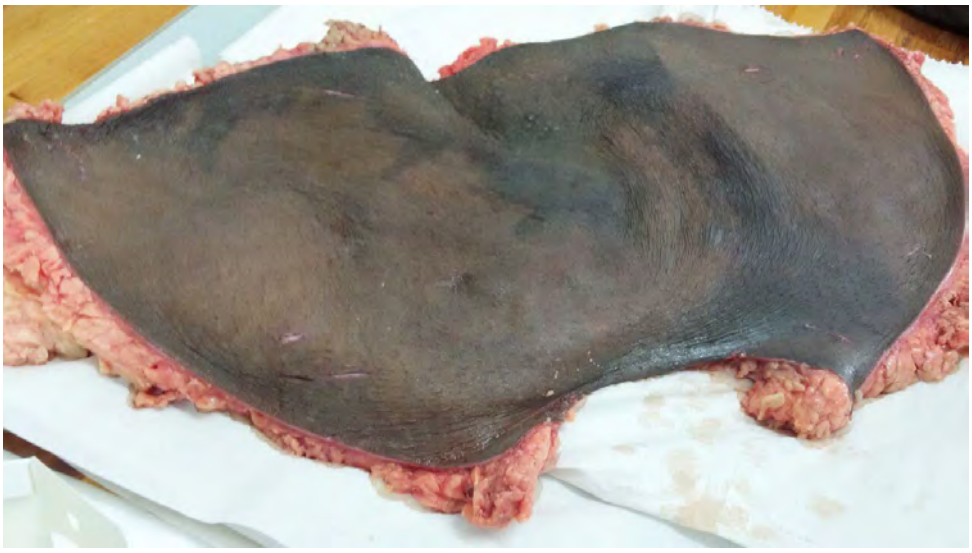

Figure D.2: Full thickness black skin as received from donor

Figure D.3: Zimmer® electric dermatome model 8821

D.2.3. SKIN DIFFUSION STUDIES

Ten amber vertical Franz diffusion cells were used, of which eight contained a specific vesicle dispersion and the other two a placebo dispersion containing no API. A punched skin circle was mounted between the two compartments with the stratum corneum facing up towards the donor compartment, with an available diffusion area of approximately 1.075 cm^2. The receptor phase consisted of 2 ml phosphate buffer solution (pH 7.4) with a Teflon-coated magnetic stirrer; the receptor compartment was filled, ensuring that no air bubbles which could possibly hinder diffusion, formed underneath the skin. The donor phase contained 1 ml of the dispersion and was closed with Parafilm® and a cap (Figure D.1) to prevent evaporation of the dispersion applied. The cell system's temperature was maintained at 37°C using a water bath (Figure D.4). Dow Corning® high vacuum grease was used to seal the Franz cells to avoid leakage of the buffer and dispersion; where after the Franz cell was securely fastened using a horse-shoe clamp (Figure D.1).

Figure D.4: A Grant® JB series water bath equipped with a magnetic stirrer plate

A pilot study was conducted using three Franz cells to determine buffer extraction times for these dispersions, since the APIs used are highly lipophilic and no diffusion of these APIs into the receptor phase (hydrophilic) was anticipated. The entire receptor phase was extracted every two hours for twelve hours and replaced with fresh buffer (37°C) to ensure sink conditions. No API concentrations were noted in the receptor phase; however, concentrations were measured in the skin. It was thus decided to extract the receptor phase only once at twelve hours. The samples were assayed immediately using an Agilent® 1100 series HPLC to determine the API concentration in the receptor phase.

D.2.4. TAPE STRIPPING

After the 12 h diffusion studies, the skin circles were removed from the Franz cells and fixed onto a board on Whatman® filter paper. All excess dispersion was dabbed from the skin surface with a clean paper towel. For the tape stripping, fifteen strips of clear 3M Scotch® tape were used and an extra two strips for cleaning. The strips were used to remove the stratum corneum-epidermis, which was then put in a polytop containing 5 ml absolute ethanol and placed in the fridge for approximately 8 h. The remaining dermis of the diffusion area (circular imprint of the donor phase is indicative of this area) was cut into small pieces, to enlarge the surface area, and also transferred into a polytop with 5 ml absolute ethanol and stored between 2-8°C for approximately 8 h. Thereafter, the ethanol was filtered, using a 45 µm filter, and analysed by HPLC.

D.2.5. HPLC ANALYSIS

The analysis of the samples was performed using an Agilent® 1100 Series HPLC in a controlled environment (25°C). The instrument was equipped with an Agilent® 1100 pump, diode array detector, an autosampler injector module and Chemstation Rev. A.10.02 software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA). A Restek Ultra C_{18} fully endcapped reversed phase column (250 x 4.6 mm, 5 µm) was used with 100 Å pores, a 20% carbon load (Restek corporation, Bellefonte, US); a pH range of 2.8–8; and a temperature limit of 80°C.

Gradient elution was used with mobile phase A consisting of 0.005 M sodium octanesulphonic-acid (pH 3.5) and mobile phase B of HPLC grade acetonitrile. The flow rate was set to 1.0 ml/min with mobile phase A at 35% and mobile phase B at 65% for 2 min, followed by a gradient shift 10% (mobile phase A) / 90% (mobile phase B) over an interval of 4 min. After 10 min the initial gradient conditions were regained to 35% (mobile phase A) / 65% (mobile phase B). A default injection volume of 20 µl was used, the runtime was set to 15 min and the UV detection was set to 210 and 284 nm. Ethanol (99.7%) was used as solvent during method validation.

The sample injections which had contact with the skin, blocked the flow cell a few times and the method was subsequently amended for skin studies. After every two skin sample injections, deionised water was injected. Mobile phase C contained pure deionised water and was set to 1 ml/min and a runtime of 5 min.

D.3. RESULTS AND DISCUSSION

D.3.1. SKIN DIFFUSION STUDIES AND TAPE STRIPPING

Skin diffusion studies were only conducted on the combination dispersions containing 1% APIs, as a severe scarcity of skin prohibited further studies. Upon investigation of the APIs to be used

it was clear that diffusion through the skin would be difficult due to the high log P values of these APIs. This was also the reason for utilising vesicles as a carrier system, though the main focus was only for topical delivery, because that is where cutaneous tuberculosis resides. Of all the skin diffusion studies conducted, no API was obtained in the receptor fluid for any of the different dispersions. The data represented in this section will, thus, only be for the tape stripping done thereafter. Figures D.5 and D.6 illustrate the concentrations (µg/ml) of clofazimine and decoquinate, respectively, that were present in the stratum corneum-epidermis (SCE) and epidermis (ED) for the liposome dispersion.

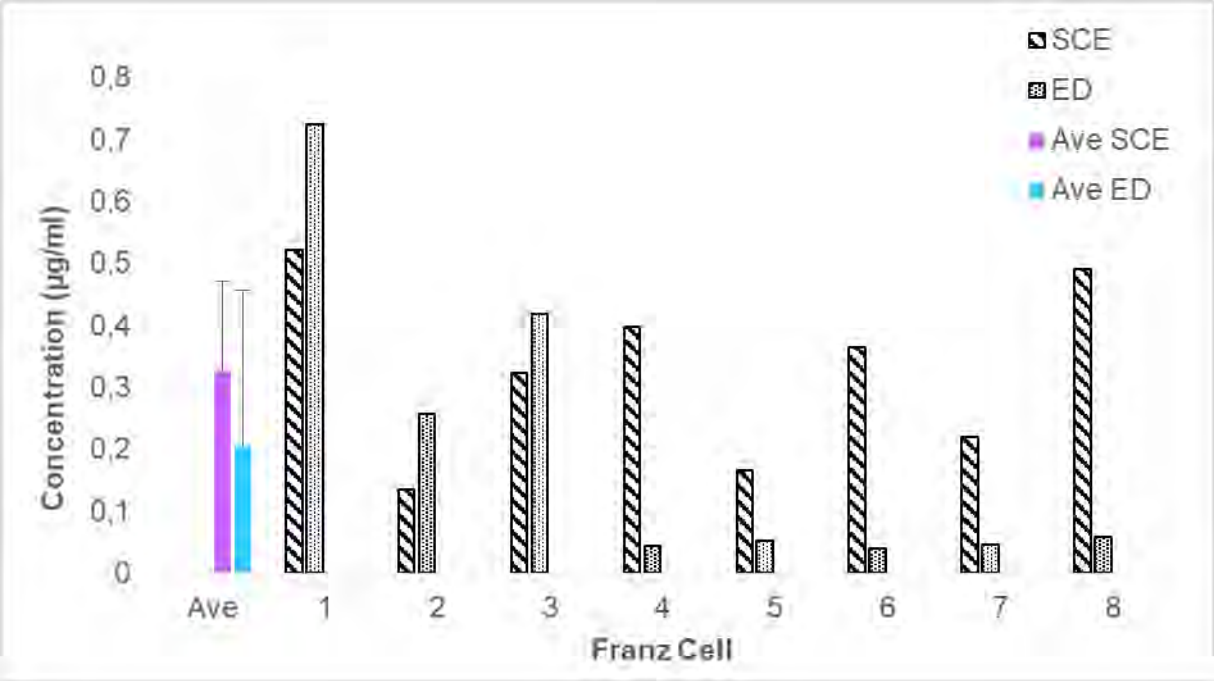

Figure D.5: Average concentration of clofazimine for the liposome dispersion in the individual Franz cells

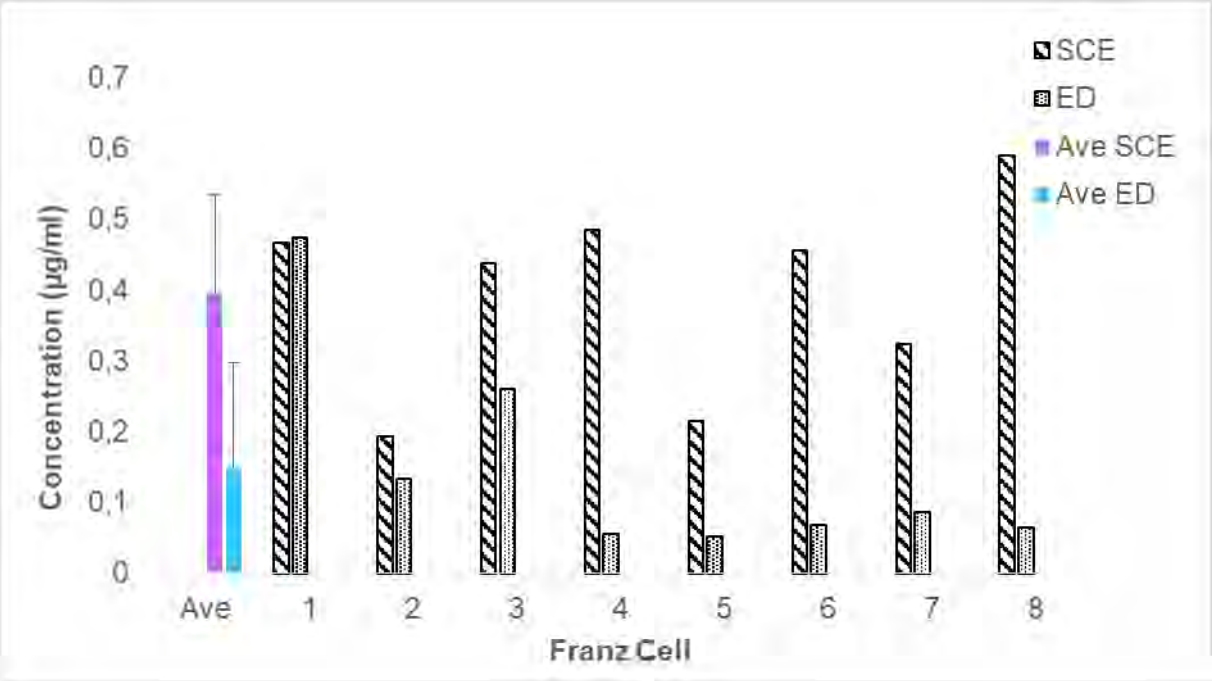

Figure D.6: Average concentration of decoquinate for the liposome dispersion in the individual Franz cells

The average decoquinate concentration in the SCE (0.396 µg/ml) was higher than the clofazimine concentration (0.328 µg/ml). Overall, the API concentrations in the SCE were higher compared to the concentrations present in the ED. This may be partly explained by the fact that the 1% dispersions created significant sediment on the skin during skin diffusion studies, which was extremely difficult to clean, and more than the designated one tape-strip for cleaning was suggested. The concentration of clofazimine present in the ED (0.205 µg/ml) was higher than the concentration of decoquinate in the ED (0.1497 µg/ml). Figures D.7 and D.8 illustrate the data obtained from the transferosome dispersion.

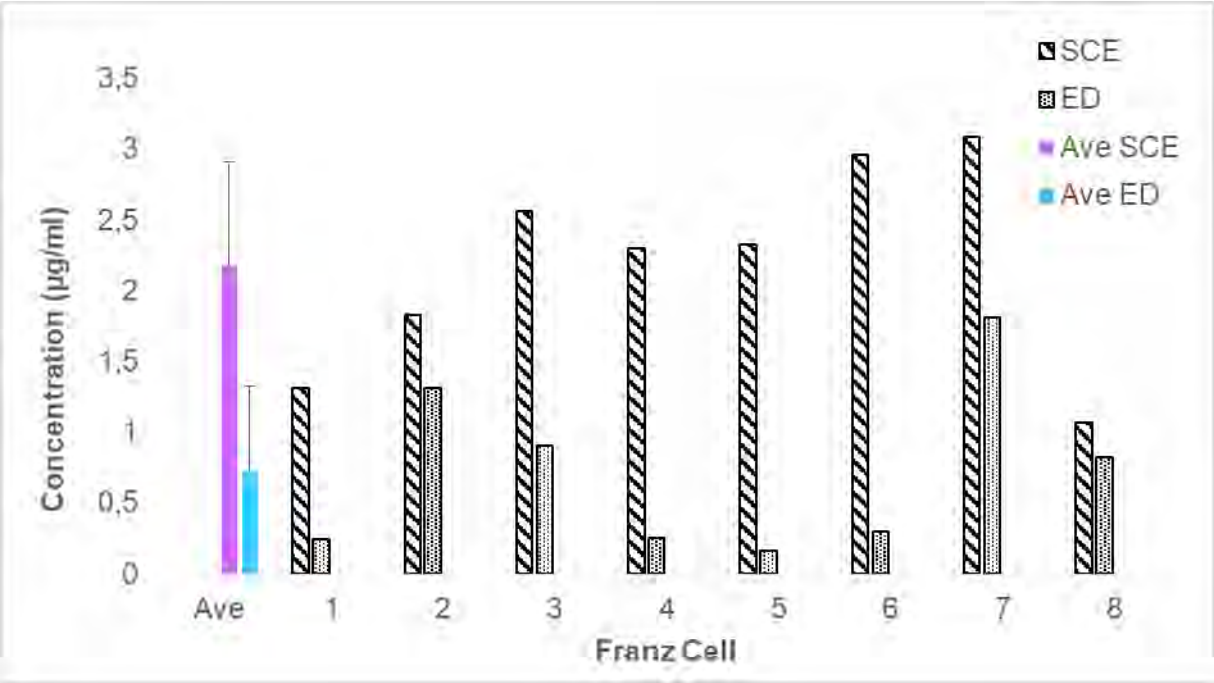

Figure D.7: Average concentration of clofazimine for the transferosome dispersion in the individual Franz cells

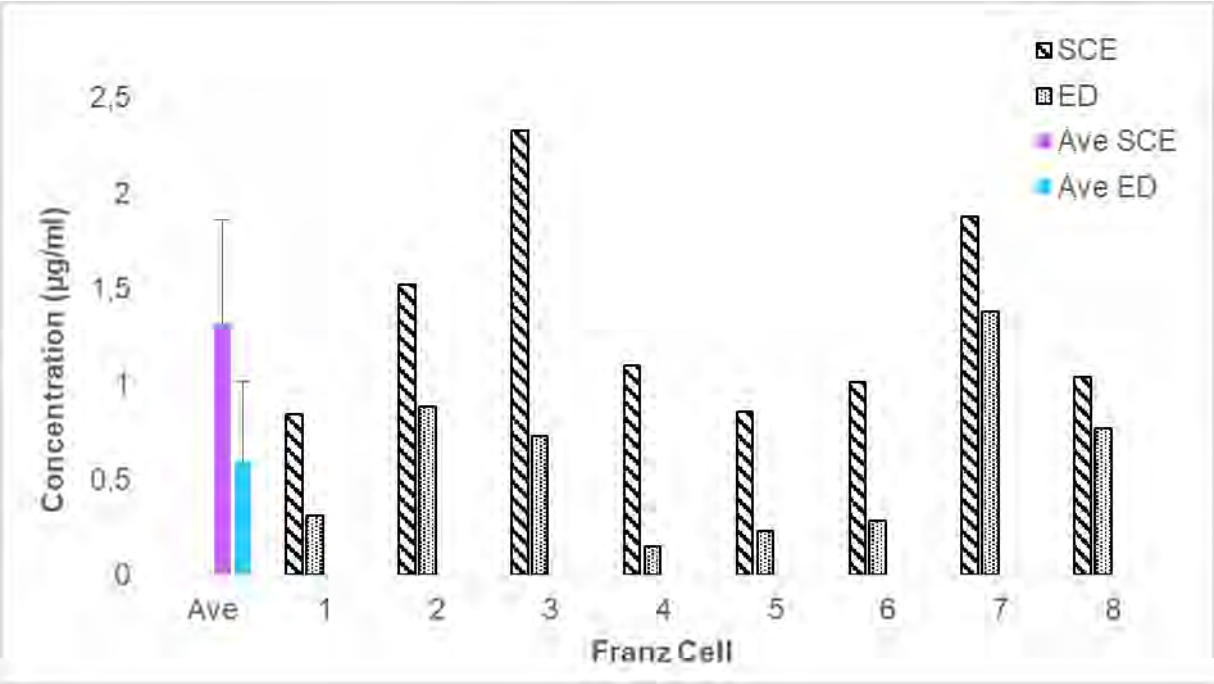

Figure D.8: Average concentration of decoquinate for the transferosome dispersion in the individual Franz cells

Overall, more API was found in the SCE, than in the ED, with the average clofazimine concentration (2.183 µg/ml) being higher than the average decoquinate concentration (1.322 µg/ml) present in the SCE. The average clofazimine concentration (0.726 µg/ml) in the ED was also higher than the average concentration decoquinate (0.594 µg/ml) for the

transferosome dispersion. Figures D.9 and D.10 depict data gathered from the niosome dispersion.

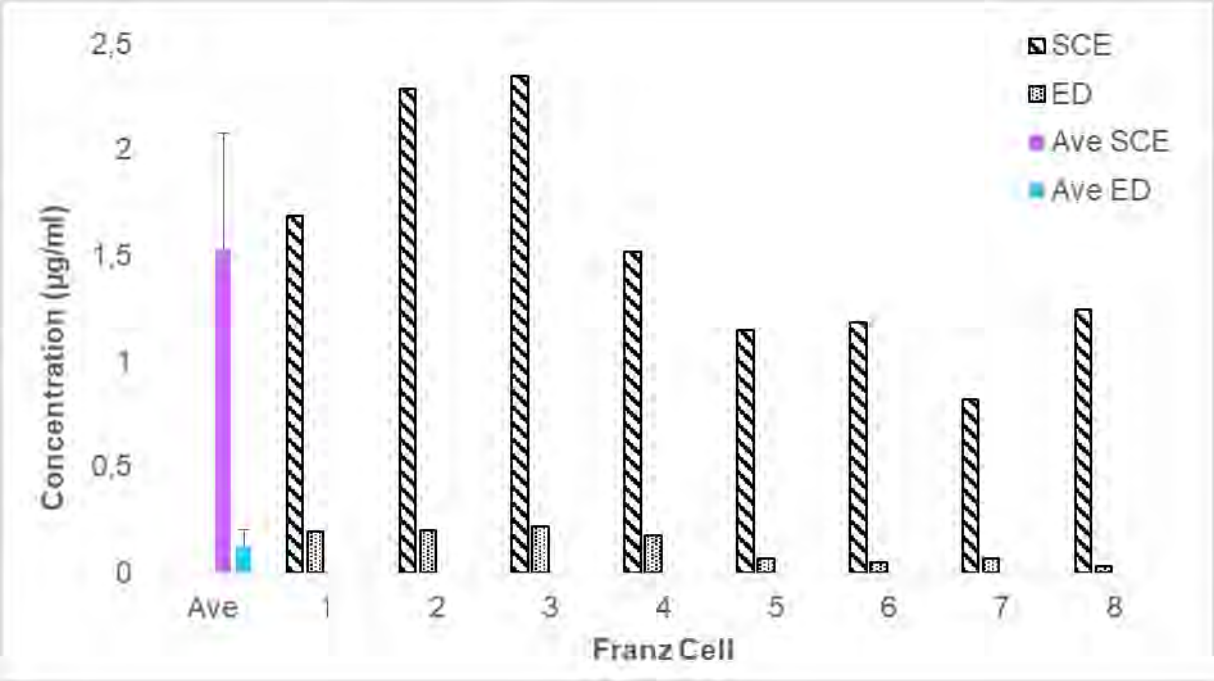

Figure D.9: Average concentration of clofazimine for the niosome dispersion in the individual Franz cells

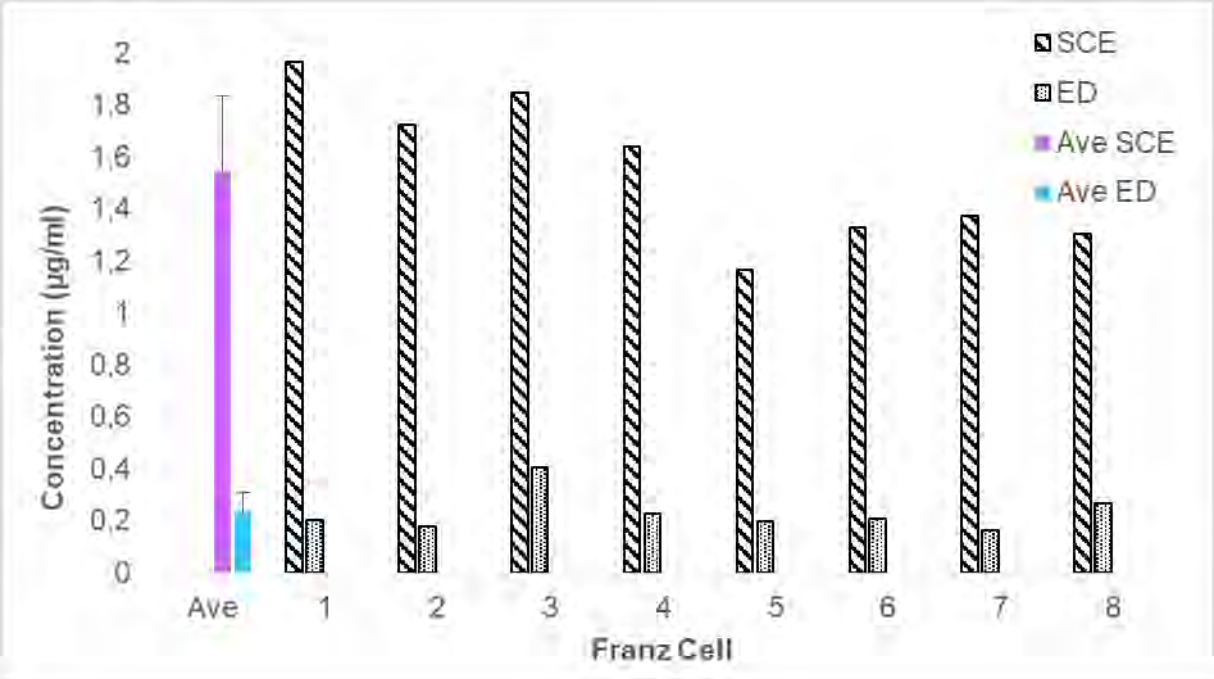

Figure D.10: Average concentration of decoquinate for the niosome dispersion in the individual Franz cells

The average concentration of API present in the SCE was relatively higher than the concentration in the ED. The average clofazimine concentration (1.531 µg/ml) in the SCE (Figure D.9) was

slightly lower than the average decoquinate concentration (1.544 µg/ml, Figure D.10), whereas the average concentration decoquinate (0.231 µg/ml) in the ED was relatively higher than the average clofazimine concentration (0.126 µg/ml). Figure D.11 and D.12 depict data obtained from the dispersion containing only the APIs and no vesicles.

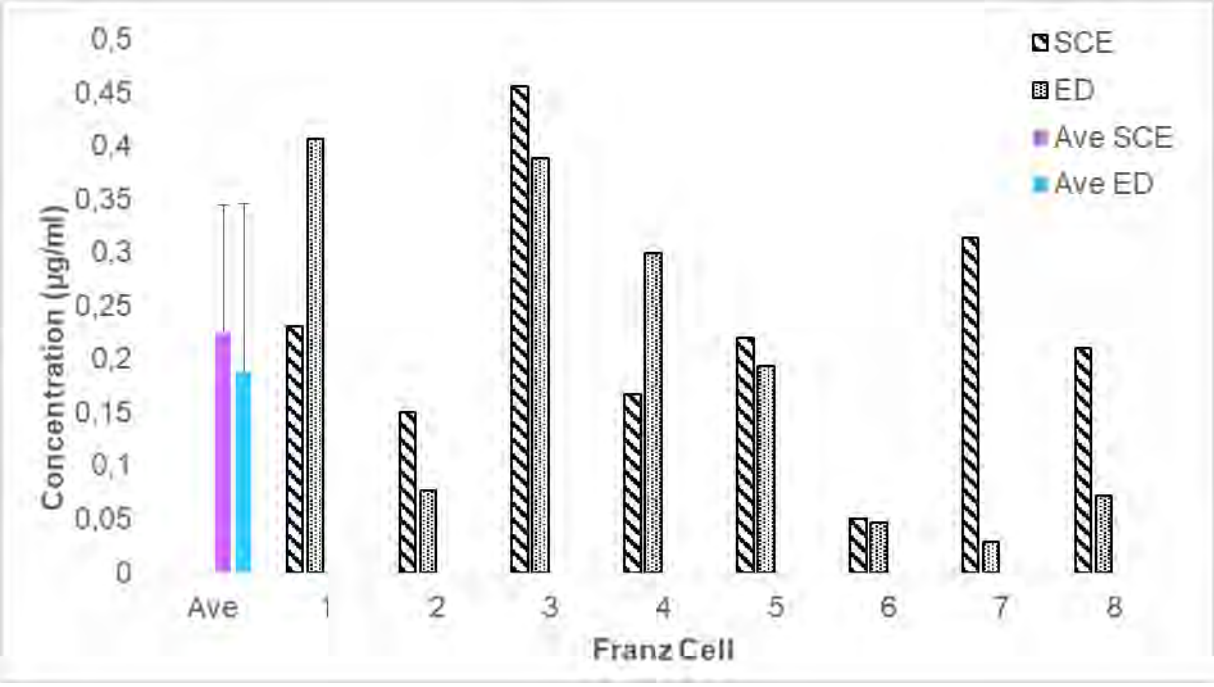

Figure D.11: Average concentration of clofazimine for the no vesicles dispersion in the individual Franz cells

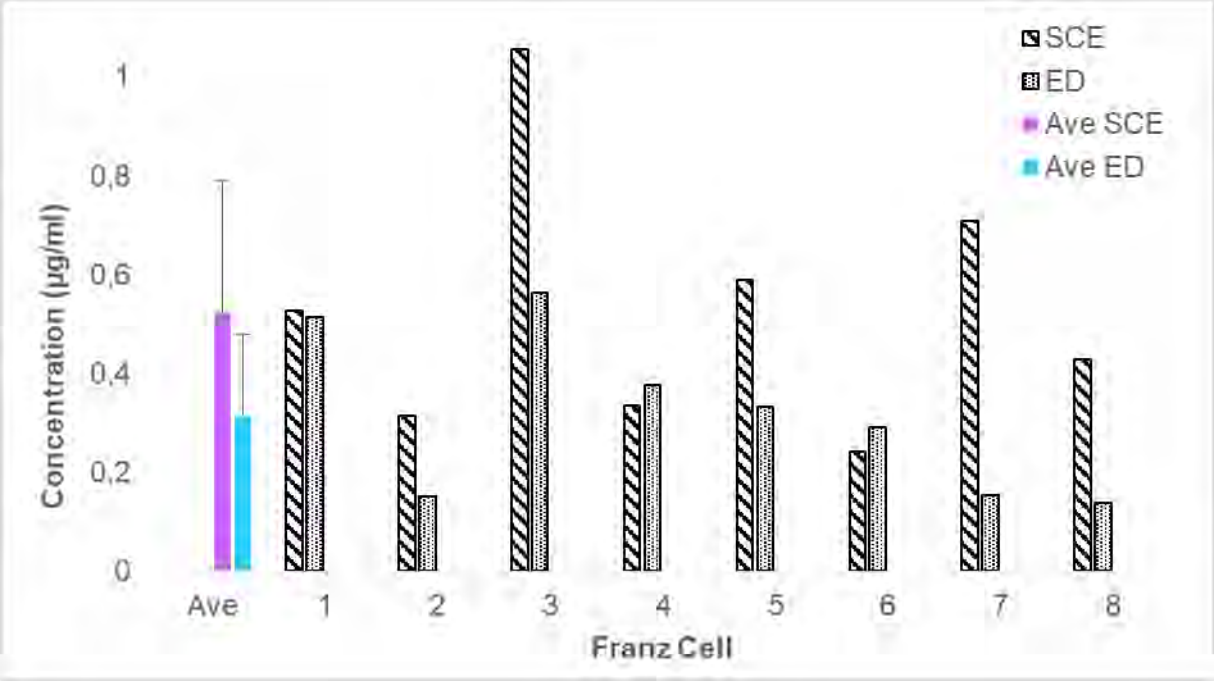

Figure D.12: Average concentration of decoquinate for the no vesicles dispersion in the individual Franz cells

The dispersion containing no vesicles did show some API present in the skin, though it was considerably lower than in the other dispersions. The SCE still had higher concentrations of API present after application of the dispersion containing no vesicles, as was also observed from the vesicle dispersions. The average concentration of decoquinate (0.526 µg/ml) present in the SCE was relatively higher than the average concentration of clofazimine (0.225 µg/ml), and the concentration of decoquinate (0.315 µg/ml) in the ED was noticeably higher than the concentration of clofazimine (0.189 µg/ml). Table D.13 illustrates the data obtained from all four dispersions.

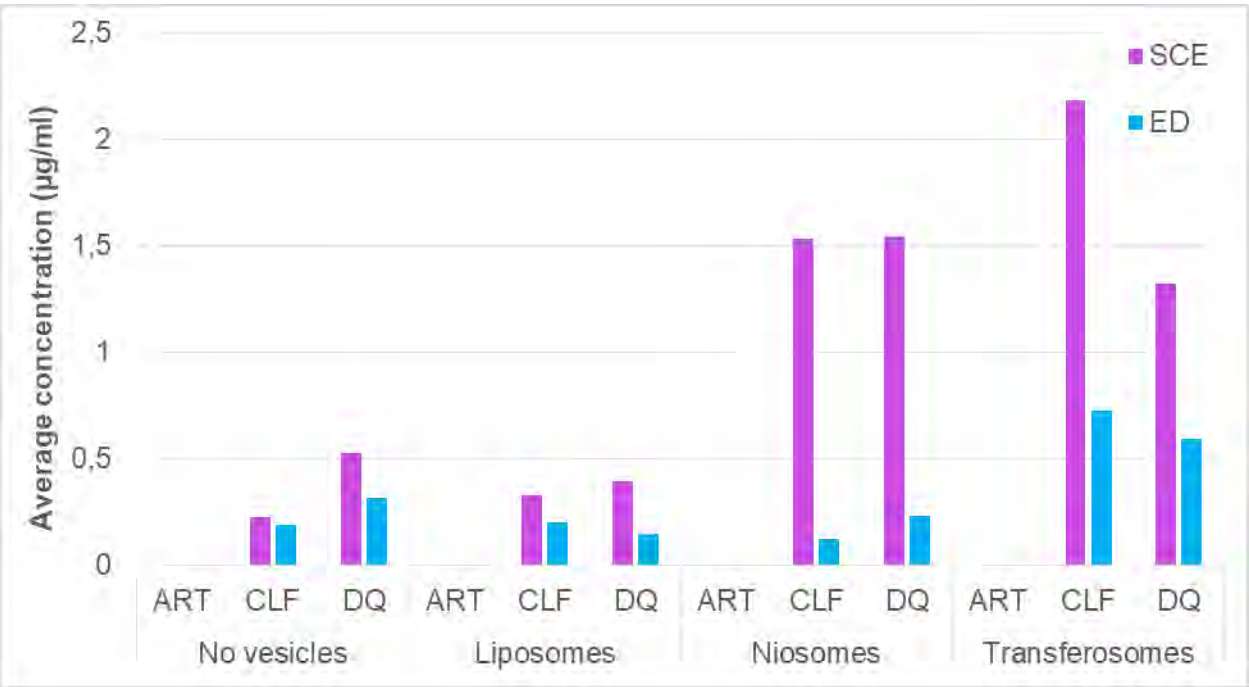

Figure D.13: Average concentrations of APIs present in the SCE and ED for the different dispersions investigated

In Figure D.13 there is a definite difference between the liposome and no vesicles dispersions and the niosome and transferosome dispersions. The SCE concentrations of both APIs detected in the transferosome dispersion and niosome dispersion were considerably higher than the concentrations detected in the liposome and no vesicle dispersions. Decoquinate delivered higher concentrations in the ED with the no vesicle dispersion compared to both the liposome and niosome dispersions. The transferosome dispersion overall depicted the most favourable results. The limits of detection (LOD) for artemisone, clofazimine and decoquinate were determined as 4.42 µg/ml, 0.042 µg/ml and 0.703 µg/ml, respectively, as indicated in Annexure A. This may explain why no artemisone was detected for any of the dispersions. Most of the data collected for decoquinate also fall below its LOD and thus the data may be considered inaccurate.

D.4. CONCLUSION

The data collected are promising since no studies have been performed on this combination of APIs and this study indicated that it is possible to deliver decoquinate and clofazimine into the skin. Artemisone was not measured in the skin, but it does not necessarily mean that it was not present. It might be that the concentration present was below the limit of quantitation. The transferosome dispersion displayed the highest concentrations decoquinate and clofazimine present in the skin, making it the most promising dispersion. Transferosomes contain no cholesterol, unlike the niosomes and liposomes. This may suggest that transferosomes are less lipophilic, and thus, these highly lipophilic APIs are less likely to remain in the vesicles and would rather be inclined to penetrate more freely into the skin. This could have been confirmed by conducting membrane studies, but since these APIs are so lipophilic the receptor phase would have had to consist of mostly ethanol to produce any results, which is unrealistic since the receptor phase should represent human blood.

Though the concentration of APIs was 1%, the concentration of vesicles in the aqueous phase was 5% (preparation of vesicles, Annexure C). If the concentration of vesicles in the aqueous phase can be increased from the 5% used, entrapment of the APIs may be increased; and higher concentrations of APIs may be present in the skin. This may promote the presence of artemisone. Ideally, the concentrations of the APIs present in the skin should be increased to above their LOD for accurate data acquisition. Moreover, it is advisable that other penetration enhancers and delivery systems should also be investigated for the delivery of these APIs.

REFERENCES

AHAD, A., AL-SALEH, A.A., AL-MOHIZEA, A.M., AL-JENOOBI, F.I., RAISH, M., YASSIN. A.E.B. & ALAM, M.A. 2017. Formulation and characterization of novel soft nanovesicles for enhanced transdermal delivery of eprosartan mesylate. *Saudi pharmaceutical journal*, article in press: http://dx.doi.org/10.1016/j.jsps.2017.01.006

ASHTIKAR, M., NAGARSEKAR, K. & FAHR, A. 2016. Transdermal delivery from liposomal formulations – Evolution of the technology over the last three decades. *Journal of controlled release.*, 242: 126-140.

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. *International journal of pharmaceutics*, 503:1-7.

HAINE, A.T., KOGA, Y., HASHIMOTO, Y., HIGASHI, T., MOTOYAMA, K., ARIMA, H. & NIIDOME, T. 2017. Enhancement of transdermal protein delivery by photothermal effect of gold nanorods coated on polysaccharide-based hydrogel. *European journal of pharmaceutics and biopharmaceutics*, 119: 91-95.

LUO, L. & LANE, M.E. 2015. Topical and transdermal delivery of caffeine. *International journal of pharmaceutics*, 490: 155-164.

MALINOVSKAJA-GOMEZ, K., ESPUELAS, S., GARRIDO, M.J., HIRVONEN, J. & LAAKSONEN, T. 2017. Comparison of liposomal drug formulations for transdermal iontophoretic drug delivery. *European journal of pharmaceutical sciences*, 106: 294-301.

RICHARDS, G.M., ORESAJO, C.O. & HALDER, R.M. 2003. Structure and function of ethnic skin and hair. *Dermatologic clinics*, 21:595-600.

Annexure E

AUTHOR'S GUIDE FOR DIE PHARMAZIE

INSTRUCTIONS FOR AUTHORS

E.1. AIM

The journal Die**Pharmazie** publishes reviews, experimental studies, letters to the editor, as well as book reviews.

The following fields of pharmacy are covered: Pharmaceutical and medicinal chemistry, pharmaceutical analysis and drug control, pharmaceutical technology, biopharmacy (biopharmaceutics, pharmacokinetics, and biotransformation), experimental and clinical pharmacology, pharmaceutical biology (pharmacognosy), clinical pharmacy, history of pharmacy.

Articles are published in English and are classified as:

- **Reviews**
  A summarizing presentation encompassing the current state of our knowledge and providing comprehensive interpretation with citation of the literature.

- **Original articles**
  Publications from all fields mentioned above.

- **Short communications**
  Brief publications about the fields mentioned above (see Preparation of manuscripts)

- **Letters to the editor**

E.2. CONDITIONS

For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be determined and classified in a suitable way, problems must be formulated in view of the data, hypotheses should be suggested an/or the author should give possible explanations for any inconsistencies.

If possible, the author(s) should perform mathematical or statistical calculations, fit the curves appropriate, and carry out the experiments under controlled conditions. Studies involving animals or human volunteers must include details of ethical approval.

Authors are requested to submit all manuscripts **online.** Paper copy submissions are no longer acceptable. Articles are considered for publication depending on their value and pharmaceutical relevance and with the understanding that they have not been published previously and are submitted exclusively to the journal Die**Pharmazie**.

All manuscripts are subject to experts review. Additional corrections may be done by the editors.

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As the journal is publishing exclusively original research, significant changes in authorship after submission will lead to manuscript rejection. Processing charges will not be refunded in these cases.

E.3. ETHICAL CONSIDERATIONS

Editors of PHARMAZIE fully support internationally accepted high ethical standards in publication, as particularly outlined by the Committee on Publication Ethics (COPE; publicationethics.org), the International Committee of Medical Journal Editors (ICMJE; www.icmje.org), the Council of Scientific Editors (CSE; www.councilscienceditors.org), and the European Association of Science Editors (http://www.ease.org.uk/wp-content/uploads/ease_toolkit_seven_sins.pdf). Authors are requested to inform themselves from these publications.

Most important aspects are presented here in brief:

E.3.1. CONFLICTS OF INTEREST

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this: 'Conflicts of interest: none declared'. Submission of manuscripts is not possible without a conflict of interest declaration.

E.3.2. INFORMED CONSENT

Patients have a right to privacy that should not be violated without informed consent. Identifying information, including names, initials, or hospital numbers, should not be published in written descriptions, photographs, or pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent for this purpose requires that an identifiable patient be shown the manuscript to be published. Authors should disclose to these patients whether any potential identifiable material might be available via the Internet as well as in print after publication. Authors need to provide the journal with a written statement that attests that they have received and archived written patient consent.

E.3.3. 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. This has to be stated in the manuscript. Authors should also 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 (http://www.nc3rs.org.uk/arrive-guidelines), the EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm) or similar regulations. The authors should clearly indicate in the manuscript that such guidelines have been followed.

E.4. PREPARATION OF MANUSCRIPTS

In order to achieve uniform presentation and to avoid unnecessary delays because of further inquiries, all authors are requested to observe the following guidelines:

Below the title, the surname(s) of the author(s) with initials should be given without academic and professional degrees. The full address of the author for correspondence should appear below

author names. Details on the institution where the work was done are requested and should be given above the title.

Each manuscript should start with an abstract, containing the most essential results of the study. Papers should be subdivided into chapters and subchapters according to the decimal system (e.g. 2.1.3.).

To achieve clarity and brevity of the presentation, original contributions should be subdivided after the abstract (see **2.**) as follows:

1. *Introduction:* This should indicate the question under investigation which is generally based on a brief interpretation of the literature considering the current state of knowledge in the subfield and explaining the necessary theoretical foundations.
2. *Investigations and results:* Methods should only be described generally (see "Experimental"), referring to previous or analogous studies. The presentation of results should be precise, with necessary formulas (numbered in sequence with Arabic numerals), diagrams, tables and figures added separately (together with the legend) to the manuscripts. Numerical values of results should generally be presented either in tables or curves (please mark statistical limits).
3. *Discussion* (unless covered by ***2.*** as *Investigations, results and discussion*): It should not repeat results already given, but should state the conclusions drawn from the results or provide a theoretical debate and comparison with literature citations.
4. *Experimental:* This part describes briefly the detailed experimental conditions. Unless directions taken from literature have been modified, it suffices to refer to the original source. In the case of well-known inorganic or organic compounds chemical formulae or common abbreviations may be used (e.g. NaCl, H2SO4, CH3OH, C6H6: Ac, Eth, Me, Phe, DMSO) under "Experimental". In other parts of the paper this is not desirable. Results of elemental analyses can be omitted if it is stated that all the results were in an acceptable error range.

Short communications are limited to 100 lines (including short summary; subdivisions are not required; the "Experimental" - if there is one - should be marked), up to 15 citations of literature and a maximum of 2 supplementary materials (schemes, figures, tables) are allowed.

Only the surnames of authors are given in the text. When there are more than two authors, only the name of the first one is used, followed by *et al*.

References in the text have to be cited by author and year, if there are three or more authors, use et al. (Miller 1997; Miller and Smith 2000; Miller et al. 2001). If the year is the same for several references identify these with a, b, c etc (Smith 1998a; Smith 1998b etc.) both in the text and in the reference list. At the end of the paper, references are listed in alphabetical order under the

first author's surname. All authors should be given here. If there are several references to items with the same first author, arrange these chronologically regardless the alphabetical order of the co-authors ("alphabetic-chronological" order).

Journal names should be abbreviated according to "Index Medicus" (Medline) or "Chemical Abstracts Service Source Index".

Quotations have to follow the following style:

Journal articles:

Lee J (2002) Formulation development of epidermal growth factor. Pharmazie 57: 787 - 790.

Lee EB, Shin KH, Woo WS (1984) Pharmacological study on piperine. Arch Pharm Res 7: 127 - 132.

Books/Book chapters

Krishan K, Andersen ME (1994) physiologically based pharmacokinetic modelling in toxicology. In: Hayes W (ed.) Principles and methods of toxicology, 3rd ed., New York, p. 149 – 187

For the identification of pharmaceutical substances, the International Non-proprietary Names (INN) proposed or recommended by the WHO should be used. Registered Trade Marks (usually indicated with R; in an article this sign should only be used when it is first mentioned or used in the summary), trivial names and chemical nomenclature can be added.

Nomenclature and spelling should conform to the directions given by IUPAC and IUB. Units of measurement are determined by the directions of the International Units System SI as symbols; M instead of mol/l or mol * l-1 is allowed.

Botanical names (species, genus) should be marked in italics.

The following abbreviations should be used consequently (except in the title and all subtitles). All other abbreviations have to be explained in the manuscript at first usage, if aforementioned directions are not applicable. Abs. = absolute: anh. = anhydrous; b.p.; b.r. = boiling point, -range; calcd. = calculated; CC = column chromatography; conc. = concentrated; dec. = decomposition, eq. = equation; Fig. = figure; GC = gas chromatography, - chromatogram, HPLC = high performance liquid chromatography, -chromatogram; i.m. = intramuscular; i.p. = intraperitoneal; IR = infrared; i.v. = intravenous; m.p.; m.r. = melting point, -range; MS = mass spectrometry, mass spectrum; NMR = nuclear magnetic resonance spectrum; PC = paper chromatography, -

chromatogram, % = per cent, percentage, p.o. = peroral; s.c. = subcutaneous; TLC = thin layer chromatography, - chromatogram; UV = ultraviolet.

Footnotes must be numbered consecutively and are to be added separately to the manuscript. They are printed following the "Experimental".

Dedications (e.g., on the occasion of the 60th or higher birthday) should be inserted between author(s) and summary.

Additions to legends of table should be marked by *, **, *** or a, b, c, d etc.

Figures have to be of sufficient quality for reproduction process. Even after size reduction the figures' key has to be easy to read. Manuscripts containing figures of insufficient quality cannot be accepted.

ANNEXURE F

AUTHOR'S GUIDE TO THE JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

GUIDE FOR AUTHORS

F.1. INTRODUCTION

This journal is an international medium directed towards the needs of academic, clinical, government and industrial analysis by publishing original research reports and critical reviews on pharmaceutical and biomedical analysis. It covers the interdisciplinary aspects of analysis in the pharmaceutical, biomedical and clinical sciences, including developments in analytical methodology, instrumentation, computation and interpretation. Submissions on novel analytical methods focusing on drug purity and stability studies, pharmacokinetics, therapeutic monitoring, metabolic profiling; drug-related aspects of analytical biochemistry and forensic toxicology; quality assurance in the pharmaceutical industry are also welcome.

Studies from areas of well established and poorly selective methods, such as UVVIS spectrophotometry (including derivative and multi-wavelength measurements), basic electroanalytical (potentiometric, polarographic and voltammetric) methods, fluorimetry, flowinjection analysis, etc. are accepted for publication in exceptional cases only, if a unique and substantial advantage over presently known systems is demonstrated. Similarly, assays of simple drug formulations by any methods, as well as, the determination of drugs in biological samples based merely on spiked samples are not acceptable. Drug purity/stability studies should contain information on the structure elucidation of the impurities/degradants. Papers dealing with the analytical aspects of traditional folk medicines are acceptable if the results are expected to attract the interest of readers also outside the area of origin. Bioanalytical papers (pharmacokinetic, bioequivalence, protein and DNA binding studies) are accepted if the focus is on innovative analytical methodology. Bioanalysis of new investigational drugs that are currently in the preclinical phase are only acceptable if their pharmacological activity is well documented in an international medium.

In determining the suitability of submitted articles for publication, particular scrutiny will be placed on the degree of novelty and significance of the research and the extent to which it adds to existing knowledge in pharmaceutical and biomedical analysis. In all submissions to the journal, authors

must address the question of how their proposed methodology compares with previously reported methods. A substantial body of work cannot be fractionated into different shorter papers.

F.1.1. Types of Paper

The following types of papers will be considered for publication:

Reviews: Authors wishing to submit a review should send a short synopsis to one of the editors before starting detailed work on a manuscript. The structure and presentation of a review article will normally be at the author's discretion. Reviews may be relatively short, i.e. dealing with a limited subject, or longer and more general in nature.

Full Length Research Papers: These papers should describe in detail original and important pieces of work in the fields covered by the Journal.

Short Communications: These should describe complete and original pieces of research whose length and/or importance do not justify a full-length paper. The format is the same as that for a full-length Research Paper, except that the total number of figures and/or tables should not normally exceed six. This approximate length of the manuscript should be 10 pages excluding Tables and Figures, Keywords are essential. The Editors reserve the right to publish as a Short Communication a paper originally submitted as a full-length Research Paper.

F.1.2. Submission checklist

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

Ensure that the following items are present:

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

- E-mail address
- Full postal address

All necessary files have been uploaded:

Manuscript:

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

Graphical Abstracts / Highlights files (where applicable)

Supplemental files (where applicable)

Further considerations

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

For further information, visit our Support Center.

F.2. BEFORE YOU BEGIN

F.2.1 Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

F.2.2. Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. If there are no conflicts of interest then please state this: 'Conflicts of interest: none'. More information.

F.2.3. 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

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F.2.4. 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.

F.2.5. Copyright

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F.2.7. Open access

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F.2.8. Submission

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All manuscripts will be assessed by two independent Reviewers. Reviews will be assessed by one Reviewer. Authors will be informed of the Reviewers' comments. Authors are required to ensure that each line of their manuscript is numbered consecutively.

F.3. PREPARATION

F.3.1. Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

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.

F.3.2. Article structure

Page and line numbers

Add page and line numbers. This is useful for reviewers, who need to refer to specific pages and lines in your manuscript.

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. When describing mixed solvents for chromatography, extraction or other purposes, the following convention must be adopted: solvent A-solvent B-solvent C (a:b:c, v/v/v) or (a:b:c, w/w/w) where a:b:c are the proportions (by volume or weight as appropriate) of the components A, B and C, respectively.

The method of preparation of buffers should be clearly expressed, with the pH value and molarity stated in parentheses, e.g. sodium acetate (pH 4.7; 0.1 M). For mixed solvent systems, it should be clearly stated whether the pH value quoted is the pH of the original aqueous component or the apparent pH (i.e. pH*) of the mixed solvent system. Typical examples of mobile phases employed in liquid chromatography might be:acetonitrile-sodium octylsulphate (10 mM)-sodium acetate (pH 4.7;0.1 M) (25:25:50, v/v/v), and acetonitrile-sodium octylsulphate (10 mM)-sodium acetate (0.1 M)(25:25:50, v/v/v)(pH* 4.7). Discussion of the optimisation procedure for the proposed method / assay should be given in detail.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

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.

F.3.3. 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 lower case 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.

F.3.4. Abstract

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

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Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. 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. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

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Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

F.3.5. 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.

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.

List of Journal of Pharmaceutical and Biomedical Analysis abbreviations.

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.

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.

F.3.6. Artwork

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- 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.
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A detailed guide on electronic artwork is available.

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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.

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- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

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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 and shading in table cells.

F.3.8. 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.

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Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given. Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.

Reference to a book:

[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

Reference to a dataset:

[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/xwj98nb39r.1.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations.

F.3.9. 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 labelled 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 in total. Any single file should not exceed 50 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.

F.3.10. Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

RESEARCH DATA

This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

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Annexure G

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