The use of grapeseed oil emulsions for the transdermal delivery of selected statins

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In medicine, history is largely told as a chronicle of magnificent people doing extraordinary things. For most of us, life is not made up of big moments, but small moments…

Someone once told me the pursuit of happiness is our greatest source of unhappiness. When the unimaginable happened, my mother adapted and found strength from our Heavenly Father. I hope that when my life does not go as planned (which it certainly will not), I can handle myself with the same strength and grace that my mother has taught me. She may not be a Nobel Prize winner or a scientist, but my mother is definitely my hero.

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ABSTRACT

Statins are used for the medical treatment of hypercholesterolemia, but after oral administration, it may cause gastro-intestinal disturbances and first-pass hepatic metabolism may decrease the bioavailability. Hence, the aforementioned may be avoided through transdermal administration. During this study, four statins (atorvastatin, mevastatin, pravastatin and pitavastatin) were investigated. Not all statins have ideal physicochemical properties for transdermal delivery and therefore, a drug delivery vehicle, i.e. nano-emulsions were utilised and compared to nano-emulgels. Grapeseed oil (penetration enhancer) was utilised as the oil phase during the formulation of the nano-formulas (nano-emulsions and nano-emulgels). High performance liquid chromatography (HPLC) was utilised to analyse the statins and membrane release studies were performed to determine if statins were released from the nano-formulas. Transdermal and topical drug delivery was determined by means of skin diffusion studies and tape stripping, respectively to determine whether the statins were delivered through the skin and into the systemic circulation and/or into the skin. The Franz diffusion cell method was used to conduct membrane release and skin diffusion studies. Cytotoxicity studies were performed on premalignant human immortalised keratinocytes (HaCaT). Two methods were used to indicate possible cell damage, e.g. neutral red (NR) assay and methylthiazol tetrazolium (MTT) assay. Statistical analyses were performed to analyse the variances in the data obtained from the membrane release studies, skin diffusion studies (transdermal and topical) and cytotoxicity studies.

Membrane release studies concluded that pravastatin had the highest median flux amongst all the nano-formulas of which the nano-emulgel containing pravastatin had a higher median flux than the nano-emulsion containing pravastatin. Skin diffusion studies revealed that the nano-emulgels improved the transdermal delivery of the statins more than their respective nano-emulsions, and that pitavastatin had the highest median amount per area diffused of all the tested nano-formulas. The nano-emulsions delivered higher median concentrations of the statins in the stratum corneum-epidermis (SCE) than the nano-emulgels. The nano-emulsion containing atorvastatin and the nano-emulgel containing pitavastatin delivered the highest concentration statin from the respective nano-formulas in both the SCE and epidermis-dermis. When the plasma concentrations of the statins after oral administration were compared to the average concentration diffused after transdermal delivery, it was revealed that the nano-formulas had reached higher concentrations for all the statins transdermally than the plasma concentrations, except mevastatin (plasma concentrations are unknown). The half maximal inhibitory concentration (IC_{50}) values from the MTT- and NR-assays indicated that mevastatin (when compared to the other statins) was the most toxic to the HaCaT cells.

Keywords: Statins; grapeseed oil; nano-emulsion; nano-emulgel; transdermal; cytotoxicity

UITTREKSEL

Statiene word vir die mediesebehandeling van hipercholesterolemie gebruik, maar dit kan na orale toediening lei tot gastro-intestinale versteurings en die biobeskikbaarheid kan verminder, as gevolg van die eerstedeurgangseffek in die lewer. Gevolglik kan die voorafgaande vermy word deur middel van transdermale toediening. Vier statiene (atorvastatien, mevastatien, pravastatien en pitavastatien) is tydens hierdie studie ondersoek. Aangesien alle statiene nie oor die ideale fisieschemiese eienskappe beskik vir transdermale aflewering nie, is 'n geneesmiddel-afleweringssisteem, bv. nano-emulsies, gebruik en met nano-emuljelle vergelyk. Druiwepitolie (penetrasie-bevorderaar) is tydens die formulering van die nano-formules (nano-emulsies en nano-emuljelle) gebruik as die olie-fase. Hoëdrukvloeistofchromatografie (HDVC) is gebruik om die statiene te analiseer en membraanvrystellingstudies is uitgevoer om te bepaal of die statiene vanuit die nano-formules vrygestel is. Transdermale en topikale geneesmiddelaflewering is respektiewelik bepaal deur middel van veldiffusiestudies en kleefbandstroping om vas te stel of die statiene deur die vel en in die sistemiese sirkulasie en/of in die vel, afgelewer is. Sitotoksisiteitstudies is op premaligne menslike geïmmortaliseerde keratonisietselle (HaCaT) uitgevoer. Twee metodes is gebruik om moontlike selbeskadiging aan te dui, bv. neutraalrooi (NR) en metieltiasoltetrasolium (MTT) toetse. Statistiese ontledings is uitgevoer om die afwykings in die data, wat verkry is tydens die membraanvrystellingstudies, veldiffusiestudies (transdermaal en topikaal) en sitotoksisiteitstudies, te ontleed.

Membraanvrystellingstudies het getoon dat pravastatien die hoogste mediaan vloed van al die nano-formules gehad het, waarvan 'n hoër mediaan vloed verkry is vanaf die nano-emuljel wat pravastatien bevat, eerder as die nano-emulsie wat pravastatien bevat. Veldiffusiestudies het bewys dat die transdermale aflewering van die statiene verbeter is deur die nano-emuljelle, meer as hul teenoorstaande nano-emulsies, en dat die diffusie van pitavastatien die hoogste mediaan hoeveelheid per oppervlakte van al die getoetsde nano-formules getoon het. Die nano-emulsies het hoër mediaankonsentrasies van die statiene in die stratum korneum-epidermis (SKE) afgelewer as die nano-emuljelle. Die nano-emulsie wat atorvastatien bevat en die nano-emuljel wat pitavastatien bevat, het die hoogste konsentrasie statiene uit die onderskeie nano-formules gelewer in beide die SKE en epidermis-dermis. Toe die plasmakonsentrasies van die statiene na orale toediening vergelyk is met die gemiddelde diffusiekonsentrasie na transdermale aflewering, is gevind dat die konsentrasies vir al die statiene vanaf die nano-formules hoër was na transdermale aflewering as tydens die plasma-konsentrasies, behalwe vir mevastatien (plasmakonsentrasies is onbekend).Die half-maksimale inhibisiekonsentrasie (IK_{50}) waardes van die MTT- en NR-toetse het aangedui dat mevastatien (in vergelyking met die ander statiene) die toksieste was vir die HaCaT-selle.

Sleutelwoorde: Statiene; druiwepitolie; nano-emulsie; nano-emuljel; transdermaal; sitotoksisiteit

ABBREVIATIONS

ACN	Acetonitrile
AFB	Aktiewe farmaseutiese bestanddele
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
Apo B	Apolipoprotein B
APVMA	Australian Pesticides and Veterinary Medicines Authority
ATL	Analytical Technology Laboratory
ATO	Atorvastatin
BT474A	Human ductal carcinoma cells
CHD	Coronary heart disease
C_{max}	Peak concentration
CO_2	Carbon dioxide
CSUR	Competitive Support for Unrated Researchers
CVD	Cardiovascular disease
CYP	Cytochrome P (Hepatic enzyme)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ED	Epidermis-dermis
EDTA	Trypsin-Versene®
%EE	Entrapment efficiency
FBS	Foetal bovine serum

FC	Franz cells
FDA	Food and Drug Administration
FH	Familial hypercholesterolemia
H+	Hydrogen ions
H_2O_2	Hydrogen peroxide
HaCaT	Human keratinocytes/menslike keratinosiete
HCl	Hydrochloric acid
HeFH	Heterozygous familial hypercholesterolemia
HeLa cells	Human cervix cancer cells
HEp-2	Human epithelial type 2 carcinoma cells - HeLa contaminant
HLB	Hydrophilic-lipophilic balance
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HoFH	Homozygous familial hypercholesterolemia
HPLC	High performance liquid chromatographic
HREC	Health Research Ethics Committee
HRTEM	High-resolution transmission electron microscopy
HSD	Honestly significant difference
IC_{50}	The half-maximal inhibitory concentration
ICH	International Conference of Harmonisation
IQR	Interquartile range
KH_2PO_4	Potassium dihydrogen orthophosphate
LAMB	Laboratory for Applied Molecular Biology
LDH	Lactate dehydrogenase

LDL	Low density lipoprotein
LDLr	Lipoprotein receptor gene
LLOD	Lowest limit of detection
LLOQ	Lowest limit of quantification
LOD	Limit of detection
Log D	Octanol-buffer distribution coefficient
Log P	Octanol-water partition coefficient
LOQ	Limit of quantification
MEV	Mevastatin
MTT	Methylthiazol tetrazolium
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NE	O/w nano-emulsion
NE1	O/w nano-emulsion with a Tween® 80:Span® 60 ratio of 3:3
NE2	O/w nano-emulsion with a Tween® 80:Span® 60 ratio of 3:2
NE3	O/w nano-emulsion with a Tween® 80:Span® 60 ratio of 2:3
NEAA	Non-Essential Amino Acid
NE-ATO1	O/w nano-emulsion with atorvastatin and Tween® 80:Span® 60 ratio of 3:3
NE-ATO2	O/w nano-emulsion with atorvastatin and Tween® 80:Span® 60 ratio of 3:2
NE-ATO3	O/w nano-emulsion with atorvastatin and Tween® 80:Span® 60 ratio of 2:3
NE-MEV1	O/w nano-emulsion with mevastatin and Tween® 80:Span® 60 ratio of 3:3
NE-MEV2	O/w nano-emulsion with mevastatin and Tween® 80:Span® 60 ratio of 3:2
NE-MEV3	O/w nano-emulsion with mevastatin and Tween® 80:Span® 60 ratio of 2:3

NE-PIT1	O/w nano-emulsion with pitavastatin and Tween® 80:Span® 60 ratio of 3:3
NE-PIT2	O/w nano-emulsion with pitavastatin and Tween® 80:Span® 60 ratio of 3:2
NE-PIT3	O/w nano-emulsion with pitavastatin and Tween® 80:Span® 60 ratio of 2:3
NE-PLAS1	O/w nano-emulsion placebo with Tween® 80:Span® 60 ratio of 3:3
NE-PRA1	O/w nano-emulsion with pravastatin and Tween® 80:Span® 60 ratio of 3:3
NE-PRA2	O/w nano-emulsion with pravastatin and Tween® 80:Span® 60 ratio of 3:2
NE-PRA3	O/w nano-emulsion with pravastatin and Tween® 80:Span® 60 ratio of 2:3
NG	Nano-emulgel
NG1	Nano-emulgel with Tween® 80:Span® 60 ratio of 3:3
NG-ATO1	Nano-emulgel with atorvastatin (2%) and Tween® 80:Span® 60 ratio of 3:3
NG-MEV1	Nano-emulgel with mevastatin (2%) and Tween® 80:Span® 60 ratio of 3:3
NG-PIT1	Nano-emulgel with pitavastatin (2%) and Tween® 80:Span® 60 ratio of 3:3
NG-PLAS1	Nano-emulgel placebo with Tween® 80:Span® 60 ratio of 3:3
NG-PRA1	Nano-emulgel with pravastatin (2%) and Tween® 80:Span® 60 ratio of 3:3
NR	Neutral Red
NRF	National Research Foundation
NRS	Neutral Red Solution
NWU	North-West University
OECD	Organisation for Economic Co-operation and Development
OH-	Hydroxide ions
o/w	Oil-in-water/olie-in-water
PBS	Phosphate buffer solution
PCS	Photon correlation spectroscopy

PCSK9	Proprotein convertase subtilisin/kexin type 9
PdI	Polydispersity index
Pen/Strep	Penicillin/Streptomycin
PIT	Phase inversion temperature
PIT	Pitavastatin
PRA	Pravastatin
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
R^2	Coefficient of determination
%RSD	Percentage relative standard deviation
SCE	Stratum corneum-epidermis
SD	Standard deviation
SS1s	Statin stock solutions
SS-ATO1	Statin stock solution with atorvastatin
SS-MEV1	Statin stock solution with mevastatin
SS-PIT1	Statin stock solution with pitavastatin
SS-PRA1	Statin stock solution with pravastatin
TAM	Thermal activity monitor
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
UNODC	United Nations Office on Drugs and Crime
USP	United States Pharmacopeia
UV	Ultraviolet

VLDL	Very low density protein
w/o	Water-in-oil
w/w	Weight per weight

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Fig 2: Chromatogram representation of: A) atorvastatin, B) fluvastatin, C) lovastatin, D) mevastatin, E) pitavastatin, F) pravastatin, G) rosuvastatin and H) simvastatin; in addition: a) standard solution, b) receptor phase extraction, c) tape-stripping (SCE) and d) skin samples (ED)

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TRANSDERMAL NOVEL DRUG DELIVERY IN HYPERCHOLESTEROLAEMIA

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Equation B.1: %EE = [(Ct - Cf)/Ct] x 100

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Equation E.1: $C_1V_1 = C_2V_2$

Equation E.2: MTT (mg) = Total volume (ml) x 0.5 mg/ml

Equation E.3: $\%\text{viable cells} = \frac{((\text{absorbance 560 nm - 630 nm}) - \text{blank absorbance})}{(\text{Negative control absorbance - blank absorbance})} \times 100$

Equation E.4: y = mx + c

Equation E.5: Amount NRS = Total amount x 10%

CHAPTER 1

INTRODUCTION, RESEARCH PROBLEM AND AIMS

1.1 Introduction

Hypercholesterolaemia is a chronic condition caused by elevated plasma low-density lipoprotein (LDL) cholesterol levels and increases the risk of premature cardiovascular disease, stroke and other vascular diseases (CVD) (Marais, 2016; Nordestgaard *et al.*, 2013; Rohilla *et al.*, 2012). Based on several guidelines, elevated LDL cholesterol levels indicate a primary risk factor for hypercholesterolemia (Last *et al.*, 2011). In contrast, the body requires a minimum level of LDL to accomplish certain functions (Bandyopadhyay *et al.*, 2018). However, the body will generate sufficient cholesterol without any additional dietary cholesterol (Ma, 2004).

Approximately 4.4 million deaths are associated worldwide with elevated serum cholesterol each year (Venkitachalam *et al.*, 2012). Hypercholesterolemia affects up to 53% of the global population (Marais, 2016). A historic target has been sanctioned by the World Health Organization (WHO) to lessen premature mortality from non-communicable diseases (NCD) by 25% by the year 2025 (Murphy *et al.*, 2017). Science has shifted from an era in which hypercholesterolemia was not identified as abnormal to one in which controlling hypercholesterolemia is known to reduce total mortality (Steinberg & Gotto, 1999).

Serum cholesterol can be decreased by controlling the risk factors, of which controlling high LDL is of paramount importance, implementing lifestyle modifications and initiating drug therapy (Istvan & Deisenhofer, 2001). There are several different classes of lipid-modifying drugs available, including, nicotinic acid (niacin), bile acid-binding resins (e.g. colestipol, cholestyramine, colesevelam), the fibrates (e.g. clofibrate, bezafibrate, fenofibrate, gemfibrozil), cholesterol-absorption inhibitors (e.g. ezetimibe), PCSK9 (proprotein convertase subtilisin/kexin type 9) inhibitors (e.g. alirocumab, evolocumab) (Earl, 2019) and finally, the statins (e.g. atorvastatin, mevastatin, pravastatin and pitavastatin) (Schachter, 2005).

Statins, also known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are the preferred initial pharmacological therapy for hypercholesterolaemia (Braamskamp *et al.*, 2012; O'Sullivan, 2007; Schachter, 2005). Statins are used to target cholesterol levels and are responsible for decreasing major cardiovascular events by 25 – 40% (Marais, 2016). HMG-CoA reductase is the rate limiting enzyme, responsible for the synthesis of cholesterol (Braamskamp *et al.*, 2012; O'Sullivan, 2007). Inhibition of the HMG-CoA reductase enzyme results in a decrease cholesterol biosynthesis and hepatic cholesterol concentrations; additionally, clearance

of LDL-cholesterol particles from the blood will increase due to the enhanced expression in LDL-receptors (Bilheimer *et al.*, 1983).

Statins are generally well tolerated (Black, 2002), but are responsible for a range of adverse effects, including mild to rare, but life-threatening conditions, such as rhabdomyolysis (Das *et al.*, 2015; O'Sullivan, 2007; Rao *et al.*, 2011), which follows after the progression of myopathy (Furberg & Pitt, 2001; Staffa *et al.*, 2002). Other adverse effects reported and associated with the use of statins include gastrointestinal side-effects, which is fully discussed in Chapter 2. The aforementioned can be minimised by implementing managing factors, e.g. specific administration dosage and the use of combination therapy (Ballantyne *et al.*, 2003). Poor patient compliance and discontinuation of therapy can be expected when these adverse effects occur after oral administration of statins (Mancini *et al.*, 2013).

Drug-induced liver injury is a major problem with the use of these active pharmaceutical ingredients (APIs). Documented data indicates that statins are generally responsible for hepatotoxicity, and there are other related issues regarding their usage in humans that require additional research (Karahalil *et al.*, 2017). More studies should be conducted to assess the pharmacokinetics of specific statins at different doses, long-term effects of prolonged use of statins on the hepatic histology and genetic polymorphisms (Karahalil *et al.*, 2017). Generally, hepatotoxicity mechanisms have not been entirely understood, but elevated levels of alanine aminotransferase (ALT) can be used as a possible guide (Karahalil *et al.*, 2017). Due to poor predictive values, ALT tests has been unsuccessful in preventing serious liver disease (Karahalil *et al.*, 2017; Tolman, 2002). Thus, by investigating other routes of administration, the adverse effects might be avoided regarding the liver.

The oral administration of statins, as a daily dose, are well absorbed from the intestine however they undergo significant extensive first-pass metabolism within the liver, which decreases systemic bioavailability to about 5 – 50% (McFarland *et al.*, 2014). Refining the drug release method through transdermal drug delivery of these drugs, it is possible in some cases to enhance their bioavailability and reduce side-effects. Statins are mainly metabolised by cytochrome P450 (CYP450) enzymes, but pravastatin is less considerable through this pathway (Bottorff & Hansten, 2000; Feidt *et al.*, 2010). CYP450 activity differs from patient to patient due to low or non-existent activity of a specific isoform (Bottorff & Hansten, 2000). This could explain why the same drug-drug combinations are effective for some patients and toxic for others.

Statins are recommended as first-line treatment as they are well tolerated and have a good safety record (Maron *et al.*, 2000; Schaiff *et al.*, 2008). The amount of cholesterol is influenced by its synthesis and catabolism, in which the liver plays an important role (Onwe *et al.*, 2015). With the use of a lipid profile serum test the amount of cholesterol and triglycerides in the blood can be

established to initiate the most suitable treatment regarding the patients' health (Onwe *et al.*, 2015). During this study the focus will be on four statins, namely atorvastatin, mevastatin, pitavastatin and pravastatin. Considering limitations and problems regarding statins via the oral route, the transdermal route of administration will be focused on, as first-pass metabolism is prevented utilising the skin for administration and possible reduced side-effects (Cho *et al.*, 2009).

The skin is the largest organ of the human body and covers the entire external surface (Arda *et al.*, 2014; Goodwin, 2011; Johnsen, 2010), with an average body surface area of approximately 2 m^2 (Lai-Cheong & McGrath, 2009). The skin consists of three layers: epidermis, dermis, and the hypodermis (Lai-Cheong & McGrath, 2009). Observing the deepest part of the epidermis towards the surface of the skin, these layers include the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and finally, the stratum corneum (Arda *et al.*, 2014).

The stratum corneum serves as the main barrier between the the body and the environment (Arda *et al.*, 2014; Wickett & Visscher, 2006; Wilbur, 2017) and is known to be the rate-limiting barrier regarding transdermal delivery of APIs (Barry, 1983). As the outermost layer of the skin, it is well accepted that the stratum corneum is non-viable, lipophilic and exceptionally impermeable (Shah, 1994) and plays the primordial role by participating in functional properties of the skin (Levi & Dauskardt, 2012). The main lipids that occur in the stratum corneum include fatty acids, ceramides, cholesterol and triglycerides (Akhtar, 2014; Benson, 2005).

Corneocytes and keratin microfibrils are considered the 'bricks,' while the lipids found between the cells of the layers are the 'mortar' (Nemes & Steinert, 1999; Uchida & Park, 2016; Wickett & Visscher, 2006). These lipids will act as flux regulators, allowing an API through the skin (Williams, 2003). Certain physiochemical characteristics are essential parameters that govern API particle delivery across the skin layers by means of transdermal delivery (Alkilani *et al.*, 2015).

Pravastatin is clinically referred to as a hydrophilic statin (Fong, 2014; Schachter, 2005) and does not depend on the CYP450 pathway (Schachter, 2005). Hydrophilic statins, such as pravastatin, rely mainly on active transport using the OATP (organic anion transporting polypeptide) (Fong, 2014), opposed to atorvastatin, mevastatin, and pitavastatin, which are greater lipophilic compounds (Kim *et al.*, 2010; Schachter, 2005). Statins that are considered as lipophilic, tend to achieve greater levels of exposure in non-hepatic tissues (Fong, 2014). These particular APIs are not completely compliant with the ideal physiochemical characteristics for transdermal delivery therefore the APIs need to be formulated within a carrier system that will enhance skin permeation (Gaber *et al.*, 2017).

Various carrier systems are available (Gaber *et al.*, 2017), but an appropriate carrier system, e.g. nano-emulsions (**NEs**), needs to be selected to successfully facilitate transdermal delivery of

statins. **NEs** encompass high solubilisation capacity for both lipophilic and hydrophilic APIs (Tsai *et al.*, 2014). A **NE** has become a widely applied formulation for the delivery of APIs (Özgün, 2013). Its small droplet size presents some useful properties, such as robust stability, high surface area per unit volume, optically transparent appearance and adjustable rheology (Gupta *et al.*, 2016). **NEs** are kinetically stable over long periods of time and relatively insensitive to chemical and physical changes (Gupta *et al.*, 2016). This formulation provides rapid penetration of APIs through the skin due to the large surface area of droplets (Sharma *et al.*, 2010). These systems consist of two phases (water and oil phase), in which either droplets are carefully dispersed into the opposite phase, and by means of an appropriate co-surfactant and surfactant, the system can be stabilised (Morrow *et al.*, 2007) within the nanometre scale (20 – 500 nm) (Sharma *et al.*, 2010). Stable **NEs** are critical, for the wrong selection of components (e.g. surfactants, oil) in each phase can influence *in vivo* and *in vitro* delivery system studies. Penetration enhancers adjust the barrier of the skin, improving the permeation and absorption of APIs (Alexander *et al.*, 2012; Balázs *et al.*, 2016).

Natural penetration enhancers interact with cellular corneocytes, disrupt lipophilic bilayers and eliminate matters of lamellar bodies, which can increase the flux across the stratum corneum (Vermaak *et al.*, 2011). According to literature, fatty acid esters are more beneficial than fatty alcohols due to their flexibility of lipids in terms of tailored globule size and unique solubilisation capacity (Pawar & Babu, 2014). In this study, grapeseed oil, categorised as a natural penetration enhancer, was investigated. Regarding skin structure and permeability enhancement relationship, unsaturated fatty acids are more efficient in increasing the permeability of the skin barrier compared to saturated fats (Čižinauskas *et al.*, 2017). The main characteristic of grapeseed oil is its high content in polyunsaturated fatty acids, vitamin E and phenolic compounds (Fiori *et al.*, 2010). It is a polyunsaturated oil, and contains beneficial compounds, e.g. oleic acid (15.8%) and linoleic acid (69.6%) (AL-Bayati & Enaad, 2015). This is suitable, because of the C_{18}-fatty acids that are also present in the human skin, which will increase skin permeability and allow the delivery of both lipophilic and hydrophilic drugs (Barry, 1983a; Čižinauskas *et al.*, 2017) and lower the possibility of skin irritation (Büyüktimkin *et al.*, 1997; Vermaak *et al.*, 2011). Additionally, grapeseed oil is high in proanthocyanidins, a unique compound rich in antioxidants that are 20 times stronger than vitamin C and 50 times more efficient than vitamin E (AL-Bayati & Enaad, 2015).

In this study, the formulation of oil-in-water (o/w) **NE** was compared to a nano-emulgel (**NG**). Transdermal **NGs** are formulated by adding a gelling agent to the novel **NEs** and it exhibits characteristics of both **NEs** and **NGs** (Basera *et al.*, 2015; Chellapa *et al.*, 2015; Eid *et al.*, 2014) that are discussed Appendix B and C. **NGs** decrease interfacial and surface tension, which can

ultimately increase skin permeability due to the enhanced viscosity of the formulation (Chellapa *et al.*, 2015; Eid *et al.*, 2014).

The utilisation of high performance liquid chromatography (HPLC) analysis were implemented to successfully identity the API and provide quantitative results (Katsanidis *et al*., 1999). The analyses of drugs are one of the most demanding but one of the most general uses of high performance of liquid chromatography and analyte concentrations are often low in the case of transdermal drug delivery (Chen *et al*., 2004) of selected statins.

Cytotoxicity studies were also performed, which is a useful initial step in the research process to ultimately determine the potential toxicity of a test substance, e.g. selected statins. Minimal to no toxicity is essential for the successful development of a pharmaceutical or transdermal preparation and in this regard, cellular toxicity studies play a crucial role (Moharamzadeh *et al* ., 2007).

1.2 Research problem

Statins administered as an oral daily dose undergo extensive hepatic first-pass metabolism (Betteridge, 2010), which increases the risk for hepatic toxicity (McKenney *et al.*, 2006), and therefore have low systemic bioavailability, while causing gastro-intestinal and hepatic effects (Mancini *et al.*, 2013).

Statins do not have ideal physiochemical properties for transdermal delivery and therefore systemic delivery will be challenging (Ng & Lau, 2015). Moreover, the stratum corneum is considered lipophilic, while the subsequent layers are hydrophilic, thus, creating barriers preventing API penetration through the skin (Foldvari, 2000).

1.3 Aims and objectives

The aim of this study was to investigate the transdermal delivery of the selected statins, namely atorvastatin, mevastatin, pitavastatin and pravastatin, after incorporation into both a **NE** and a **NG**, respectively. Both the **NEs** and the **NGs** consisted of grapeseed oil in the oil phase. Furthermore, the potential toxicity of the **NEs** were determined with and without the API.

The specific objectives were to:

- Develop and validate a method of analysis, i.e. high performance liquid chromatography (HPLC) to determine the concentrations of the selected statins.
- Formulate both **NEs** and **NGs** containing the selected statins separately together with natural oil, e.g. grapeseed oil, as the oil phase.

- Characterise both **NEs** and **NGs** with respect to the zeta-potential, viscosity, droplet size, pH, visual examination, entrapment efficacy and morphology.
- Determine the release of the selected statins from the **NEs** and **NGs** by using membrane release studies.
- Use Franz cell skin diffusion studies and tape stripping to determine the transdermal and topical delivery of selected statins from the **NEs** and **NGs**, respectively.
- Evaluate the cytotoxic effects of each selected statin (separately) and in combination with the **NE** using *in vitro* cell cultures (premalignant human immortalised keratinocytes (HaCaT)) via neutral red (NR) assay and methylthiazol tetrazolium (MTT) assay.

References

Akhtar, N. 2014. Microneedles: an innovative approach to transdermal delivery—a review. *International journal of pharmacy and pharmaceutical sciences*, 6:18-25.

AL-Bayati, A.J. & Enaad, D.F. 2015. Histopathological study and surgery the effect of grape seed oil on wound healing in rabbits. *International journal of science and research*, 4(6):959-962.

Alexander, A., Dwivedi, S., Giri, T.K., Saraf, S., Saraf, S. & Tripathi, D.K.J.J.o.C.R. 2012. Approaches for breaking the barriers of drug permeation through transdermal drug delivery. *Journal of controlled release*, 164(1):26-40.

Alkilani, A., McCrudden, M.T. & Donnelly, R. 2015. Transdermal drug delivery: innovative pharmaceutical developments based on disruption of the barrier properties of the stratum corneum. *Pharmaceutics*, 7(4):438-470.

Arda, O., Göksügür, N. & Tüzün, Y. 2014. Basic histological structure and functions of facial skin. *Clinics in dermatology*, 32(1):3-13.

Balázs, B., Vizserálek, G., Berkó, S., Budai-Szűcs, M., Kelemen, A., Sinkó, B., Takács-Novák, K., Szabó-Révész, P. & Csányi, E. 2016. Investigation of the efficacy of transdermal penetration enhancers through the use of human skin and a skin mimic artificial membrane. *Journal of pharmaceutical sciences*, 105(3):1134-1140.

Ballantyne, C.M., Corsini, A., Davidson, M.H., Holdaas, H., Jacobson, T.A., Leitersdorf, E., März, W., Reckless, J.P. & Stein, E.A.J.A.o.i.m. 2003. Risk for myopathy with statin therapy in high-risk patients. *Archives of internal medicine*, 163(5):553-564.

Bandyopadhyay, D., Qureshi, A., Ghosh, S., Ashish, K., Heise, L.R., Hajra, A. & Ghosh, R.K. 2018. Safety and efficacy of extremely low LDL-cholesterol levels and its prospects in hyperlipidemia management. *Journal of lipids*, 2(1):122-132.

Barry, B. 1983. Skin transport. Dermatological formulations. *Percutaneous absorption*, 2(2):95-126.

Basera, K., Kothiyal, P. & Gupta, P. 2015. Nanoemulgel: a novel formulation approach for topical delivery of hydrophobic drugs. *World journal of pharmacy and pharmaceutical sciences*, 4(10):1871-1886.

Benson, H.A.J.C.d.d. 2005. Transdermal drug delivery: penetration enhancement techniques. *Current drug delivery*, 2(1):23-33.

Bilheimer, D.W., Grundy, S.M., Brown, M.S. & Goldstein, J.L.J.P.o.t.N.A.o.S. 1983. Mevinolin and colestipol stimulate receptor-mediated clearance of low density lipoprotein from plasma in familial hypercholesterolemia heterozygotes. *Proceedings of the national academy of sciences*, 80(13):4124-4128.

Black, D.M.J.C.a.r. 2002. A general assessment of the safety of HMG CoA reductase inhibitors (statins). *Current atherosclerosis reports*, 4(1):34-41.

Bottorff, M. & Hansten, P.J.A.o.i.m. 2000. Long-term safety of hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitors: the role of metabolism—monograph for physicians. *Archives of internal medicine*, 160(15):2273-2280.

Braamskamp, M.J., Wijburg, F.A. & Wiegman, A. 2012. Drug therapy of hypercholesterolaemia in children and adolescent. *Drugs*, 72(6):759-772.

Bucks, D., Maibach, H.I.J.D. & sciences, t.p. 1999. Occlusion does not uniformly enhance penetration *in vivo*. *Occlusion report*, 97:81-106.

Büyüktimkin, N., Büyüktimkin, S. & Rytting, J. 1997. Chemical means of drug permeation enhancement. *Interpharmacy*, 6:357-475).

Chellapa, P., Mohamed, A.T., Keleb, E.I., Elmahgoubi, A., Eid, A.M., Issa, Y.S. & Elmarzugi, N.A. 2015. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR journal of pharmacy*, 5:2250-3013.

Chen, H., Chang, X., Weng, T., Zhao, X., Gao, Z., Yang, Y., Xu, H. & Yang, X., 2004. A study of microemulsion systems for transdermal delivery of triptolide. *Journal of controlled release*, 98(3):427-436.

Cho, C.-W., Choi, J.-S., Kim, S.-J. & Shin, S.-C.J.D.d. 2009. Enhanced transdermal delivery of loratadine from the EVA matrix. *Drug delivery*, 16(4):230-235.

Čižinauskas, V., Elie, N., Brunelle, A. & Briedis, V. 2017. Skin penetration enhancement by natural oils for dihydroquercetin delivery. *Molecules*, 22(9):1536.

Das, S., Ghosh, S. & Sharma, A.J.M.J.O.D.D.P.U. 2015. Late onset rhabdomyolysis developing secondary to atorvastatin therapy in a coronary artery disease patient. *Medical journal of Dr. Dy Patil University*, 8(2):244-244.

Dayan, N. 2005. Pathways for skin penetration. *Cosmetics and toiletries*, 120(6):67-76.

Earl, G.J.N.c.c. 2019. PCSK9 inhibitors: Add-on therapy to reduce stroke risk. *Nursing... critical care*, 14(1):42.

Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti-inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of nanomedicine and nanotechnology*, 5(2):1.

Feidt, D.M., Klein, K., Hofmann, U., Riedmaier, S., Knobeloch, D., Thasler, W.E., Weiss, T.S., Schwab, M., Zanger, U.M.J.D.M. & Disposition. 2010. Profiling induction of cytochrome p450 enzyme activity by statins using a new liquid chromatography-tandem mass spectrometry cocktail assay in human hepatocytes. *Drug Metabolism and disposition*, 38(9):1589-1597.

Fiori, L., Solana, M., Guella, G., Strim, C. & Tosi, P. 2010. Grape seed oil: Supercritical extraction, chemical analysis and fractionation. 12th euro meet supercritical fluids, Graz (Austria).

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

Fong, C.W.J.E.j.o.m.c. 2014. Statins in therapy: understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies. *European journal of medicinal chemistry*, 85:661-674.

Furberg, C.D. & Pitt, B.J.T. 2001. Withdrawal of cerivastatin from the world market. *Trials*, 2(5):205.

Gaber, M., Medhat, W., Hany, M., Saher, N., Fang, J.-Y. & Elzoghby, A.J.J.o.C.R. 2017. Protein-lipid nanohybrids as emerging platforms for drug and gene delivery: challenges and outcomes. *Journal of controlled release*, 254:75-91.

Goodwin, C. 2011. Anatomy and Physiology of the Skin. *Journal of the dermatology nurses' association*, 3(4):203-213.

Gupta, A., Eral, H.B., Hatton, T.A. & Doyle, P.S.J.S.m. 2016. Nanoemulsions: formation, properties and applications. *Research*, 12(11):2826-2841.

Istvan, E.S. & Deisenhofer, J. 2001. Structural mechanism for statin inhibition of HMG-CoA reductase. *Science of pharmaceutics*, 292(5519):1160-1164.

Johnsen, G.K. 2010. Skin electrical properties and physical aspects of hydration of keratinized tissues.

Karahalil, B., Hare, E., Koç, G., Uslu, İ., Şentürk, K., Özkan, Y.J.A.o.I.H. & Toxicology. 2017. Hepatotoxicity associated with statins. *Science*, 68(4):254-260.

Katsanidis, E. & Addis, P.B., 1999. Novel HPLC analysis of tocopherols, tocotrienols, and cholesterol in tissue. *Free radical biology and medicine*, 27(11-12):1137-1140.

Kim, M.S., Jeong, M.H., Hwang, S.H., Ko, J.S., Lee, M.G., Sim, D.S., Park, K.-H., Yoon, N.S., Yoon, H.J. & Kim, K.H.J.T.K.J.o.M. 2010. Clinical pattern changes in elderly patients who underwent percutaneous coronary intervention in the last ten years. *The korean journal of medicine*, 79(6):661-672.

Lai-Cheong, J.E. & McGrath, J.A. 2009. Structure and function of skin, hair and nails. *Medicine*, 37(5):223-226.

Last, A.R., Ference, J.D. & Falleroni, J. 2011. Pharmacologic treatment of hyperlipidemia. *American family physician*, 84(5).

Levi, K. & Dauskardt, R.H. 2012. Biomechanics of the barrier function of human stratum corneum. Treatment of dry Skin Syndrome. Springer, p. 233-254).

Ma, H. 2004. Cholesterol and human health. *Nature and science*, 2(4):17-21.

Mancini, G.J., Tashakkor, A.Y., Baker, S., Bergeron, J., Fitchett, D., Frohlich, J., Genest, J., Gupta, M., Hegele, R.A. & Ng, D.S. 2013. Diagnosis, prevention, and management of statin adverse effects and intolerance: Canadian working group consensus update. *Canadian journal of cardiology*, 29(12):1553-1568.

Marais, A. 2016. Hypercholesterolaemia: from inauspicious to appropriate to detrimental: case study. *South African pharmaceutical journal*, 83(1):30-32.

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

Maron, D.J., Fazio, S. & Linton, M.F. 2000. Current perspectives on statins. *Circulation*, 101(2):207-213.

McFarland, A., Anoopkumar-Dukie, S., Arora, D., Grant, G., McDermott, C., Perkins, A. & Davey, A.J.I.j.o.m.s. 2014a. Molecular mechanisms underlying the effects of statins in the central nervous system. *International journal of molecular sciences*, 15(11):20607-20637.

McFarland, A.J., Anoopkumar-Dukie, S., Arora, D.S., Grant, G.D., McDermott, C.M., Perkins, A.V. & Davey, A.K. 2014b. Molecular mechanisms underlying the effects of statins in the central nervous system. *International journal of molecular sciences*, 15(11):20607-20637.

McKenney, J.M., Davidson, M.H., Jacobson, T.A. & Guyton, J.R. 2006. Final conclusions and recommendations of the national lipid association statin safety assessment task force. *The american journal of cardiology*, 97(8):S89-S94.

Moharamzadeh, K., Van Noort, R., Brook, I.M. & Scutt, A.M., 2007. Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes. *Dental materials*, 23(1):40-44.

Morrow, D., McCarron, P., Woolfson, A. & Donnelly, R. 2007. Innovative strategies for enhancing topical and transdermal drug delivery. *The open drug delivery journal*, 1:36-59.

Murphy, A., Faria-Neto, J.R., Al-Rasadi, K., Blom, D., Catapano, A., Cuevas, A., Lopez-Jimenez, F., Perel, P., Santos, R. & Sniderman, A. 2017. World heart federation cholesterol roadmap. *Global heart*, 12(3):179-197. e175.

Nemes, Z. & Steinert, P.M. 1999. Bricks and mortar of the epidermal barrier. *Experimental & molecular medicine*, 31(1):5.

Ng, K.W. & Lau, W.M. 2015. Skin deep: the basics of human skin structure and drug penetration. Percutaneous penetration enhancers chemical methods in penetration enhancement. Springer. p. 3-11).

Nordestgaard, B.G., Chapman, M.J., Humphries, S.E., Ginsberg, H.N., Masana, L., Descamps, O.S., Wiklund, O., Hegele, R.A., Raal, F.J. & Defesche, J.C. 2013. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidance for clinicians to prevent coronary heart disease: consensus statement of the European Atherosclerosis Society. *European heart journal*, 34(45):3478-3490.

O'Sullivan, S. 2007. Statins: A review of benefits and risks. *TSMJ*, 8:52-56.

Onwe, P., Folawiyo, M., Okike, P., Balogun, M., Umahi, G., Besong, E., Okorocha, A. & Afoke, A. 2015. Lipid Profile and the Growing Concern on Lipid Related Diseases. IOSR *Journal of pharmacy and biological sciences (IOSJPBS)*, 10:2278-3008.

Özgün, S.J.A.U. 2013. Nanoemulsions in cosmetics. *European cosmetics journal*, 1(6):3-11.

Pathan, I.B. & Setty, C.M. 2009. Chemical penetration enhancers for transdermal drug delivery systems. *Topical journal of pharmaceutical researc*h, 8(2):16-24.

Pawar, K.R. & Babu, R.J. 2014. Lipid materials for topical and transdermal delivery of nanoemulsions. *Critical Reviews™ in therapeutic drug carrier systems*, 31(5).

Prausnitz, M.R., Elias, P.M., Franz, T.J., Schmuth, M., Tsai, J.-C., Menon, G.K., Holleran, W.M. & Feingold, K.R.J.D. 2012. Skin barrier and transdermal drug delivery. *Dermatology*, 3:2065-2073.

Rao, S., Prasad, T., Mohanta, G. & Manna, P. 2011. An overview of statins as hypolipidemic drugs. *International journal of pharmaceutical sciences and drug research*, 3(3):178-183.

Rohilla, A., Dagar, N., Rohilla, S., Dahiya, A. & Kushnoor, A. 2012. Hyperlipidemia-a deadly pathological condition. *International journal of current pharmaceutical research*, 4(3):15-18.

Schachter, M. 2005. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundamental & clinical pharmacology*, 19(1):117-125.

Schaiff, R.A.B., Moe, R.M. & Krichbaum, D.W. 2008. An overview of cholesterol management. *American health & drug benefits*, 1(9):39.

Shah, V.J.D. & sciences, t.p. 1994. Skin penetration enhancers: scientific perspectives. *Drugs and the pharmaceutical sciences*, 62:19-23.

Sharma, N., Bansal, M., Visht, S., Sharma, P. & Kulkarni, G.J.C.o.Y.S. 2010. Nanoemulsion: A new concept of delivery system. *Chronicles of young scientists*, 1(2):2.

Staffa, J.A., Chang, J. & Green, L.J.N.E.J.o.M. 2002. Cerivastatin and reports of fatal rhabdomyolysis. *New England journal of medicine*, 346(7):539-540.

Steinberg, D. & Gotto Jr, A.M. 1999. Preventing coronary artery disease by lowering cholesterol levels: fifty years from bench to bedside. *The journal of the american medical association*, 282(21):2043-2050.

Tolman, K.G.J.T.A.j.o.c. 2002. The liver and lovastatin. *The journal of american association*, 89(12):1374-1380.

Tsai, M.-J., Fu, Y.-S., Lin, Y.-H., Huang, Y.-B. & Wu, P.-C.J.P.o. 2014. The effect of nanoemulsion as a carrier of hydrophilic compound for transdermal delivery. *PloS one*, 9(7):6-14.

Uchida, Y. & Park, K. 2016. Stratum Corneum. Immunology of the Skin. *Springer*, 4(1):15-30.

Venkitachalam, L., Wang, K., Porath, A., Corbalan, R., Hirsch, A.T., Cohen, D.J., Smith Jr, S.C., Ohman, E.M., Steg, P.G. & Bhatt, D.L. 2012. Global variation in the prevalence of elevated cholesterol in outpatients with established vascular disease or 3 cardiovascular risk factors according to national indices of economic development and health system performance. *Circulation*, 125(15):1858-1869.

Vermaak, I., Kamatou, G.P.P., Komane-Mofokeng, B., Viljoen, A. & Beckett, K. 2011. African seed oils of commercial importance—Cosmetic applications. *South African journal of botany*, 77(4):920-933.

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

Wilbur, R.L. 2017. The Difference Between Topical and Transdermal Medications. https://genscopharma.com/wp-content/uploads/2017/05/Topical-v-Transdermal-White-Paper-05-26-17-EMAIL.pdf Date of access: 15 Feb. 2019.

Williams, A. 2003. Transdermal and topical drug delivery: from theory to clinical practice: Pharmaceutical press London.

CHAPTER 2

TRANSDERMAL NOVEL DRUG DELIVERY IN HYPERCHOLESTEROLAEMIA

2.1 Introduction

Studies have revealed that children 8 – 10 years old are being diagnosed with hypercholesterolaemia and hypertension at an alarming rate (Tolani *et al.*, 2013). Although cholesterol is essential for normal body and mind function, abnormal elevation of cholesterol levels can occur due to various factors that can result in hypercholesterolaemia, which has been connected to serious health complications, e.g. atherosclerosis and CVDs. In atherosclerosis, plaques form and cause restriction of blood flow leading to CVDs. There are different classes of lipid-modifying treatments available, however this study focused on statins, as discussed in Chapter 1.

Statins are the preferred first-line pharmacological therapy for hypercholesterolaemia (Athyros *et al.*, 2017; Braamskamp *et al.*, 2012; O'Sullivan, 2007; Robinson & Goldberg, 2011; Schachter, 2005). Currently, oral administration of statins (Robinson & Goldberg, 2011) presents with inadequacies, such as gastrointestinal side effects (Mancini *et al.*, 2013), reduced systemic bioavailability and increased risk of hepatic toxicity (McKenney *et al*., 2007; Schierwagen *et al*., 2017).

Due to these inadequacies and side effects of oral administered statins, the transdermal route is investigated. It is possible that APIs can reach systemic blood circulation after penetrating various skin layers (Kala & Juyal, 2018). However, these unique physical properties of the skin can influence drug delivery across the skin (Ng & Lau, 2015). The epidermis forms the outermost layer and constitutes a chemical and diffusional barrier towards the environment (Johnsen, 2010), where the stratum corneum is the most specialised layer (Baibhav *et al*., 2011). The stratum corneum is known to be the rate-limiting barrier that influences transdermal drug delivery (Arda *et al*., 2014; Wickett & Visscher, 2006; Wilbur, 2017) due to the lipophilic construction of the stratum corneum, which can ultimately pose challenges to permit API absorption (Baibhav *et al*., 2011).

To accomplish transdermal delivery successfully, literature has provided the ideal physicochemical characteristics an API should possess (Naik *et al*., 2000). The selected statins (atorvastatin, mevastatin, pitavastatin and pravastatin) in this study do not meet these ideal physicochemical requirements for transdermal delivery (Naik *et al*., 2000) with regard to the octanol-buffer distribution coefficient (log D) or octanol-water partition coefficient (log P), melting point, molecular weight, diffusion coefficient, aqueous solubility, pKa (Williams, 2013), polarity

and pH (Rastogi & Yadav, 2012), therefore to commence transdermal delivery the statins need to be formulated within a carrier system (**NEs** and **NGs**), despite the unfavourable physicochemical characteristics of statins, to overcome the stratum corneum (Gabera *et al*., 2017).

An **NE** is a particular carrier system widely used for its eco-friendly, non-immunogenic and biocompatible nature (Gabera *et al*., 2017). These systems can bypass hepatic metabolism, decrease toxicity, extend half-lives and enhance absorption (Gabera *et al*., 2017). **NEs** are used for the transdermal delivery of the statins and compared to **NGs** (semi-solid formulations), which are formulated from the adjusted **NE** formula. An **NG** is also known as hydrogel-thickened **NE**, since the system is based on an **NE** foundation integrated with a gelling-agent, which may lead to better skin penetration (Basera *et al*., 2015; Chellapa, 2015; Eid *et al*., 2014) with the addition of penetration enhancers.

Penetration enhancers, also known as sorption promoters, are utilised in the formulation of this study to enhance API flux and skin permeability (Kala & Juyal, 2018). Penetration enhancers function by reversibly altering the obstacles posed by the skin (Alexander *et al*., 2012; Boglarka *et al*., 2016; Kala & Juyal, 2018). According to Charoo *et al*. (2008), many chemicals that have shown effective penetration enhancing capabilities unfortunately possess localised and systemic side effects. It is therefore imperative to investigate possible penetration enhancers that are safe, effective and easy to attain, such as grapeseed oil.

Grapeseed oil is considered a natural vegetable oil that contains both antioxidants and fatty acids, which are frequently used as penetration enhancers to endorse skin permeation (Fang *et al.*, 2004). Natural oils are worldwide available, therefore they could signify more acceptable, natural, economical alternatives compared to other penetration enhancers (Fang *et al.*, 2004).

2.2 Defining cholesterol

Cholesterol is a waxy, fat-like substance that occurs in all the cells of the human body (Robinson & Goldberg, 2011). Cholesterol is essential for synapse, dendrite and neuron development (Orth & Bellosta, 2012) to maintain normal brain function and assist with axonal guidance (Zhang & Liu, 2015). Cholesterol also serves as a forerunner of steroid hormones, vitamin D and bile acids in the human body (Scherr & Zidenberg-Cherr, 2016). However, cholesterol exhaustion leads to synaptic and dendritic spine degeneration, neurotransmission failure, and reduced synaptic plasticity (Orth & Bellosta, 2012). Furthermore, defects in cholesterol metabolism lead to central nervous system (CNS) diseases, such as Smith-Lemli-Opitz syndrome, Niemann-Pick type C (NPC) disease, Huntington's disease, and Alzheimer's disease. More importantly, cholesterol forms an essential part of somatic cells in the human body, more specifically the cell walls, where

it sustains cell communication and stability to ensure the somatic cells act as a unit (Scherr & Zidenberg-Cherr, 2016).

Cholesterol requires certain transporters to travel in the bloodstream due to its insoluble properties in blood (Rohilla *et al*., 2012). Lipoproteins are transporters that consist of a central part, made up of cholesterol and triglycerides, with an outward surface covering its protein (Rohilla *et al*., 2012). Lipoproteins are constructed by the liver to enable the transport process of lipids from the intestines to the liver and between the liver and somatic cells (Jonas, 2002).

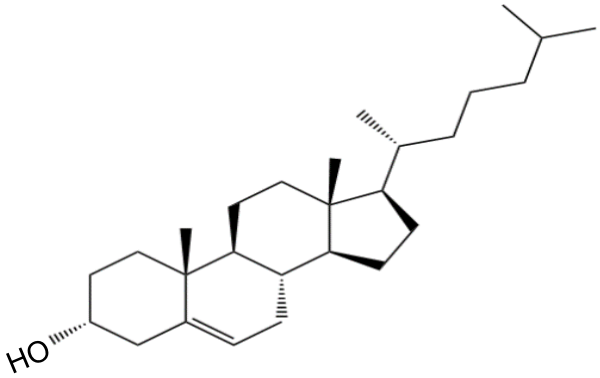

Figure 2.1: Chemical structure depiction of cholesterol

2.2.1 Lipoproteins

Lipoproteins are categorised according to their specific density, as the density of these proteins establishes lipoprotein-cholesterol bindings, e.g. high-density lipoproteins (HDL), LDL, and very-low-density lipoprotein (VLDL) (Rohilla *et al*., 2012).

2.2.1.1 High density lipoprotein

HDL, also known as “good cholesterol,” inhibits the binding of extra fat on the artery walls. HDL is responsible for the transport, binding and metabolism of cholesterol (Rohilla *et al*., 2012). The ideal level of HDL is more than 60 mg/dl, while less than 40 mg/dl indicates a great risk for developing CVD. Research implies that HDL can be used as topical wound healing administration, since it entails pleiotropic effects aside from arterial functions to operate as an anti-inflammatory, atheroprotective cell protector with additional healing characteristics to the human body (Gordts *et al*., 2014).

2.2.1.2 Low density lipoprotein

LDL is responsible for the transport of intestinal and hepatic cholesterol to specific organs, muscles, tissue and the heart, in the human body (Onwe *et al*., 2015; Rohilla *et al*., 2012). The

ideal level of LDL to maintain optimal health is less than 100 mg/dl and should preferably be 0 – 70 mg/dl, whereas 160 – 189 mg/dl are considered as elevated levels (Onwe *et al*., 2015). Increased LDL levels can promote the risk for developing CVDs and atherosclerosis (Mahmood *et al*., 2009; Onwe *et al*., 2015).

2.2.1.3 Very low-density lipoprotein

VLDL predominantly comprises of fat and transports cholesterol similar to LDL (Onwe *et al*., 2015). Elevated levels of VDL and triglycerides are also associated with CVDs and atherosclerosis (Onwe *et al*., 2015). Excess sugar, alcohol and calories are distorted to triglycerides and transported by VLDL, where it accrues in fat cells of the body, heightening the risk of developing diabetes (Smelt, 2010). The ideal triglyceride concentration is less than 150 mg/dl, values between 200-499 mg/dl are regarded as high concentration levels, 500 mg/dl and above, are considered abnormal and will increase the risk for developing CVDs (Onwe *et al*., 2015). Lipoprotein-cholesterols, e.g. LDL, HDL, VLDL, all add to the total cholesterol (TC). Guidelines of the National Cholesterol Education Programme (NCEP) stated the desired levels of TC for children and adults should be less than 180 and 200 mg/dl, respectively. Potential cardiac episodes can decrease if TC concentration levels decrease to 150 mg/dl. A level higher than 240 mg/dl is regarded as hyperlipidaemia (Rohilla *et al*., 2012).

2.2.2 Hyperlipidaemia

Hyperlipidaemia can be classified as a chronic illness and is defined as an unusual high amount of lipids present in blood plasma (Churchill-Livingstone's Dictionary, 2008). Hyperlipidaemia has a mortality figure of about 4.4 million, and has been associated with CVDs, thus dominating mortality rate globally (Santaniello & Giannini, 2015).

2.2.2.1 Pathophysiology of hyperlipidaemia

The pathophysiology of hyperlipidaemia can be classified as primary hyperlipidaemia and secondary hyperlipidaemia (Rohilla *et al*., 2012).

2.2.2.1.1 Primary hyperlipidaemia

Familial hypercholesterolaemia (FH), which is primary hyperlipidaemia, is a congenital metabolic disorder caused by a LDL receptor gene-coding defect. Due to this defect, raised serum LDL cholesterol will be present (Rohilla *et al*., 2012), which contributes to coronary heart disease, atherosclerosis, xanthomas and the accumulation of cholesterol in peripheral tissues (Fahed & Nemer, 2011).

2.2.2.1.2 Secondary hyperlipidaemia

Several underlying illnesses may contribute to secondary hyperlipidaemia, e.g. diabetes mellitus (due to the high VLDL levels), hyperadrenocorticism (due to induced VLDL production), nephrotic syndrome (increased production of cholesterol), hypothyroidism (due to the decrease of cholesterol degradation), liver illness hypercholesterolaemia, polycystic ovary syndrome and high oestrogen concentration levels in females (Rohilla et al., 2012).

Additionally, an unhealthy lifestyle, such as high saturated fat diets, passiveness, obesity, menopause, alcohol abuse and medication, e.g. beta-blockers, oral contraceptives, thiazide diuretics, glucocorticosteroids, retinoic acid, and cyclosporine can contribute to elevated cholesterol concentration levels (Fatima *et al*., 2017). Considering the different categories of hyperlipidaemia, primary hyperlipidaemia will be focused on during this study.

2.2.2.2 Primary hyperlipidaemia diagnoses

Regarding the diagnoses of primary hyperlipidaemia, a serum test, known as a lipid profile, can be performed to reveal the amount of cholesterol (HDL and LDL) and triglycerides present in the blood (Onwe *et al.*, 2015). A lipid profile serum test is utilised to measure the concentration of cholesterol and triglycerides in the blood to help assess the risk of CVDs and to start the most suitable and available treatment (Onwe *et al*., 2015).

2.2.2.3 Primary hyperlipidaemia medical treatment

There are several different classes of lipid-modifying drugs available, including, nicotinic acid (niacin), bile acid-binding resins (e.g. colestipol, cholestyramine, colesevelam), fibrates (e.g. clofibrate, bezafibrate, fenofibrate, gemfibrozil), cholesterol-absorption inhibitors (e.g. ezetimibe), and finally, statins (e.g. atorvastatin, mevastatin, pravastatin and pitavastatin) (Schachter, 2005). Statins are the preferred medical treatment for hyperlipidaemia due to their LDL lowering properties. Combination therapy is uncommon, but can be initiated if the LDL levels do not decrease to the desired level. Combination therapy consists of a prescribed statin in combination with nicotinic acid, cholesterol absorption inhibitors, fibric acid and bile acid binding resins and may possibly contribute to severe myopathy and rhabdomyolysis (Zodda *et al*., 2018).

Novel lipid-reducing alternatives, e.g. lomitapide and mipomersen and PCSK9 inhibitors (e.g. alirocumab, evolocumab) (Earl, 2019) can be used for the treatment of FH by reducing LDL levels and the risk of CVDs. However, the high financial impact of this alternative medication is costly and therefore remains a concern. This study therefore focused on the first line treatment, e.g. statins.

2.3 Statins

Statins are used to lower cholesterol levels and are responsible for decreasing major cholesterol-associated cardiovascular events by 25 – 40% (Marais, 2016). HMG-CoA reductase is the rate-limiting enzyme that is responsible for the synthesis of cholesterol (Braamskamp *et al.*, 2012; McFarland *et al.*, 2014; O'Sullivan, 2007). Statins ultimately reduce the amount of intracellular cholesterol, hence the up-regulation of hepatic LDL receptors (Braamskamp *et al.*, 2012).

Statins increase HDL cholesterol and decrease the amount of LDL cholesterol, including triglycerides in the body (Braamskamp *et al.*, 2012). According to O'Sullivan (2007), and based on data available, current registered statins, with the exception of cerivastatin, have a favourable safety profile. Statins have a wide range of pleiotropic effects that may significantly contribute to the treatment of other non-cardiovascular conditions as well (Gazzerro *et al.*, 2012; McFarland *et al.*, 2014; McFarlane *et al.*, 2002).

Bioavailability after oral administration is mainly affected by the physicochemical properties of the API and the functional status of the patient's gastrointestinal transporters regarding ontogeny and genetics (Wagner & Abdel-Rahman, 2016). Statins include mild gastro-intestinal side effects, e.g. abdominal cramps, constipation, diarrhoea, difficulty swallowing, indigestion, flatulence, nausea and vomiting (Mancini *et al.*, 2013), and decreased systemic bioavailability due to statin-stomach content interaction or extensive clearance of statins by liver enzymes (CYP450) (Marrow *et al*., 2007; Naik et al., 2000). It should be mentioned that rare, but life-threatening conditions, such as rhabdomyolysis, might occur (Corsini *et al.*, 1999; O'Sullivan, 2007; Rao *et al.*, 2011). Statin levels can increase due to the occurrence of drug interactions, which can lead to adverse effects (Piepho, 2000). The administration of statins via the oral route can also be responsible for side effects such as elevated serum aminotransferase levels, thus increasing the risk for hepatic toxicity (McKenney *et al*., 2007; Schierwagen *et al*., 2017). Orally administered statins are well absorbed from the intestine, however they undergo significant extensive first-pass hepatic metabolism, which decreases the systemic bioavailability to approximately 5 – 50% (McFarland *et al.*, 2014; Ose, 2010). Therefore, by refining the drug release method of statins via transdermal drug delivery, it may reduce oral induced side effects and enhance their overall bioavailability.

Over 40 million patients diagnosed with elevated cholesterol levels are on statin-induced medical treatment (Hennessy *et al*., 2016). Statins were regarded as a major discovery in the 20th century (Hennessy *et al*., 2016; Santaniello & Giannini, 2015) for these APIs can reduce cholesterol produced in the liver by inhibiting HMG-CoA reductase (Fong, 2016; Hennessy et al., 2016). Consequently, by decreasing intracellular cholesterol levels, the LDL receptors on the surface of the hepatocytes can multiply. Unbound LDL found in the blood will bind to these specific receptors, thus lowering blood LDL concentration levels (Fong, 2016). Additionally, the available

receptors will also lower LDL forerunners, e.g. VLDL and IDL (intermediate-density lipoprotein) (Zodda *et al*., 2018). Statins can also enhance HDL concentration and decrease the amount of triglycerides in the blood (Fong, 2016).

Statins made it possible to reduce the occurrence of CVDs, for raised cholesterol levels are related to atherosclerosis and CVDs (Fatima *et al*. 2017; Fong, 2016). This API has a dual function that is unrelated to lipid altering properties. Statins also constrain the inflammation process (Hennessy *et al*., 2016) by inhibiting the production of nonsteroidal isoprenoid complexes, which originated from mevalonic acid (Fong, 2016). Additionally, statins promote immunomodulatory and anti-microbial functions (Hennessy *et al*., 2016), and show promising effects regarding decreasing brain ischemia due to inhibition of LDL oxidation and their ability to decrease platelet accumulation and cell proliferation (Schultz *et al*., 2018).

2.3.1 Administration of statins

Statins are currently administered via the oral route, usually at night as cholesterol synthesis occurs during early mornings (Cruz *et al*., 2008). The preferred route for medicine is the oral route due to patient compliance, but this route has several shortcomings regarding the use of statins (Zodda *et al*., 2018).

2.3.2 Oral statin disadvantages

There are limitations that may occur in the oral administration of statins as a result of decreased bioavailability (atorvastatin (12%), pitavastatin (> 60%) and pravastatin (18%)) due to hepatic first-pass clearance, or CYP450 liver enzymes (atorvastatin (CYP450 3A4), mevastatin (CYP450 3A4), pitavastatin (CYP450 2C9) (Sahebzamani *et al*., 2014), or the interaction with stomach substances (Marrow *et al*., 2007; Naik *et al*., 2000; Zodda *et al*., 2018). Potential drug interactions can lead to increased statin levels in the circulatory system, as certain drugs can inhibit CYP450 liver enzymes, which will lead to possible adverse effects, mainly in patients with liver diseases (Piepho, 2000; Zodda *et al*., 2018).

Statins have an excessive affinity for plasma proteins and are extremely bound worthy (e.g. atorvastatin (98%) and pitavastatin (96%)), which can reduce pharmacological affects due to minimal exposure of the API to activate the receptors (Fong, 2016; Zodda *et al.,* 2018). Pravastatin (50%) allows the unbound drug to be up to 10 times greater than other statins due to low plasma protein binding. Pravastatin has hydrophilic properties and ensures that distribution is limited in the human body (Zodda *et al*., 2018).

2.3.3 Alternative route of administration of statins

The transdermal route will be investigated, instead of the oral statin route due to the potential side effects (as mentioned in Section 2.3) (Zodda *et al*., 2018).

2.4 Transdermal route

Centuries ago, the transdermal route was considered a possible route for systematically delivering APIs through integrated skin (Kala & Juyal, 2018). Transdermal delivery can be described as non-invasive delivery of medications or APIs through the superficial layers of the skin. In the early 20th century, a homemade medicinal preparation intended for topical delivery was mustard plasters used for chest congestion; therefore, transdermal delivery is not a modern concept (Scheindlin, 2004).

The first generation of drug delivery technology dealt with physicochemical problems, while the second and third generations struggled with biological barriers, e.g. stratum corneum (Yun *et al.,* 2015). In the past few years, new interest occurred in the development of drug delivery systems to deliver existing molecules beyond biological barriers and their current routes of administration. Transdermal drug delivery, therefore, remains a possible alternative to oral or parental drug delivery.

Transdermal delivery can offer significant advantages and is well-known to minimise and avoid many limitations of other administration routes due to the fact it: 1) is non-invasive and improves patient compliance; 2) allows drug absorption in the absence of pre-systemic metabolism, thus improving bioavailability; 3) provides a route of administration for drugs that can otherwise not be absorbed via the gastrointestinal tract; 4) ensures prolonged and constant plasma concentrations for prolonged time periods, lessens inter-/intra-variations between patients (Kala & Juyal, 2018) and tolerates the possibility of intervention, such as removal of the APIs applied on integrated skin to avoid overdosing (Rastogi & Yadav, 2012); 5) provides an alternative approach to the treatment of conditions in the deeper dermal tissues; 6) may require lower concentrations of the substance administered compared to traditional drug delivery routes, e.g. oral administration (Prausnitz & Langer, 2008). Consequently, it would be ideal to incorporate statins into a transdermal formulation. It should be taken into consideration that transdermal drug delivery is not suited for all drugs, as drug properties, e.g. molecular size and structure, play a major role in the efficacy of transdermal drug delivery systems (Prausnitz & Langer, 2008). In order to elucidate the fundamentals of transdermal drug delivery and design appropriate drug delivery systems, the anatomy and physiological principles of the skin need to be explained.

2.4.1 Integumentary system

The skin accounts for nearly 16% of the total body weight of an adult (Wickett & Visscher, 2006). Skin is a dynamic organ in a continuous state of change and is self-renewing, as cells of the outer layers are constantly shedding and replaced by inner cells moving up to the surface (Baroni *et al*., 2012; Goodwin, 2011). The skin's vital role is to prevent the loss of water and other essential substances, necessary to maintain optimal health (Madison, 2003). It provides protection against external environmental elements, such as mechanical and chemical insults, infections, heat, water, and electromagnetic radiation (Baroni *et al*., 2012; Wickett & Visscher, 2006). The regulation of blood pressure and body temperature adds to the significance of the skin. In addition, the skin supports synthesis and metabolism, such as vitamin D production, and contains anti-oxidative and antimicrobial properties (Flynn, 2002; Mostafa *et al*., 2015).

Drug penetration and permeation through the skin are extensively affected by the physical properties of the skin layers (Ng & Lau, 2015). As mentioned in Chapter 1, the skin consists of three layers, namely the epidermis, dermis, and the hypodermis (Lai-Cheong & McGrath, 2009). The dermis is situated beneath the epidermis and is constructed from connective tissue, containing proteins, elastin, collagen and reticulum. Additionally, the dermis is thicker than the epidermis and presents as a protective layer against any external mechanical influence (Goodwin, 2011; Johnsen, 2010). The dermis hosts sweat glands, hair follicles, sebaceous glands, nerves, blood vessels and sensory receptors (Arda *et al*., 2014). Beneath the dermis is the hypodermis, containing mostly adipose tissue with the purpose of isolating heat (Johnsen, 2010). Consequently, the hypodermis is the deepest layer that connects the skin to underlying structures, e.g. bone and muscles (Arda *et al*., 2014; Johnsen, 2010).

The epidermis consists of keratinised stratified squamous epithelium (Arda *et al*., 2014; Goodwin, 2011). Due to constant differentiation and cytomorphosis of keratinocytes, thick skin consists of five layers and thin skin has four layers (Arda *et al*., 2014). Observing the deepest part of the epidermis towards the surface of the skin, the viable epidermis includes the stratum basale, stratum spinosum, stratum granulosum and stratum lucidum; the non-viable epidermis consists of the stratum corneum (Arda *et al*., 2014; Kute & Saudagar, 2013; Ng & Lau, 2015). The epidermis also consists of appendageal elements such as hair follicles and sweat ducts (Ng & Lau, 2015).

2.4.1.1 The non-viable epidermis

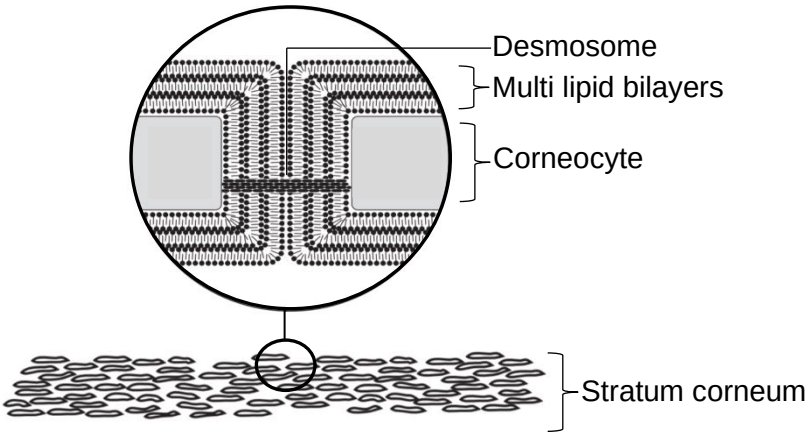

Figure 2.2: The 'brick and mortar' model of the human stratum corneum (Baroni *et al.*, 2012)

The stratum corneum is 25 – 45 µm in diameter and 0.3 – 0.7 µm in thickness and consists of 15 – 25 layers of hydrophilic corneocytes with intercellular spacing (0.1 – 0.3 µm) and thickness (10 – 25 µm), which are flat cells covered with lipids and desmosomes (Geerligs, 2006). These lipids function to prevent transepidermal water loss from beneath the epidermis and consist of long chain fatty acids, ceramides and cholesterol, together with small amounts of phospholipids and glucosylceramides (Geerligs, 2006; Menon *et al*., 2012; Williams, 2013). However, depletion of ceramides with very long chain fatty acids can result in a defective skin permeability barrier function (Gaur *et al*., 2014), which can possibly influence flux (API transport) through the skin barrier (El Maghraby *et al*., 2008; Jepps *et al*., 2013). Regarding the barrier function of the stratum corneum, the unique composition of the stratum corneum's intercellular lipids and their structural arrangements in multiple lamellar layers within a continuous lipid domain, is critical for the stratum corneum to function properly (Wilbur, 2017).

Keratinocytes synthesise major structural proteins, namely keratins (Baroni *et al.*, 2012; Taichman & Prokop, 1982). Keratinocyte differentiation is a process that occurs in the skin and is associated with physical cell changes. The cells then transform into flat squamous cells of the upper epidermal layer, which is eventually enclosed by a cell envelope that includes cross-linked proteins and a covalently bound lipid envelope (Baroni *et al.*, 2012). Desmosomes are intercellular junctions that provide strong adhesion between cells, connecting keratinocytes (Baroni *et al.*, 2012; Garrod & Chidgey, 2008). Corneocytes comprise of resistant cell envelopes and keratin microfibrils that are considered the 'bricks,' while the lipids found between the cells of the layers are considered the 'mortar' (Nemes & Steinert, 1999; Uchida & Park, 2016; Wickett & Visscher, 2006). The composition of the "brick and mortar" model sets challenges to hydrophilic and lipophilic absorption (Baibhav *et al*., 2011). Keratinocytes uses the mortar to release lipids

in the open spaces between the cells, as they are being transformed into corneocytes (Wickett & Visscher, 2006).

2.4.1.2 Viable epidermis

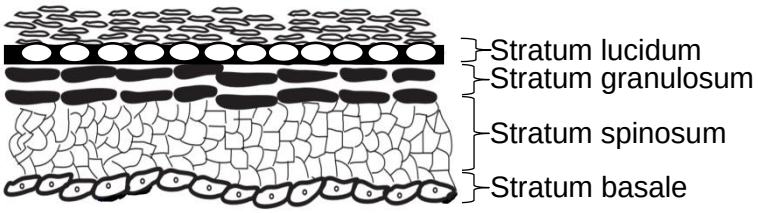

Figure 2.3: The viable epidermis and sub-layers e.g. stratum lucidum, stratum granulosum, stratum spinosum and stratum basale (Baroni *et al.*, 2012)

The viable epidermis, e.g. stratum lucidum, stratum basale, stratum spinosum and stratum granulosum, is a hydrophilic layer between the dermis and the non-viable epidermis (stratum corneum) and primarily consists of water and protein (Jepps *et al*., 2013; Kute & Saudagar, 2013) and small amounts of melanocytes, Langerhans cells and Merkel cells (Geerligs, 2006). Keratinocytes travel from the stratum basale (where the migration process starts) to the surface of the skin, where they adapt in form, size and physical characteristics (Geerligs, 2006). Following cell division, these particular cells migrate to the stratum spinosum, once they have transformed into bigger three dimensional (polyhedral) cells (Geerligs, 2006). Polyhedral cells in the stratum spinosum will start to flatten as they reach the stratum granulosum (Geerligs, 2006); at this point, the process of nucleic and mitochondrial deterioration occurs and when they reach the stratum corneum, they are entirely altered to non-viable (Geerligs, 2006). Shedding is a referred term that defines when old cells are discarded on the surface and replaced with new cells (Geerligs, 2006); this process occurs over a period of 6 to 30 days (Geerligs, 2006).

2.4.1.3 Dermis

The dermis is 3 – 5 mm thick and located between the hypodermis and the epidermis (Jepps *et al*., 2013; Kute & Saudagar, 2013). The dermis consists of collagen fibrils and elastin, which are enclosed by thick hydrophilic mucopolysaccharide gel (Geerligs, 2006; Jepps *et al*., 2013; Williams, 2013). The dermis can function as protection by using elastic fibres to ensure protection of vessels and cells, which play a role in the healing process (Geerligs, 2006). The dermis also sustains the epidermis (Geerligs, 2006) and includes nerve endings, blood vessels and lymph vessels (Foldvari, 2000).

2.4.1.4 Hypodermis

The thickness of the hypodermis varies with race, gender, anatomical location and age. The hypodermis (adipose tissue) is situated beneath the dermis and above the muscles (Geerligs, 2006). The hypodermis is joined to the dermis with nerve and vascular complexes, and epidermal appendages (hair and nerve endings) (Geerligs, 2006). Adipose tissue contains fibroblastic tissue, macrophages, leukocytes and premature adipocytes (Geerligs, 2006). There is adequate blood supply in the hypodermis for adipocytes to function effectively (Geerligs, 2006). The core function of the hypodermis is to defend the body against physical trauma, to offer isolation against temperature alterations and to store high-energy molecules (Williams, 2013).

2.4.2 Skin penetration mechanism

Any substance applied, with the purpose to penetrate the skin, has three possible routes across the epidermis: a) follicular route, which is through the appendages, b) through the corneocytes, which is the transcellular route, and c) through the matrix layers, representing the intercellular route (Ng & Lau, 2015; Wilbur, 2017). These routes are not mutually exclusive, therefore most compounds infusing the skin through a combination of pathways are grounded on their unique physicochemical properties of the permeating molecule (Wilbur, 2017).

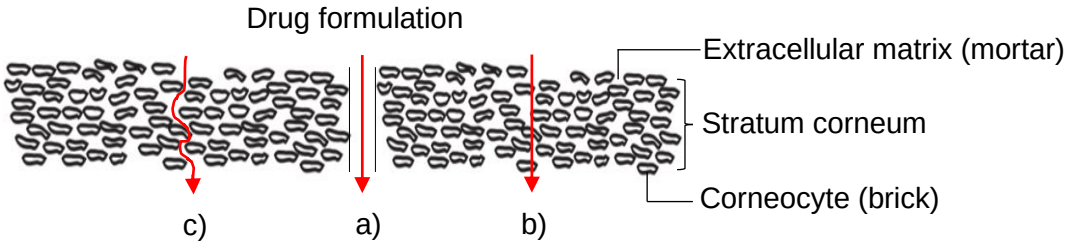

Figure 2.4: Drug permeation pathways in the skin: a) follicular, b) transcellular and c) intercellular route (Baroni *et al.*, 2012)

Most molecules penetrate the skin through the intercellular route and consequently, many enhancing techniques aim to disrupt or bypass its elegant molecular architecture (Pathan & Setty, 2009). Molecules that are lipophilic and polar are able to travel through the intercellular route, however the amount and rate of diffusion are obstacles to be taken into consideration in the physicochemical properties of the permeant (Ng & Lau, 2015; Wilbur, 2017).

API permeation involves several processes, including the release of the specific drug from the dosage form, followed by diffusion of the permeant through the stratum corneum (Wilbur, 2017). Therefore, these processes are extremely dependant on the solubility and diffusivity of the drug within each environment (Wilbur, 2017).

2.4.2.1 Follicular route

This route is known as the transappendageal route, or shunt route, responsible for the permeation of the API via hair follicles, apocrine glands and sweat channels (Gaur *et al*., 2014; Williams, 2003). The follicular route avoids the challenges of the stratum corneum with regard to a lipophilic API (Marrow *et al*., 2007; Williams, 2003). However, it should be mentioned that these follicular channels, as illustrated in Figure 2.4, only exist in approximately 0.1% of the skin surface, making this route unpopular (Gaur *et al*., 2014; Jepps *et al*., 2013; Marrow *et al*., 2007).

2.4.2.2 Transcellular route

This pathway requires transportation (diffusion and partition) of the API through hydrophilic corneocyte layers that consist of hydrated keratin and lipophilic lipid coatings of the stratum corneum (Ng & Lau, 2015; Rastogi & Yadav, 2012). This route is favourable for hydrophilic APIs (pravastatin), however corneocytes are rich within the stratum corneum, therefore challenges occur due to the lipophilic nature of lipid coatings (Marrow *et al*., 2007). Many challenges are faced by a molecule that crosses the stratum corneum by this route (Williams, 2003), mostly because this pathway involves a variety of diffusion and partitioning stages (Morrow *et al*., 2007).

2.4.2.3 Intercellular route

The intercellular route transports the API through non-polar lipophilic lipid coatings of the stratum corneum (Geethu *et al*., 2014; Marrow *et al*., 2007; Williams, 2003). Even though this route is advantageous for non-polar (uncharged) APIs, it remains difficult to transport the API across the stratum corneum (Marrow *et al*., 2007; Ng & Lau, 2015), because the API must diffuse and partition in the continuous aqueous together with the lipid environment (Rastogi & Yadav, 2012).

The API requires specific ideal characteristics (Rastogi & Yadav, 2012) to overcome challenges the skin presents when attempting to penetrate the skin through the aforementioned routes, such as restraining permeation of API (Williams, 2013), which affects the flux of APIs (Williams, 2013). This particular pathway is considered the most significant regarding the permeation of an API across the lipophilic stratum corneum. Manipulation of the solubility from the lipid domain and modification of the ordered structure, as techniques to improve penetration, is therefore the primary focus (Benson, 2005; El Maghraby *et al*., 2008).

2.4.3 Physicochemical properties that influence transdermal delivery

The appropriate nominee intended for transdermal drug delivery can be determined by comparing the physicochemical properties of the intended API to that of the properties considered ideal for transdermal delivery (Liu *et al*., 2016). It is important to consider the physicochemical parameters

to predict drug skin permeability (Liu *et al*., 2016). Table 2.1 displays some of these physicochemical characteristics for the selected statins regarding this study. These properties will also be discussed in more detail in the subsequent section.

Table 2.1: The physicochemical properties of pitavastatin, pravastatin, atorvastatin and mevastatin compared to the ideal physicochemical properties for transdermal delivery

	Mevastatin	Pitavastatin	Pravastatin	Atorvastatin	Ideal properties
Log D (pH 7.4)	3.570[i]	1.50[e]	- 0.47[e]	1.00 – 1.25[e]	1.00 – 3.00[h]
Molecular weight (Da)	390.520[b]	421.46[g]	446.50[c]	1155.34[a]	< 500.00[h]
Melting point (°C)	152.000[b]	No data	171.20 – 173.00[c]	159.20 – 160.70[f]	< 200.00[h]
Aqueous solubility (mg/ml)	0.032[d]	0.50[j]	10.00[k]	0.19[l]	> 1.00[h]

a) Drugbank, 2005a; b) Pubchem, 2005b; c) Drugbank, 2005b; d) Drugbank, 2005c; e) Fong, 2016; f) Pubchem, 2005a; g) Drugbank, 2005d; h) Naik *et al.,* 2000; i) Kim *et al*., 2011; j) Cayman chemical, 2014; k) Cayman chemical, 2015; l) Rodde *et al*., 2014

Table 2.1 indicates that statins used in this study do not comply with all the ideal physicochemical characteristics, consequently statins will not be able to overcome this barrier and penetrate the skin without the assistance of a carrier system, also known as a transport agent (Gabera *et al*., 2017).

2.4.3.1 Log P and log D

Considering Fick's first law, a high partition coefficient (K) is indicative of an increased flux (J). Implying that APIs with high partition coefficients are more lipophilic and will therefore partition into the stratum corneum fluently, however, it will be susceptible to obstacles regarding partitioning into aqueous tissue (Barry, 2007).

The partition coefficient of an API indicates its division between the hydrophilic and lipophilic phase (Williams, 2013). To determine the API distribution, the log P and log D value can be of assistance when considering the brick (hydrophilic) and mortar (lipophilic) structure of the stratum corneum (Williams, 2013). If the API is too hydrophilic, it is incapable of diffusing through the stratum corneum, however lipophilic APIs will remain in the lipophilic part of the stratum corneum as they permeate the skin with more efficiency (Sharma *et al*., 2011; Williams, 2003). It is thus ideal for an API to contain both lipophilic and hydrophilic characteristics to encourage successful permeation (Naik et al., 2000; Williams, 2003). The required log P and log D of an API is 1 – 3, signifying both oil and water solubility (Subedi *et al*., 2010; Williams, 2003; Wiedersberg & Guy,

2014). Log P values above 3 indicate high lipophilicity, whereas log P values under 1 indicate the compound is more hydrophilic (Kim *et al*., 2011).

2.4.3.2 Molecular weight

It is suggested the API flux can be influenced by molecular weight (Barry, 2002; Williams 2003), therefore the size and the molecular weight of the API molecules directly affect the transdermal permeation process (Williams, 2003). The size and the molecular weight should not surpass 500 g/mol or 500 Da, to enable successful transdermal delivery (Naik *et al*., 2000; Williams, 2013). Mevastatin (390.520 g/mol) (O'Neil, 2001), pitavastatin (421.46 g/mol) and pravastatin (446.52 g/mol) have molecular weights below 500 g/mol (Ruan *et al*., 2012). These selected statins fall within this suggested standard as their molecular weights vary between 390.520 – 446.52 g/mol (Bhagat & Sakhare, 2014; O'Neil, 2006; O'Neil, 2001; Tabassum *et al*., 2017), while atorvastatin (1155.34 g/mol) (Al-Adl *et al*., 2017) does not meet the required parameter. Considering the characteristics of the skin, larger molecules can be obstructed due to the barrier of the stratum corneum (Williams, 2013), hence atorvastatin could be limited for transdermal administration regarding this study (Prausnitz *et al*., 2004).

2.4.3.3 Melting point

Melting point can be defined as the specific temperature to which a solid material transforms into a liquid state at atmospheric pressure (Reddy *et al*., 2016). According to studies, a lower melting point will result in higher extent of skin permeation (Mohammadi-Samani *et al*., 2014). There is a direct relationship between the melting point and the aqueous solubility of an API (Williams, 2003). A higher melting point will result in lower aqueous solubility (Williams, 2003). The ideal melting point is < 200 °C (Naik *et al*., 2000) to acquire transdermal delivery of the selected statins; atorvastatin (± 151 °C) (Satpute *et al*., 2018), mevastatin (no data), pitavastatin (190 – 192 °C) (Van der Schaaf *et al*., 2006) and pravastatin (171.2 – 173 °C) (LKT labs, 2018), all succeed in the required melting point parameter.

2.4.3.4 Aqueous solubility

The term solubility, or salute, indicates the ability of a chemical compound (gas, liquid or solid) to dissolve in a solvent (gas, liquid or solid) to create a homogenous solution (Savjani *et al*., 2012). API solubility is greatly dependent on the applied solvent, and is measured as the concentration where further saturation of the solution will not increase the obtained concentration (Savjani *et al*., 2012). Solubility can influence the bioavailability of an API (Naik *et al*., 2000), since poor aqueous solubility can result in undesirable permeation (Kulkarni & Nagarsenker, 2008). According to the Encyclopaedia Britannica (2016), solubility is a fundamental physicochemical

characteristic of an API due to the close correlation between skin permeability and solubility, hence higher solubility results in better skin penetration. Therefore, aqueous solubility will be influenced by lipophilic APIs (Williams, 2003). The ideal aqueous solubility is > 1 mg/ml for an API to penetrate the skin successfully for transdermal delivery (Naik *et al*., 2000). According to literature, the aqueous solubility of selected statins is indicated as follows:

- Atorvastatin (0.190 mg/ml) with phosphate buffered solution (PBS) at pH 6.8 (Rodde *et al*., 2014)
- Mevastatin (0.032 mg/ml) with PBS at pH 6.8 (Drugbank, 2005)
- Pitavastatin (of 0.500 mg/ml) with PBS at pH 7.2 (Cayman chemical, 2014)
- Pravastatin (10.000 mg/ml) with PBS at pH 7.2 (Cayman chemical, 2015)

2.4.3.5 Diffusion coefficient

Diffusion can be defined as the relocation of molecules from a high concentration (initially concentrated) to a low concentration (area without/low concentration) (Comsol, 2017; Williams, 2003). The diffusion coefficient indicates the extent of the molecule flux beyond a plane (high concentration gradient) to another plane with a different/low concentration gradient (Comsol, 2017). Data from the diffusion coefficient progresses linearly over time, thus has a unit of area (cm^2) per time (h or s) (Comsol, 2017; Williams, 2003). Diffusion becomes more complex when a porous medium is involved, since molecules must steer through porous material, therefore Fick's laws for passive diffusion is applicable (Comsol, 2017). Fick's law is used to establish passive diffusion of APIs through the skin (Williams, 2013). The stratum corneum is a complex structure, which can ultimately lead to restriction of the diffusion process (Barry, 2002). The rate of diffusion can be influenced by the specific phase of formulation (Barry, 2002), where diffusivity is dependent on the number of hydrogen (H^+) bonds at hand in the APIs' chemical structure, amongst other aspects (Williams, 2013). The ideal extent of hydrogen (H^+) bonds is ≤ 5 donor H^+-bonds (HBD) and ≤ 10 acceptor H^+-bonds (HBA) to diffuse successfully (Ammar, 2017). By exploiting Molinspiration (computational tool), atorvastatin (HBD = 4, HBA = 7), mevastatin (HBD = 1, HBA = 5) (Pubchem, 2005), pitavastatin (HBD = 3, HBA = 5) (De Brito, 2011) and pravastatin (HBD = 4, HBA = 7) (IUPHAR/BPS Guide to pharmacology, 2017) are within the hydrogen bond requirements (HBD & HBA); statin hydrogen bonds should therefore not interfere with diffusion.

2.4.3.6 pH, pKa and ionisation

The human skin exhibits an acidic environment at a pH of ± 5 (Ng & Lau, 2015; Williams, 2013). It has been proposed that pH value beneath 3, or exceeding 9 can cause irritation and harm the

skin, which can ultimately affect skin penetrability (Akula *et al*., 2018; Naik *et al*., 2000). The specific pH has the aptitude to alter the ratios to which weakly acid and basic APIs dissociate; they are dependent on the pKa or pKb values of the API (Akula *et al*., 2018; Barry, 2002). The degree of ionisation at a specific pH has considerable value in topical and transdermal delivery (Nair *et al*., 2013) and is found exceptionally significant due to the lipophilic barrier observed from the skin (Williams, 2003).

It has been insinuated that API permeation and diffusion beyond the dermis is influenced by the ionic state, consequently ionised species permeate poorly compared to unionised species (Akula *et al*., 2018; Li *et al*., 2012; Williams, 2003), as dictated by the pH-partition hypothesis. As a result, high amounts of unionised API can improve skin permeability (Akula *et al*., 2018). It can be hypothesised that unionised species permeate lipid layers of the stratum corneum more efficiently than ionised species (Barry, 2002; Williams, 2003). Definite concentrations can be established regarding ionised and unionised species by the pH of the delivery system and ionisation constant of a weak acid or base (Wiechers, 1989). The Henderson-Hasselbalch equation was altered and used to establish the ionisation of an API/formulation (Equations 2.1 and 2.2)

$$\%\text{ionised} = \frac{100}{1 + \text{antilog}\,(pKa - pH)} \qquad \textbf{Equation 2.1}$$

$$\%\text{unionised} = 100 - \%\text{ionised} \qquad \textbf{Equation 2.2}$$

The following pKa values were revealed regarding selected statins (acids and bases): atorvastatin 4.6 (Fong, 2016), mevastatin 13.49 (Wong *et al*., 2008), pitavastatin 4.7 and pravastatin 4.7 (Fong, 2016); these selected statins are therefore considered weak acids. Due to these pKa values, the unionised species of the statins were calculated. At skin pH 5, atorvastatin (28.475%), pitavastatin (33.386%) and pravastatin (33.386%) displayed low unionised species, with the exception of mevastatin (99.999%) which presented a high amount of unionised species. Considering the ionic state of atorvastatin, pitavastatin and pravastatin, this can lead to poor penetration of the skin, thus penetration enhancers are utilised together with selected statins in attempt to overcome this problem. Consequently, the degree of ionisation at a specific pH has considerable value in topical and transdermal delivery.

2.5 Drug delivery vehicles

In this study, **NEs** and **NGs** were selected as the transport agent for the transdermal delivery of the statins (atorvastatin, mevastatin, pitavastatin, pravastatin). Transport agents are expected to be biocompatible, biodegradable and non-immunogenic (Gabera *et al*., 2017).

2.5.1 Nano-emulsions

An **NE** can be defined as a transport system that has two immiscible liquid phases (oil phase and water phase) to create a single-phase formulation. The characteristics of a **NE** includes adjustable rheology, high surface area per unit volume, and robust stability (Gupta *et al*., 2016), hence, kinetically stable (extended period) and generally insensitive to physical and chemical changes (Gupta *et al*., 2016).

These dispersions have been utilised since the 1940s (Kumar, 2014). **NEs** have three different types of composition, e.g. o/w **NE** (oil droplets dispersed in water medium), water-in-oil (w/o) **NE** (water droplets dispersed in oil medium) and bicontinuous **NE**. The composition of bicontinuous **NEs** includes water, as well as oil droplets that are dispersed and surrounded by a continuous system (Kumar, 2014; Verma *et al*., 2018). Consequently, o/w **NEs** will be formulated during this study.

NEs are also known as ultrafine-emulsions, submicron-emulsions, mini-emulsions and nano-dispersed systems (Kumar, 2014), and are an effective and safe option for pharmaceutical formulation development (Chime *et al*., 2014). **NEs** are regarded as isotropic systems, for they measure identical property values when inspected from different angles (Kumar, 2014) with high physical and thermodynamic stability (Abolmaali *et al*., 2011; Lu *et al*., 2014; Tadros *et al*., 2004). **NEs** increase the solubility of lipophilic APIs (Chen *et al*., 2011; Nanjwade *et al*., 2013; Verma *et al*., 2018). A lesser degree of side effects is perceived with these transport systems, for a minimum dose is required (Verma *et al*., 2018). **NEs** can sustain their satisfactory aesthetic form due their droplet size range (Lu *et al*., 2014; Tadros *et al*., 2004) therefore avoiding the occurrence of sedimentation, creaming and flocculation. The droplet size range (100 – 500 nm) (Nalini *et al*., 2017) of an **NE** decreases tension between water and oil surface particles, with no gravitational interference and limited size distribution (Kumar, 2014). These dispersions provide rapid penetration of APIs through the skin due to the large surface area of these droplets (Sharma *et al*., 2010), which can be compared to an **NG**. The bioavailability and permeation also increases due to tiny particles across skin barriers (large surface area) with minimal effort when applied (Kela & Kaur, 2013; Klang *et al*., 2015; Lu *et al*., 2014; Nalini *et al*., 2017; Tadros *et al*., 2004). In order to produce nano-scale size droplets within an **NE**, emulsification methods are required (Abolmaali *et al*., 2011; Tadros *et al*., 2004; Verma *et al*., 2018). **NEs** require energy input, either from chemical potential of the components or from mechanical devices, e.g. ultrasound emulsification generators, high shear stirring and high-pressure homogenisers (Uson *et al*., 2004).

2.5.1.1 Emulsification

There are two validated methods to formulate **NEs**, e.g. high-energy emulsification and low energy emulsification. High-energy emulsification includes, sonication and high amplitude ultrasonic emulsification (Ochoa *et al*., 2016), jet dispersion, microfluidisation (Kumar, 2014), high-energy stirring emulsification, high-pressure homogenisation and membrane emulsification (Verma *et al*., 2018). Low energy emulsification involves, emulsion inversion point emulsification, spontaneous emulsification and phase inversion temperature emulsification (Verma *et al*., 2018). This study followed high-energy emulsification utilising an ultrasonicator.

An ultrasonicator fitted with a probe, generates mechanical vibrations producing cavitation, which is the breakdown of particles in a formulation (Kumar, 2014). Cavitation creates crucial tremors throughout the entire formulation therefore dispersed droplets (nano-particles) are located in the solution with a droplet size range of 100 – 500 nm.

2.5.1.2 Penetration enhancement

Most formulas contain penetration enhancers to achieve exactly what the referred term states, penetration enhancement. It improves the permeation rate to reach the necessitated therapeutic effect and the permeation of non-permeable APIs through the barriers of the skin, increasing transdermal absorption (Kala & Juyal, 2018). Conversely, the quantity and the type of penetration enhancer differs with the APIs utilised (Kala & Juyal, 2018). Penetration enhancers should be one-directional and pharmacologically inactive in a formula with the only purpose to enhance API penetration. The penetration enhancers and other compounds in the formula must be compatible with each other to be considered effective (Parhi *et al*., 2012). There are six categories of penetration enhancers, i.e. natural penetration enhancers, physical enhancers, chemical enhancers, particulate systems, biochemical approach and drug vehicle based (Lakshmi *et al*., 2017).

Natural enhancers are generally used to disrupt lipophilic bilayers of the skin by interacting with cellular corneocytes (Wang *et al*., 2003; Williams, 2013). This phenomenon produces an increased API flux across the stratum corneum (Vermaak *et al*., 2011). This interaction is made possible via fatty acids within the chemical structure of natural penetration enhancers by modification of the intercellular lipid structure of the stratum corneum to reduce barrier resistance, that are known to increase skin permeation regarding APIs (Kala & Juyal, 2018; Vermaak *et al*., 2011). The permeation of both lipophilic and hydrophilic APIs can be increased, though the flux of ionised APIs is higher (Kala & Juyal, 2018). Additionally, long chain unsaturated fatty acids (C_{18}) can raise penetration enhancing effects (Kala & Juyal, 2018). Penetration enhancers comprising linoleic and oleic fatty acids, which are also found in the human skin (Wang, 2012),

are the most advantageous to transdermal delivery by reducing the probability of skin irritations (Vermaak *et al*., 2011). Grapeseed oil is the natural penetration enhancer that was used in this study.

Grapeseed oil is a great source of fundamental fatty acids (Hussein *et al.*, 2015). This oil comprehends essential unsaturated fatty acids, such as α-linolenic acid (ω-3) and γ-linolenic acid (ω-6) for these fatty acids cannot be created by the human body (Hussein *et al.*, 2015). The fatty acids regarding grapeseed oil are adjusted to the following values: 0.0 – 0.2% myristic acid (C_{14}:0), 7.0 – 13.0% palmitic acid (C_{16}:0), 3.0 – 6.0% stearic acid (C_{18}:0), 0.0 – 0.9% palmitoleic acid (C_{16}:1), 14.0 – 25.0% oleic acid (C_{18}:1), 61.0 – 73.0% linoleic acid (C_{18}:2), and 0.0 – 0.6% linolenic acid (C_{18}:3), classifying it as a high quality nutritional oil (Hussein *et al.*, 2015). This oil also has the ability to serve as a free radical scavenger beyond the antioxidant properties of polyphenols. Grapeseed polyphenols are more potent compared to other well-known antioxidants, e.g. vitamin C, vitamin E, and β-carotene (Hussein *et al.*, 2015).

Grapeseed extract has great antioxidant beneficial potential, for instance the protection against oxidative damage in cells, as well as the modulation of antioxidant enzyme expression in both animal and human models. During the winemaking process, grape seeds are considered as a by-product and are thus deemed economical and easy to attain. The method of oil extraction is cold pressing, which includes no chemical treatment or heat and can hold more health benefits. With cold pressing, there is no concern about solvent residues in the oil, making it a safer and more consumer-desired product compared to other extraction methods. Grape seeds hold 8 – 20% of oil (dry basis). The interest in the use of grapeseed oil has increased due to its organoleptic properties. In Europe, this oil has been manufactured in Italy, France and Germany since 1930 and has been classified as a culinary oil (Garavaglia *et al.*, 2016).

Hydrophilic constituents of grapeseed oil comprise phenolic compounds, such as flavonoids, carotenoids, tannins, phenolic acids and stilbenes; it also contains gallic acid, which is also known for its antioxidant properties. The main polyphenols characterised in grapeseed oil are trans-resveratrol, procyanidin B1, catechins and epicatechins (Garavaglia *et al.*, 2016), while the lipophilic constituents of grapeseed oil consist of linoleic acid, oleic acid, tocotrienols and vitamin E. Linoleic is the most copious fatty acid (FA) in cold-pressed grapeseed oils, obtaining between 66.0 - 75.3% of the total FA, and is part of the polyunsaturated FA group (PUFA), known for the promotion of human health. Grapeseed oil is high in PUFA, about 85 – 90%. Oleic acid is a monounsaturated FA (MUFA) found in grapeseed oil with minimal quantities of saturated fatty acids (SFAs) present. Grapeseed oil is rich in tocotrienols (unsaturated forms of vitamin E), of which γ-tocotrienol is the most plentiful compared to α-tocotrienol; according to reports, γ-tocotrienol is claimed to be one of the most potent antioxidants. Grapeseed oil contains a large

amount of vitamin E, which is higher than in olive oil and soybean oil. Vitamin E has beneficial effects due to its anti-tumoural, neuroprotective and high antioxidant activity (Garavaglia *et al.*, 2016).

2.5.1.3 Decreasing surface tension

Surfactants act as an interfacial film between the different phases of a formulation to stabilise the system to which it is applied (Kumar, 2014), by reducing surface tension, enhancing emulsification (Baibhav *et al*., 2011; Hyma *et al*., 2014), easing droplet splitting (decreased particle size) (Huyn, 2012) and improving **NE** stability (Setya *et al*., 2014) as discussed in Appendix B. Surfactants form an amphipathic molecule and contain two segments, e.g. the head (lipophilic) that interacts with the water phase, and the tail (hydrophilic), which interacts with the oil phase (Huyn, 2012). Surfactants possess various chemical properties, hence, suitable hydrophilic-lipophilic balance (HLB) values are essential (Kaur *et al*., 2017).

2.5.2 Semi-solid formulations

Semi-solid formulations, such as gels, creams and ointments, are advantageous and suitable for the delivery of APIs through the skin. These formulations are mostly effortless to apply or to remove from the skin due to their high viscosity (Mahalingam *et al*., 2008; Pund *et al*., 2015; Williams, 2013) and have an affinity to be non-irritating. Semi-solid formulations are tolerably stable and can therefore avoid unnecessary recurrent application (Brown *et al*., 2018). Semi-solid formulations applied on the skin have a dual function, acting as an appropriate carrier system, which increases contact between the API and the skin and simultaneously encourages API delivery (Gupta & Garg, 2002). Transdermal administration of **NEs** can be enhanced by converting nano-sized emulsions into a semi-solid dosage form, **NG** (Rai *et al*., 2018).

Gel preparations contain a liquid phase within a three dimensional polymeric matrix, which is found by adding a gelling agent that results in cross-linkage. Gel preparations can be classified as hydrophilic or hydrophobic. Hydrophilic gels contain a water base, whereas hydrophobic gels usually contain a liquid paraffin base (Bora *et al*., 2014). Gels propose benefits viz. long-term stability, simple formulation, non-greasy and trouble-free application (Nabi *et al*., 2014). For the purpose of this study, an **NG** was investigated and formulated.

2.5.2.1 Nano-emulgels

An **NG** (semi-solid formulation) can be described as an **NE**-based-hydrogel with droplet sizes between 100 - 500 nm (Nalini *et al*., 2017), by combining an **NE** with a suitable gelling agent (Chellapa, 2015). **NGs** have a consistency between a solid and a liquid and exhibit the characteristics of both gels and nano-sized emulsions (Kaur *et al*., 2017).

NGs operate as a drug storage system that influences API delivery from the inner to the outer phase of the system. Conversely, specific delivery of an **NG** depends on the arrangement of the polymer bindings of the structure along with the crosslink density (Chellapa, 2015). The affinity of the API in the **NG** determines whether the API can penetrate the skin efficiently (Chellapa, 2015).

NGs produce dispersed oil drops from the gel structure that permeate through the stratum corneum to release the API and not via the aqueous phase, as seen in **NEs** (Chellapa, 2015). An **NE** merged with a gelling agent reduces interfacial and surface tension compared to an ordinary **NE** that will finally increase skin permeability and formulation viscosity (Chellapa, 2015, Eid *et al*., 2014). Additionally, the **NG** results in swelling of the water phase and increased formulation viscosity (Chellapa, 2015; Mahalingam *et al*., 2008; Mitsui, 1997).

2.5.2.1.1 Gelling agents

Gelling agents prevent particle separation (Mitsui, 1997) due to their high molecular weights that improve formulation stability (Mahalingam *et al*., 2008). **NGs** are more stable than **NEs**, as the oil droplets encapsulate lipophilic APIs, and are better dispersed in the gel-structure (Chellapa, 2015). This gel structure regulates the release of APIs, which increases the effectiveness of limited half-life APIs (Chellapa, 2015). **NGs** increase patient compliance (Pund *et al*., 2015; Williams, 2013) due to their uncomplicated use on the skin compared to other transdermal formulations, e.g. ointments and creams (Chellapa, 2015). Carbopol® Ultrez 20 is the gelling-agent utilised in this study.

Carbopol® Ultrez 20 can be described as a white powder that wets and dissolves easily (Lubrizol, 2018). Additionally, this gelling-agent causes swelling, which increases the viscosity of the **NEs** and therefore enhances stability, as well as permeability of the formulations when applied to the skin (Chudasama, 2011). Carbopol® Ultrez 20 can remain stable over a wide pH range, which reduces potential problems.

NGs applied topically include adhesion characteristics and induced solubilisation capability, which cause higher concentration gradients of APIs; therefore, a higher concentration can penetrate the skin (Chellapa, 2015).

2.6 Conclusion

Hypercholesterolaemia or hyperlipidaemia is linked to atherosclerosis and CVDs. Currently, the first-line medical therapy for hypercholesterolaemia is statins, which are administered orally with a decreased systemic bioavailability. Therefore, the transdermal pathway will be considered in an attempt to find the most effective pathway. The physicochemical properties of statins do not

meet the required standards for transdermal delivery. Consequently, **NEs** (carrier system) should be researched and used to encapsulate statins to implement possible transdermal delivery. The formula contains a penetration enhancer to increase absorption of the statins via the transdermal route. Grapeseed oil is a natural penetration enhancer, which disarranges the lipophilic bilayers of the skin to increase the total flux of the APIs across the stratum corneum. An effective **NE** formula for statins in this study will be modified to create a suitable **NG** that can increase viscosity and, hopefully, enhance skin permeability. The **NEs** and **NGs** were compared with regard to transdermal delivery.

References

Abolmaali, S.S., Tamaddon, A.M., Farvadi, F.S., Daneshamuz, S. & Moghimi, H. 2011. Pharmaceutical nanoemulsions and their potential topical and transdermal applications. *Iranian journal of pharmaceutical sciences*, 7:139-150.

Akula, P., Lakshmi P.K. 2018. Effect of pH on weakly acidic and basic model drugs and determination of their *ex vivo* transdermal permeation routes. *Brazilian journal of pharmaceutical sciences*, 54(2):1-8.

Al-Adl, S.M., Abdel-Aziz, L.M., Mohamed, M.A.M. 2017. Spectrophotometric determination of atorvastatin calcium and rosuvastatin calcium in bulk and dosage form using P-Dimethylaminobenzaldehyde. *Journal of applied pharmacy*, 9(1):1-7.

Alexander, A., Dwivedi, S., Ajazuddin, Giri, T.K., Saraf, S., Saraf. S. & Tripathi, D.K. 2012. Approaches for breaking the barrier of drug permeation through transdermal drug delivery. *Journal of controlled release*, 164:26-40.

Ammar, O. 2017. In silico pharmacodynamics, toxicity profile and biological activities of the Saharan medicinal plant Limoniastrum feei. *Brazilian journal of pharmaceutical sciences*, 53(3):1-10.

Athyros, V.G., Katsiki, N. & Mikhailidis, D.P. 2017. Letter to the editor: Treating nonalcoholic fatty liver disease with statins. Are all statins equal? *American journal of physiology-gastrointestinal and liver physiology*, 312(6):681-G682.

Baibhav, J., Gurpreet, S., Rana, A.C., Seema, S. & Vikas, S. 2011. Emulgel: a comprehensive review on the recent advances in topical drug delivery. *International research journal of pharmacy*, 2:66-70.

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

Barry, B. 2002. Transdermal drug delivery. (*In* Aulton, M.E., *ed*. Pharmaceutics: the science of dosage form design. 2nd edition London: Churchill Livingstone. p. 499-533).

Basera, K., Bhatt, G., Kothiyal, P. & Gupta, P. 2015. Nanoemulgel: a novel formulation approach for topical delivery of hydrophobic drugs. *World journal of pharmacy and pharmaceutical sciences*, 4(1):1871-1886.

Benson, H.A.E. 2005. Transdermal drug delivery: penetration enhancement techniques. *Current drug delivery*, 2:23-33.

Boglarka, B., Gabor, V., Szilvia, B., Maria, B., Andras, K., Balint, S., Krisztina, T., Piroska, S. & Erzsebet, C. 2016. Investigation of the efficacy of transdermal penetration enhancers through the use of human skin and a skin mimic artificial membrane. *Journal of pharmaceutical sciences*, 105:1134-1140.

Bora, A., Deshmukh, S. & Swain, K. 2014. Recent advances in semisolid dosage form. *International journal of pharmaceutical sciences and research*, 5(9):3594-3608.

Braamskamp, M.J., Wijburg, F.A. & Wiegman, A. 2012. Drug therapy of hypercholesterolaemia in children and adolescents. *Drugs*, 72:759-72.

Brown, T.L., Hiu, S.P., Chan, T., Angove, M.J., Tucci, J. 2018. Semi-solid and solid dosage forms for the delivery of phage therapy to epithelia. *Pharmaceuticals*, 11(26):1-12.

Cayman chemical. 2010. Fluvastatin (sodium salt) (Material safety data sheet). https://s3-us-west-2.amazonaws.com/drugbank/msds/DB01095.pdf?1374296087 Date of access: 18 Jan. 2019.

Cayman chemical. 2014. Pitavastatin calcium salt (Product information 15414) https://www.caymanchem.com/pdfs/15414.pdf Date of access: 18 Oct. 2018.

Cayman chemical. 2015. Pravastatin (sodium salt) (Material safety data sheet). https://www.caymanchem.com/msdss/10010343m.pdf Date of access: 15 Feb. 2019.

Chudasama, A., Patel, V., Nivsarkar, M., Vasu, K. & Shishoo, C. 2011. Investigation of microemulsion system for transdermal delivery of itraconazole. *Journal of advanced pharmaceutical technology & research*, 2(1):30-38.

Charoo, N.A., Anwer, A., Kohli, K., Pillai, K., Rahman, Z.J.P.d. & technology. 2005. Transdermal delivery of flurbiprofen: permeation enhancement, design, pharmacokinetic, and pharmacodynamic studies in albino rats. *Pharmaceutical development and technology*, 10(3):343-351.

Chellapa, P., Mohamed, A.T., Keleb, E.I., Elmahgoubi, A., Eid, A.M., Issa, Y.S. & Elmarzugi, N.A. 2015. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR journal of pharmacy*, 5(10):43-47.

Chime, S.A., Kenechukwu, F.C. & Attama, A.A. 2014. Nanoemulsions: advances in formulation, characterization and application in drug delivery. (*In* Sezer, A.D., *ed*. Application of nanotechnology in drug delivery. p. 77-126). http://cdn.intechopen.com/pdfs-wm/47116.pdf Date of access: 20 Dec. 2017.

Chen, H., Khemtong, C., Yang, X., Chang, X. & Gao, J. 2011. Nanonization strategies for poorly water-soluble drugs. *Drug discovery today*, 16:354-360.

Churchill-Livingstone"s Dictionary of Sport and Exercise Science and Medicine. 2008. Hyperlipidaemia. http://medical-dictionary.thefreedictionary.com/browse/hyperlipidaemia Date of access: 29 Mar. 2017.

Comsol, 2017. Diffusion coefficient. https://www.comsol.com/multiphysics/diffusion-coefficient Date of access: 4 Feb. 2019. Cruz, M.P., Küstner, E.C., Vicente, J.A.G., Ferrero, X.M., Thio, E.B., López, J.L. 2008. Adverse side effects of statins in the oral cavity. *Medicina oral patologia oral y cirugia bucal*, 13(2):E98-101.

De Brito, M.A. 2011. Pharmacokinetic study with computational tools in the medicinal chemistry course. *Brazilian journal of pharmaceutical sciences*, 47(4):797-805.

Debnath, S., Satayanarayana & Kumar, G.V. 2011. Nanoemulsion-a method to improve the solubility of lipophilic drugs. *Pharmanest: an international journal of advances in pharmaceutical sciences*, 2:72-83.

Drugbank. 2005. https://www.drugbank.ca/drugs/DB06693 Date of access: 4 May 2019.

Earl, G.J.N.c.c. 2019. PCSK9 inhibitors: Add-on therapy to reduce stroke risk. *Nursing... critical care*, 14(1):42.

Eid, A.M., El–Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti–inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of nanomedicine and nanotechnology*, 5:1-10.

El Maghraby, G.M., Barry, B.W. & Williams, A.C. 2008. Liposomes and skin: from drug delivery to model membranes. *European journal of pharmaceutical sciences*, 34:203-222.

Encyclopaedia Britannica. 2016. Partition coefficient. http://global.britannica.com/science/partition-coefficient Date of access: 5 Jan. 2019.

Fahed, A.C., Nemer, G.M. 2011. Familial hypercholesterolemia: The lipids or the genes? *Nutrition & metabolism*, 8:23.

Fang, J.-Y., Chiu, H.-C., Wu, J.-T., Chiang, Y.-R. & Hsu, S.-H.J.I.j.o.p. 2004. Fatty acids in Botryococcus braunii accelerate topical delivery of flurbiprofen into and across skin. *International journal of pharmaceutics*, 276(1-2):163-173.

Fatima, T., Ansari, M., Naaz, H., Banu, H., Mehveen, Z. 2017. Hyperlipidemia- A critical pathological condition. *International journal of pharmacy and pharmaceutical research*, 8(3):110-124.

Ferris, H.A., Perry, R.J., Moreira, G.V., Shulman, G.I., Horton, J.D., Kahn, C.R. 2017. Loss of astrocyte cholesterol synthesis disrupts neuronal function and alters whole-body metabolism. *Journal of the national academy of sciences*, 114(5):1189-1194.

Flynn, G.L. 2002. Cutaneous and transdermal delivery - processes and system of delivery. (*In* Banker, G.S. & Rhodes, C.T., eds. Modern pharmaceutics. 4th ed. New York: Marcel Dekker. p. 187-235).

Foldvari, M. 2000. Non-invasive administration of drugs through the skin: challenges in delivery system design. *Pharmaceutical sciences and technology today*, 3:417-425.

Fong, C. 2016. Statins in therapy: Understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies. *European journal of medicinal chemistry*, 85:661-674.

Fong, C. 2016. Statins in therapy: Cellular transport, side effects, drug-drug interactions and cytotoxicity -the unrecognized role of lactones. https://hal.archives-ouvertes.fr/hal-01185910/document Date of access: 2 Nov. 2018.

Gabera, M., Medhata, W., Hanya, M., Sahera, N., Fang, J., Elzoghby, A. 2017. Protein-lipid nanohybrids as emerging platforms for drug and gene delivery: Challenges and outcomes. *Journal of controlled release*, 254:75-91.

Garavaglia, J., Markoski, M.M., Oliveira, A. & Marcadenti, A. 2016. Grape seed oil compounds: Biological and chemical actions for health. *Nutrition and metabolic insights*, 9:S32910.

Garrod, D. & Chidgey, M. 2008. Desmosome structure, composition and function. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, 1778(3):572-587.

Gaur, S., Garg, A., Yadav, D., Beg, M. & Gaur, K. 2014. Nanoemulsion gel as novel oil based colloidal nanocarrier for topical delivery of bifonazole. *Indian research journal of pharmacy and science*, 1:36-54.

Geerligs, M. 2006. *In vitro* mechanical characterization of human skin layers: stratum corneum, epidermis and hypodermis. *http://www.mate.tue.nl/mate/pdfs/11389.pdf* Date of access: 4 Oct. 2018.

Geethu, V.S., Manikandan, P., Nethaji, R., Surenderan, N.S. & Babu, G. 2014. A review on: transdermal drug delivery systems. *Indo American journal of pharmaceutical research*, 4:1809-1824.

Godin, B. & Touitou, E. 2007. Transdermal skin delivery: Predictions for humans from *in vivo*, *ex vivo* and animal models. *Advanced drug delivery reviews*, 59:1158.

Gordts, S.C., Muthuramu, I., Amin, R., Jacobs, F., De Geest, B. 2014. The impact of lipoproteins on wound healing: topical HDL therapy corrects delayed wound healing in apolipoprotein E deficient mice. *Pharmaceuticals*, 7:419-432.

Gupta, P. & Garg, S. 2002. Recent advances in semisolid dosage forms for dermatological application. *Pharmaceutical technology*, 144-162.

Hasani-Ranjbar, S., Nayebi, N., Moradi, L., Mehri, A., Larijani, B. & Abdollahi, M. 2010. The efficacy and safety of herbal medicines used in the treatment of hyperlipidemia; a systematic review. *Current pharmaceutical design*, 16:2935-47.

Healthguidance. 2017. http://www.healthguidance.org/entry/1807/1/Benefits-of-Apricot-Kernel -Oil.html Date of access: 12 Jan. 2018.

Hennessy, E., Adams, C., Reen, F.J., O"Gara, F. 2016. Statins inhibit bacterial growth and virulence. *Antimicrobial agents and chemotherapy*, 2(2):1-44.

Hoag, G.E. 2018. Topical analgesic pain relief formulations manufacture and methods of use thereof. *United states patent application publication*, 12(1):10-24.

Hussein, S., Abdrabba, S.J.I.J.o.A.S. & Mathematics. 2015. Physico-chemical characteristics, fatty acid, composition of grape seed oil and phenolic compounds of whole seeds, seeds and leaves of red grape in Libya. *International journal of applied science and mathematics*, 2(5):2394-2894.

Huynh, P.T. 2012. Solvent-free beta-carotene nanoparticles for food fortification. The State University of New Jersey: New Jersey. (Dissertation-PhD).

Hyma, P., Jahan, N., Raheemunissa, Sreelekha, G. & Babu, K. 2014. Emulgel: a review. *International journal of pharmaceutical archive*, 3:1-11.

IUPHAR/BPS. 2017. Guide to pharmacology. http:www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2953 Date of access: 2 Jan. 2019.

Jepps, O.W., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. 2013. Modelling the human skin barrier - towards a better understanding of dermal absorption. *Advanced drug delivery reviews*, 65:152-168.

Jonas, A. 2002. Lipoprotein structure. (*In* Vance, D.E. & Vance, J.E., *eds*. Biochemistry of lipids, lipoproteins and membranes. 4th ed. Urbana: Illinois. p. 483-504).

Kala, S & Juyal, D. 2018. Recent developments on natural transdermal penetration enhancers. *International journal of pharmaceutical sciences and research*, 9(6):2190-2196.

Kela, S.K. & Kaur, C.D. 2013. Pharmaceutical nanoemulsions an ardent carrier for drug delivery. *Indo american journal of pharmaceutical research*, 3:9202-9212.

Kim, M.C., Ahn, Y., Jang, S.Y., Cho, K.H., Hwang, S.H., Lee, M.G., Ko, J.S., Park, K.H., Sim, D.S., Yoon, N.S., Yoon, H.J., Kim, K.H., Hong, T.J., Park, H.W., Kim, J.H., Jeong, M.H., Cho, J.G., Park, J.C. & Kang, J.C. 2011. Comparison of clinical outcomes of hydrophilic and lipophilic statins in patients with acute myocardial infarction. *The Korean journal of internal medicine*, 26:294-303

Klang, V., Schwarz, J.C. & Valenta, C. 2015. Nanoemulsions in dermal drug delivery. (*In* Dragicevic-Curic, N. & Maibach, H.I., *eds*. Percutaneous penetration enhancers: chemical methods in penetration enhancement: drug manipulation strategies and vehicle effects. Heidelberg: Springer. p. 255-266).

Koocheki, A., Kadkhodaee, R. 2011. Effect of alyssum homolocarpum seed gum, Tween 80 and NaCl on droplets characteristics, flow properties and physical stability of ultrasonically prepared corn oil-in-water emulsions. *Food hydrocolloids*, 25:1149-1157.

Kulkarni, M. & Nagarsenker, M. 2008. A study on improvement of solubility of rofecoxib and its effect on permeation of drug from topical formulations. *Indian journal of pharmaceutical sciences*, 70(4):466-471.

Kumar, S. 2014. Role of nano-emulsion in pharmaceutical sciences. A review. *Asian journal of research in pharmaceutical sciences and biotechnology* 2(1):1-15.

Kumarn, G.P., Rajeshwarrao, P. & Padmavathi, T. 2011. India college of pharmacy, nonionic surfactant vesicular systems for effective drug delivery-an overview. *Acta pharmaceutica sinica B*, 1(4):208-219.

Kute, S.B. & Saudagar, R.B. 2013. Emulsified gel: a novel approach for delivery of hydrophobic drugs: an overview. *Journal of advanced pharmacy education and research*, 3:368-376.

Lakshmi, P.K., Samratha, K.D., Prasanthi, B., Veeresh, A.C. 2017. Oil as penetration enhancers for improved transdermal drug delivery. *International research journal of pharmacy*, 8(4):9-17.

Li, N., Wu, X., Jia, W., Zhang, M.C., Tan, F. & Zhang, J. 2012. Effect of ionization and vehicle on skin absorption and penetration of azelaic acid. *Drug development and industrial pharmacy*, 38:985-994.

Liu, C., Quan, P. & Fang, L. 2016. Effect of drug physicochemical properties on drug release and their relationship with drug skin permeation behaviors in hydroxyl pressure sensitive adhesive. *European journal of pharmaceutical sciences*, 93:437-446.

LKT labs. 2018a. Fluvastatin sodium (93957-55-2). https://www.lktlabs.com/product/fluvastatin-sodium/ Date of access: 3 Aug. 2019.

LKT labs. 2018b. Pravastatin sodium (81131-70-6). https://www.lktlabs.com/product/pravastatin-sodium/ Date of access: 7 Nov. 2018.

Lohani, A., Verma, A., Joshi, H., Yadav, N., Karki, N. 2014. Nanotechnology-based cosmeceuticals. International scholarly research notices. *Dermatology*, 1-14.

Lu, W., Chiang, B., Haung, D. & Li, P. 2014. Skin permeation of D-limonene-based nanoemulsions as a transdermal carrier prepared by ultrasonic emulsification. *Ultrasonics sonochemistry*, 21:826-832.

Lubrizol. 2018. Carbapol® Ultrez 20 polymer. http://www.lubrizol.com Date of access: 24 Aug. 2019.

Mahalingam, R., Li, X. & Jasti, B.R. 2008. Semisolid dosages: ointments, creams and gels. (*In* Gad, S.C., *ed*. Pharmaceutical manufacturing handbook: production and processes. New Jersey: John Wiley & Sons. p. 267-309).

Mahmood, Z.A., Ahmed, S.W., Sualeh, M., Mahmood, S.B.Z. 2009. Hyperlipidemia development and consequences. *Medical chan*nel, 15(3):14-17.

Mancini, G.B.J., Tashakkor, A.Y., Baker, S., Bergeron, J., Fitchett, D., Frohlich, J., Genest, J., Gupta, M., Hegele, R.A., Ng, D.S., Pearson, G.J., Pope, J. 2013. Diagnosis, prevention, and management of statin adverse effects and intolerance: Canadian working group consensus update. *Canadian journal of cardiology*, 29(2):1553-1568.

Marais, A. 2016. Hypercholesterolaemia: from inauspicious to appropriate to detrimental: case study. *South African pharmaceutical journal*, 83(1):30-32.

Marrow, D.I.J., McCarron, P.A., Woolfson, A.D. & Donnelly, R.F. 2007. Innovative strategies for enhancing topical and transdermal drug delivery. *The open drug delivery journal*, 1:36-59.

McFarland, A.J., Anoopkumar-Dukie, S., Arora, D.S., Grant, G.D., McDermott, C.M., Perkins, A.V. & Davey, A.K. 2014. Molecular mechanisms underlying the effects of statins in the central nervous system. *International journal of molecular sciences*, 15(11):20607-20637.

McKenney, J.M. Davidson, M.H. Jacobson, T.A. Guyton, J.R. 2006. National lipid association statin safety assessment task force. *American journal of cardiology*, 97(8A):89C-94C.

Menon, G.K., Cleary, G.W. & Lane, M.E. 2012. The structure and function of the stratum corneum. *International journal of pharmaceutics*, 435:3-9.

Mitsui, T. 1997. Polymers. (*In* Mitsui, T., *ed*. New cosmetic science. Netherlands: Elsevier. p. 138-140).

Mostafa, W.Z., Hegazy, R.A. 2015. Vitamin D and the skin: Focus on a complex relationship. *Journal of advanced research*, 6:793-804.

Mohammadi-Samani, S., Yousefi, G., Mohammadi, F. & Ahmadi, F. 2014. Meloxicam transdermal delivery: effect of eutectic point on the rate and extent of skin permeation. *Iranian journal of basic medical sciences*, 17(2):112-118.

Naik, A., Kalia, Y.N. & Guy, R.H. 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical science and technology today*, 3:318-325.

Nair, A., Jacob, S., Al-Dhubiab, B., Attimarad, M. & Harsha, S. 2013. Basic considerations in the dermatokinetics of topical formulations. *Brazilian journal of pharmaceutical sciences*, 49(3):423-434.

Nabi, S.A.A., Sheraz, M.A., Ahmed, S., Mustaan, N. & Ahmad, I. 2014. Pharmaceutical gels: a review. *RADS journal of pharmacy and pharmaceutical sciences*, 4(1):40-48.

Nalini, T., Kumari, V.S. & Basha S.K. 2017. Novel nanosystems for herbal drug delivery. *World journal of pharmacy and pharmaceutical sciences*, 6(5):1447-1463.

Nanjwade, B.K., Varia, P.K., Kadam, V.T., Srichana, T. & Kamble, M.S. 2013. Development and evaluation of nanoemulsion of repaglinide. *JSM nanotechnology & nanomedicine*, 1(2):1-8.

Natarajan, V.T., Ganju, P., Ramkumar, A., Grover, R., Gokhale, R.S. 2014. Multifaceted pathways protect human skin from UV radiation. https://media.nature.com/lw926/nature-assets/nchembio/journal/v10/n7/images/nchembio.1548-F1.jpg Date of access: 7 Feb. 2018.

Nemes, Z. & Steinert, P.M. 1999. Bricks and mortar of the epidermal barrier. *Experimental & molecular medicine*, 31(1):5.

Ng, K.W., Lau, W.M. 2015. Skin deep: The basics of human skin structure and drug penetration. file:///C:/Users/22819509/Downloads/ng-2015-skin-deep.pdf Date of access: 9 Jan. 2019.

Ochoa, A.A., Hernandez-Becerra, J.A., Cavazos-Garduno, A., Vernon-Carte, E.J., Garc, H.S. 2016. Preparation and characterization of curcumin nanoemulsions obtained by thin-film hydration emulsification and ultrasonication methods. http://www.scielo.org.mx/pdf/ rmiq/v15n1/1665-2738-rmiq-15-01-00079.pdf Date of access: 28 Sept. 2018.

O'Neil, M.J., *ed*. 2001. The Merck index: an encyclopedia of chemicals, drugs and biologicals. 13th ed. Whitehouse Station, NJ: Merck.

Onwe, P.E., Folawiyo, M.A., Okike, P.I., Balogun, M.E., Umahi, G., Besong, E.E., Okorocha, A.E., Afoke, A.O. 2015. Lipid profile and the growing concern on lipid related diseases. *Journal of pharmacy and biological sciences*, 10(5):22-27.

Orth, M. & Bellosta, S.J.C. 2012. Cholesterol: its regulation and role in central nervous system disorders. *Cholesterol*, 2012.

O'Sullivan, S. 2007. Statins: A review of benefits and risks. *The trinity student medical journal*, 8:52-56.

Parhi, R., Suresh, P., Mondal, S., Kumar, P.M. 2012. Novel penetration enhancers for skin applications. *Current drug delivery*, 9:219-230.

Piepho, R.W. 2000. The Pharmacokinetics and Pharmacodynamics of Agents Proven to Raise High-Density Lipoprotein Cholesterol. *The American journal of cardiology*, 86(12A): 35L-40L.

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

Pund, S., Pawar, S., Gangurde, S. & Divate, D. 2015. Transcutaneous delivery of leflunomide nanoemulgel: mechanistic investigation into physicomecanical characteristics, *in vitro* anti-psoriatic and anti-melonoma activity. *International journal of pharmaceutics*, 487:148-156.

Pubchem. 2005a. https://pubchem.ncbi.nlm.nih.gov/compound/53232 Date of access: 4 Aug 2019.

Rai, V.K., Yadav, N.M.K.S. & Yadav, N.P. 2018. Nanoemulsion as pharmaceutical carrier for dermal and transdermal drug delivery: Formulation development, stability issues, basic considerations and applications. *Journal of controlled release*, 270:203-225.

Rastogi , V., Yadav, P. 2012. Transdermal drug delivery system: An overview. *Asian journal of pharmaceutics*, 161-170.

Reddy, C.S.K., Khan, K.K.A., Nagaraja. 2016. A review on the determination of melting point measurement system. *International journal of advance research electrical electronics and instrumentation engineering*, 5(2):975-979.

Reddy, A.K., Debnath, S. & Babu, M.N. 2013. Nanoemulsions a novel approach for lipophilic drugs - a review. *Asian journal of pharmaceutical research*, 3:84-92.

Robinson, JG. & Goldberg, AC. 2011. Treatment of adults with Familial Hypercholesterolemia and evidence for treatment: Recommendations from the National Lipid Association Expert Panel on Familial Hypercholesterolemia. *Journal of clinical lipidology*, 5:S18-S29.

Rodde, M.S., Divase, G.T., Devkar, T.B., Tekade, A.R. 2014. Solubility and bioavailability enhancement of poorly aqueous soluble atorvastatin: *in vitro*, *ex vivo*, and *in vivo* studies. *BioMed research international*, 1-11.

Rohilla, A., Dagar, N., Rohilla, S., Dahiya, A., Kushnoor, A. 2012. Hyperlipidemia- A deadly pathological condition. *International journal of current pharmaceutical research*, 4(2):15-18.

Ruan, F., Zheng, Q., Wang, J. 2012. Mechanisms of bone anabolism regulated by statins. *Bioscience reports*, 32:511-519.

Sahebzamani, F.M., Munro, C.L., Marroquin, O.C., Diamond, D.M., Keller, E., Kip, K.E. 2014. Examination of the FDA warning for statins and cognitive dysfunction. *Journal of pharmacovigilance*, 2(4):1-9.

Santaniello, M. & Giannini, G. 2015. Statins solubilized in fish oil: Versatile and simple combinations by innovative formulations. *Nutrition and food technology*, 1(1):1-10.

Satpute, V.M., Shirsat, M.K., Dhobale, A.V., Wani, R.M., Kharde, S.N. 2018. Formulation and evaluation of atorvastatin calcium oral dispersible tablet. *International journal of current advanced research*, 7:14170-14174.

Savjani, K.T., Gajjar, A.K., Savjani, J.K. 2012. Drug solubility: Importance and enhancement techniques. International scholarly research network: *Pharmaceutics*, 2012:1-10.

Setya, S., Talegaonkar, S. & Razdab, B.K. 2014. Nanoemulsions: formulation methods and stability aspects. *World journal of pharmacy and pharmaceutical sciences*: 3:2214-2228.

Sharma, N., Agarwal, G., Rana, A.C., Bhat, Z.A., Kumar, D. 2011. Trasderma drug deivery system: A too for ove drug deivery system. *Iteratioa oura for drug deveopmet & research*, 3(3):70-84.

Schachter, M. 2005. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundamental & clinical pharmacology*, 19(1):117-125.

Scherr, R.E., Zidenberg-Cherr, S. 2016. Nutrition and health info sheet: Cholesterol. https://nutrition.ucdavis.edul>factsheets.pdf Date off access: 15 Jan 2019.

Scheindlin, S., 2004. Transdermal drug delivery: past, present, future. *Molecular interventions*, 4(6):308.

Schierwagen, R., Trebicka, J., Uschner, F.E., Magdaleno, F., Klein, S. 2017. Rationale for the use of statins in liver disease. *American journal of physiology gastrointestinal and liver physiology*, 312:G407-G412.

Schultz, G., Patten, D.K., Berlau, D.J. 2018. The role of statins in both cognitive impairment and protection against dementia. *Translatioal neurogeneration*, 7(5):1-11.

Smelt, A.H.M. 2010. Triglycerides and gallstone formation. *Clinica chimica acta*, 411:1625-1631.

Steele, G. & Austin, T. 2009. Preformulation investigations using small amounts of compound as an aid to candidate drug selection and early development. (*In* Gibson, M., *ed*. Pharmaceutical preformulation and formulation: a practical guide from candidate drug selection to commercial dosage form. 2nd ed. New York: Informa Healthcare. p. 17-128).

Subedi, R.K., Oh, S.Y., Chun, M. & Choi, H. 2010. Recent advantages in transdermal drug delivery. *Archives of pharmacal research*, 33:339-351.

Taichman, L.B. & Prokop, C.A. 1982. Synthesis of keratin proteins during maturation of cultured human keratinocytes. *Journal of investigative dermatology*, 78(6):464-467.

Tadros, T., Izquierdo, P., Esquena, J. & Solans, C. 2004. Formation and stability of nano-emulsions. *Advances in colloid and interface science*, 108(19):303-318.

Tolani, S., Pagler, T.A., Murphy, A.J., Bochem, A.E., Abramowicz, S., Welch, C., Nagareddy, P.R., Holleran, S., Hovingh, G. & Kuivenhoven, J.A.J.A. 2013. Hypercholesterolemia and reduced HDL-C promote hematopoietic stem cell proliferation and monocytosis: studies in mice and FH children. *Atherosclerosis*, 229(1):79-85.

Trommer, H., Neubert,R.H.H. 2006. Overcoming the stratum corneum: The modulation of skin penetration. *Skin pharmacology and physiology*, 19:106-121.

Uchida, Y. & Park, K. 2016. Stratum Corneum. Immunology of the Skin. Springer. p. 15-30.

Uson, N., Garcia, M.J. & Solans, C. 2004. Formation of water-in-oil (W/O) nano-emulsions in a water/mixed non-ionic surfactant/oil systems prepared by a low-energy emulsification method. *Colloids and surfaces a: physicochemical and engineering aspects*, 250:415-421.

Van Der Schaaf, P.A., Blatter, F., Szelagiewicz, M., Oberwil, S. 2006. United States patent application publication https://patentimages.storage.googleapis.com/5f/33/53/12e51d9490de73/US20060142582A1.pdf Date of access: 8 Feb. 2019.

Verma, S., Kumar, N., Kumar, U., Jain, G. 2018. Nanoemulsion: an exceptional mode for delivery of poorly soluble drug. *World journal of pharmacy and pharmaceutical sciences*, 7(2):374-392.

Vermaak, I., Kamatou, G.P.P., Komane-Mofokeng, B., Viljoen, A.M. & Beckett, K. 2011. African seed oils of commercial importance: cosmetic application. *South African journal of botany*, 77:920-933.

Wang, Y., Fan, Q., Song, Y. & Michnaik, B. 2003. Effects of fatty acids and iontophoresis on the delivery of midodrine hydrochloride and the structure of human skin. *Pharmaceutical research*, 20:1612-1618.

Wang, W. 2012. Evaluation of siberian apricot (Prunus sibirica L.) germplasm variability for biodiesel properties. *Journal of american oil chemists' society*, 89:1743-1747.

Wagner, J. & Abdel-Rahman, S.M. 2016. Pediatric statin administration: navigating a frontier with limited data. *The Journal of pediatric pharmacology and therapeutics*, 21(5):380-403.

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

Wiechers, J.W. 2008. Skin delivery: what it is and why we need it. (*In* Wiechers, J.W., *ed*. Science and applications of skin delivery systems. Carol Stream, IL: Allured Publishing. p. 1-21).

Wiedersberg, S. & Guy, R.H. 2014. Transdermal drug delivery: 30+ years of war and still fighting! *Journal of controlled release*, 190:150-156.

Wilbur, R.L. 2017. The Difference Between Topical and Transdermal Medications. https://genscopharma.com/wp-content/uploads/2017/05/Topical-v-Transdermal-White-Paper-05-26-17-EMAIL.pdf Date of access: 8 Jan. 2019.

Williams, A.C. 2003. Structure and function of human skin. (*In* Williams, A.C., *ed*. Transdermal and topical drug delivery: from theory to clinical practice. London: Pharmaceutical Press. p. 1-26).

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E., *ed*. Aultons pharmaceutics: the design and manufacture of medicines. 4th ed. London: Churchill Livingstone. p. 675-697).

Yun, Y.H., Lee, B.K. and Park, K., 2015. Controlled drug delivery: historical perspective for the next generation. *Journal of Controlled Release*, 219:2-7.

Zhang, D. 2009a. Polyoxyethylene sorbitan fatty acid esters. (*In* Rowe, R.C., Sheskey, P.J. & Quinn, M.E., *eds*. Handbook of pharmaceutical excipients. 6th *ed*. USA: Pharmaceutical Press. p. 549-553).

Zhang, D. 2009b. Sorbitan esters. (*In* Rowe, R.C., Sheskey, P.J. & Quinn, M.E., *eds*. Handbook of pharmaceutical excipients. 6th ed. USA: Pharmaceutical Press. p. 675-678).

Zhang, J., Liu, Q. 2015. Cholesterol metabolism and homeostasis in the brain. *Protein & cell*, 6(4):254-264.

Zhao, Q., Kuang, W., Long, Z., Fang, Liu, D., Yang, B., Zhao, M. 2013. Effect of sorbitan monostearate on the physical characteristics and whipping properties of whipped cream. *Food chemistry*, 141:1834-1840.

Zodda, D., Giammona, R., Schifilliti, S. 2018. Treatment strategy for dyslipidaemia in cardiovascular disease prevention: focus on old and new drugs. *Pharmacy*, 6(10):1-16.

CHAPTER 3

ARTICLE FOR THE PUBLICATION IN "DIE PHARMAZIE"

Chapter 3 was written for "Die Pharmazie" in article format. UK English and Arial 10 were used. This Chapter was justified for the ease of reading. The complete authors' guidelines are presented in Appendix F. This chapter was written in collaboration with Elme Oosthuysen.

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Development and validation of an HPLC method for the simultaneous detection and quantification of different statins after transdermal delivery

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Abstract

After *in vitro* skin diffusion studies were performed, samples needed to be analysed. An HPLC method was developed and validated to simultaneously analyse statins (atorvastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin). A Venusil XBP C_{18} (2) reverse phase column (150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, Newark, DE) with gradient conditions that starts at 25% acetonitrile and increased linearlly of 90% after 1.0 min, subsequently it was held at 90% until 6.5 min and then re-equilibrated at start conditions. Mobile phases consisted of acetonitrile/water and 0.005 M (0.2%) octane sulphonic acid-Na (pH 3.5). Flow rate was set at 1.0 ml/min at an injection volume of 10 µl. Linearity values of the HPLC method was indicative of concentration ranging from 0.2-206.4 µg/ml and correlated with the (R^2) coefficient of 1. The validation was proven to be accurate and statins could be quantified even in combination with other excipients used during formulation.

1. Introduction

Cholesterol can be seen as a hydrophobic molecule that intervenes with the structural integrity of plasma membranes, when cholesterol and fatty acids are esterified, the formation of cholesteryl esters are then stored in the lipid droplets of the cells and can form very low-density-lipoprotein (VLDL) in the liver [Rader et al. 2003]. Hypercholesterolemia causes endothelial dysfunction, since the lipids start accumulating in the vascular wall [Becher et al. 2017]; and the risk for hypercholesterolemia can be increased by an unhealthy diet, obesity, smoking and lifestyle or genetic factors [Lippi and Favalo, 2011]. Familial hypercholesterolemia (FH) can be described as a codominant autosomal disorder of lipoprotein metabolism that is inherited, (FH) can be characterised by high plasma levels of low-density lipoprotein cholesterol (LDL) and in turn can increase coronary heart diseases [Civeira, 2004]. Worldwide, approximately 80% males and 50% females with FH will suffer from a coronary heart disease before the age of 65 years, these statistics can be prevented with early onset of diagnosis and treatment [Bouhairie and Goldberg, 2015]. Cholesterol lowering treatment with early onset of atherosclerosis reduces risks of myocardial ischemia and coronary artery stenosis, preferred cholesterol lowering treatment are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins [Rodenburg, 2016]. Statins inhibit HMG-CoA reductase, which is the enzyme responsible for the conversion of mevalonic acid into cholesterol and changes the conformation

of the substrate that prevents the release of HMG-CoA in its functional structure [Stancu and Sima, 2001]. Statins increase high-density lipoprotein (HDL) cholesterol and decrease the amount of LDL cholesterol, including triglycerides in the body [Braamskamp et al. 2012]. According to O'Sullivan (2007) and based on data available, current registered statins (with the exception of cerivastatin) have a favourable safety profile. Statins have a wide range of pleiotropic effects that may significantly contribute to the treatment of other non-cardiovascular conditions as well [Gazzerro et al. 2012; McFarland et al. 2002; McFarlane et al. 2014].

The use of statins is associated with side-effects such as myalgia and cramps, rhabdomyolysis and autoimmune myopathy, these effects on muscles are caused by the increased creatine kinase levels [Thompson et al. 2016; Bays, 2006]. Glucose and lactate (responsible for these muscle symptoms) are transformed into pyruvate in the sarcoplasm, where it is transported to the mitochondria and forms acetyl coenzyme A (acetyl-CoA) [Du Souich et al. 2017]. Gastrointestinal side-effects caused by statins after being used orally include bloating, diarrhoea and nausea; constipation can be caused by a disturbance in the cholesterol-bile excretion [Wierzbicki et al. 2003].

All eight statins have been linked to rare instances of clinically apparent acute liver injury. Nevertheless, the most reports have been with atorvastatin and simvastatin and the fewest with pravastatin and pitavastatin [Björnsson, 2017]. The latency to onset varies considerably and can present after more than six months or even several years after taking the first dose [Karahalil et al. 2017]. The majority of cases are hepatocellular, but cholestatic hepatitis is also well described for most statins [Karahalil et al. 2017].

Orally administered statins are well absorbed from the intestine; however, they undergo significant extensive first-pass hepatic metabolism, which decreases its systemic bioavailability to approximately 5-50% [McFarland et al. 2014]. Therefore, by changing the drug release method (i.e. through transdermal drug delivery (TDD)), it may be possible to enhance their bioavailability and reduce certain side-effects. The transdermal route of delivery would not use the first-pass metabolism or cause gastrointestinal side-effects and may consequently increase patient compliance [Lee et al. 2018]. The HPLC method was developed and validated for atorvastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin. This method was validated for purposes of *in vitro* skin diffusion studies to detect the small concentrations that permeate through the skin.

2. Investigations, results and discussion

The validation of this method was done according to linearity, accuracy, precision on both intra-day and inter-day, system stability, specificity, LLOD (lowest limit of detection) and LLOQ (lowest limit of quantification); results are given in Table 1.

Linearity was performed by preparing standard solutions of each of the selected statins by dissolving ±20 mg of each statin in 100 ml methanol (200 µg/ml). After a stock solution was prepared a dilution was made by transferring 5 ml of the stock solution to a 50 ml volumetric flask and making it up to volume with 50 ml methanol (±20 µg/ml), after which a 2nd dilution was made by transferring 5 ml of the 1st dilution to a 50 ml volumetric flask and making it up to volume with methanol (±2 µg/ml). Each one

of these dilutions was then transferred into HPLC vials for analysis (in duplicate) at injection volumes of 2.5 µl, 5.0 µl, 7.5 µl and 10.0 µl. Results obtained from using this method is given in Table 1 where the statin concentration (µg/ml) and regression equations obtained from the plotting are displayed. When the linear regression was obtained from the correlation coefficient (R^2) results were observed and it can be concluded that there is a strong relationship between the peak area and the statin concentration, results were obtained within the concentration range.

After linearity, accuracy of statins was determined across a specific range of the analytical procedure [ICH, 2005]. The proposed results for accuracy and average recovery for each of the statins were as follow: atorvastatin 98.7% (%RSD = 0.7), fluvastatin 98.8% (%RSD = 1.3), lovastatin 106.4% (%RSD = 6.3), mevastatin 97.1% (%RSD = 1.0), pitavastatin 96.4% (%RSD = 2.2), pravastatin 96.2% (%RSD = 2.2), rosuvastatin 98.5% (%RSD = 1.0) and simvastatin 98.4% (%RSD = 1.2). The acceptable criteria range for recovery should fall within 90-110% [AVPMA 2004; Shabir 2005]. Hence, it can be concluded that the method of accuracy used was acceptable as values of each of the selected statins fell within the range of required limits.

Precision can be described as the closeness of individual measures; the procedure is applied for repeated measurements of multiple aliquots of a single homogenous sample [FDA 2001; Shah 1992; [Paithankar 2013 Snyder et al. 1997]. Precision for this analytical method was examined based on three principles, e.g. repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility [Huber 2007; ICH 2005; Paithankar 2013; USP 2011]. To determine precision, a minimum of five replicate samples are needed (APVMA, 2004). Precision was determined firstly as intra-day precision and the following consecutive days were inter-day precision. Intra-day precision was measured by weighing 0.6, 0.8 and 1.0 g of each selected statin formulation in triplicate in a 100 ml volumetric flask and making it up to volume. For intra-day repeatability %RSD must be <10% [Okafo and Roberts, 2003]; all the statins had a %RSD of <2% [APVMA 2004; Snyder et al. 1997]. The same method as for intra-day precision was used for inter-day precision, but only one concentration was selected, for this validation the intermediate amount of 0.8 g of each of the statins was chosen and made up to volume in triplicate, placed into HPLC vials for analysis.. The results of each of the selected statins fell within the acceptable range according to Rafeal et al. 2007 (%RSD should be ≤3%) as observed in Table 1.

System stability determines if the validation method utilised can accurately perceive degradation of the compounds [Huber 2007]. Sample stability (over 24 h) and sample repeatability was determined. System stability was performed by weighing ±10 mg of each of the selected statins in a 100 ml volumetric flask and making it up to volume with methanol. Each of the samples were injected over a 24 h period in 1 h intervals. Sample repeatability was done, with the same stock solution as prepared for 24 h, but in seven consecutive intervals under the same conditions. The acceptance criteria for sample stability and repeatability is an %RSD of ≤2% [Cowley 2012; Paithankar 2013]. It can be concluded from Table 1 that the results obtained fell within acceptable range. After sample stability and repeatability was done, the specificity of the analytical method was measured.

Specificity is the measurement that accurately investigates the concentration of the analyte in the presence of another compound to determine the effect impurities has on the analyte [ICH 2005, Huber, 2007]. There should be no interference of the compounds on the analyte [Snyders et al. 1997]. Each test tube was spiked with 200 µl of the following compounds: 1) H_2O, 2) HCl, 3) NaOH and 4) H_2O_2, as illustrated in Fig 1: A-H. The degradation results of the selected statins show that the peak retention times were slightly affected after being stressed with HCl (all statins) and NaOH (lovastatin and mevastatin).

LOD and LOQ can be described as the lowest detectable amount of analyte that is present in a sample under specific conditions that can be accurately determined [AVPMA, 2004]. To establish LOD and LOQ, ±5 mg of each of the selected statins were weighed separately in a 100 ml volumetric flask and made up to volume with 100 ml methanol. 1 ml of each of the selected statins were then transferred into a 100 ml volumetric flask and made up to volume with methanol, where after the solutions were transferred into HPLC vials for analysis. The solutions were injected six consecutive times at four different injection volumes (2.5, 5.0, 7.5 and 10.0 µl). The LOD and LOQ values were determined for each of the statins. According to [Rathmann et al. 2015], LOQ values of acceptable criteria are ≤15%, while LOD values of acceptable criteria is ≤20% [Basal and DeStefano, 2007; FDA 2001; González et al. 2014]. Results obtained as shown in Table 1 fell in the range of acceptable criteria.

After the validation of the HPLC method on the selected statins, the analytical validation method was used during membrane release studies, *in vitro* skin diffusion studies and tape stripping, since the results of each of the selected statins analysed by this HPLC method, were satisfactory (see Fig. 2).

3. Experimental

The method developed was done by using an analytical instrument (HP1100 series HPLC equipped with a gradient pump, UV detector and autosampler injector mechanisms). The software used for analysis was ChemStation Rev. A.10.03. The laboratory environment was controlled at a temperature of 25°C. The column used was a Venusil XBP C_{18} (2) reverse phase column (150 x 4.6 mm) with a particle size of 5 µm (Agela Technologies, Newark, DE) and a flow rate of 1.0 ml/min at a UV detection of 240 nm for the selected APIs, the selected run time was 10.5 min at an injection volume of 10 µl. The mobile phases used was acetonitrile and Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand, RSA) with 0.2% octane sulphonic acid-Na at pH 3.5, which started at 25% acetonitrile and was increased linearly to 90% after 1.0 min, then it was held at 90% until 6.5 min and then re-equilibrated to start conditions. Solvents used in this experiment was methanol and the retention times for each of the selected statins (as seen in Fig. 3) were ±4.17 (atorvastatin), ±3.65 (fluvastatin), ±4.09 (lovastatin), ±6.64 (mevastatin), ±3.39 (pitavastatin), ±3.85 (pravastatin), ±4.50 (rosuvastatin) and ±7.12 (simvastatin), respectively.

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Disclaimer

Any opinion, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto. The authors declare no conflict of interest.

References

Australian Pesticides and Veterinary Medicines Authority (2004) Guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products. http://apvma.gov.au/sites/default/files/docs/guideline-69-analytical-methods.pdf Date of acces: 4 Oct. 2018.

Bays H (2005) Statin safety: An overview and assessment of the data. Am J Cardiol, 97:7C-26C.

Becher T, Schulze TJ, Schmitt M, Trinkmann F, El-Battrawy I, Akin I, Kälsch T, Borggrefe M, Stach K (2017) Ezetimibe inhibits platelet activation and uPAR expression on endothelial cells. International journal of cardiology, 227:858-862.

Björnsson, E.S.J.L.I (2017) Hepatotoxicity of statins and other lipid-lowering agents. Liver international, 37(2):173-178.

Bouhairie VE, Goldberg AC (2015) Familial hypercholesterolemia. Cardiol Clin, 33(2):169-179.

Braamskamp, M.J., Wijburg, F.A. & Wiegman, A. 2012. Drug therapy of hypercholesterolaemia in children and adolescent. *Drugs*, 72(6):759-772.

Civeira F (2004) Guidelines for the diagnosis and management of heterozygous familial hypercholesterolemia. Atherosclerosis, 173:55-68.

Cowley A (2012) The effect of selected natural oils on the permeation of flurbiprofen through human skin. Potchefstroom: NWU. (Dissertation-MSc).

Du Souich P, Roederer G, Dufour R (2017) Myotoxicity of statins: Mechanism of action. Pharmacology & Therapeutics, 175:1-16.

Food and Drug Administration (2001) Guidance for Industry: bioanalytical method validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf Date of access: 1 Apr. 2019.

Gazzerro, P., Proto, M.C., Gangemi, G., Malfitano, A.M., Ciaglia, E., Pisanti, S., Santoro, A., Laezza, C. & Bifulco, M. 2012. Pharmacological actions of statins: a critical appraisal in the management of cancer. *Pharmacological reviews*, 64(1):102-146.

González, O., Blanko, M.E., Iriart, G., Bartolomé, L., Maguregui, M.I. & Alonso, R.M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. Journal of chromatography A, 2014:10-27.

Huber, L. 2007. Validation of Analytical Methods. Agilent technologies: USA. 1-76p.

International Conference of Harmonisation (2005) Validation of analytical procedures: text and methodology Q2 (R1). http://www.ich.org//fileadmin/Public_Web_Site/ICH_Products?Guidelines?Quality?Q2_R1/Step4/Q2_R1_Guideline.pdf Date of access: 5 Jun. 2018.

Karahalil, B., Hare, E., Koç, G., Uslu, İ., Şentürk, K., Özkan, Y. 2017. Hepatotoxicity associated with statins. *Global journal of pharmacology*, 68(4):254-260.

Lee H, Song C, Baik D, Kim D, Hyeon T, Kim DH 2018. Device-assisted transdermal drug delivery. *Advanced drug delivery reviews*, 127:35-45.

Lippi G, Favaloro EJ (2011) Antisense therapy in the treatment of hypercholesterolemia. *European journal of internal medicine*, 22:541-546.

McFarland, A.J., Anoopkumar-Dukie, S., Arora, D.S., Grant, G.D., McDermott, C.M., Perkins, A.V. & Davey, A.K. 2014. Molecular mechanisms underlying the effects of statins in the central nervous system. *International journal of molecular sciences*, 15(11):20607-20637.

McFarlane, S., Muniyappa, R., Francisco, R. & Sowers, J. 2002. Pleiotropic effects of statins: lipid reduction and beyond. *The Journal of Clinical Endocrinology & Metabolism*, 87(4):1451-1458.

Okafo GN, Roberts KK (2003). Development of achiral separation methods in pharmaceutical analysis. *In* Lee DC, Webb M (*eds*.) Pharmaceutical analysis. 1st ed. Stevenage: Blackwell publishing. p. 31-68.

O'Sullivan, S. 2007. Statins: A review of benefits and risks. *TSMJ*, 8:52-56.

Paithankar, H.V. 2013. HPLC method validation for pharmaceuticals. International journal of universal pharmacy and bio sciences, 2(4):229 – 240.

Rader DJ, Cohen J, Hobbs HH (2003) Monogenic hypercholesterolemia: new insights in pathogenesis and treatment. Journal of clinical investigation, 111:1795-1803.

Rafael JA, Jabor JR, Casagrande R, Georgetti SR, De Fátima Borin M, Fonseca MJV (2007) Validation of HPLC, DPPH* and nitrosation methods for mesalamine determination in pharmaceutical dosage forms. *The brazilian journal of pharmaceutical sciences*, 43:97-103.

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

Rodenburg J, Visser MN, Wiegman A, van Trotsenburg P, van der Graaf A, de Groot E, Wijnburg FA, Kastelein JJP, Hutten BA (2016) Statin treatment in children with familial hypercholesterolemia: Theyounger, the better. *Atherosclerosis*, 116:664-668.

Shabir GA (2004) A practical approach to validation of HPLC methods under current good manufacturing practices. Journal of Validation Technology, 10:210-218.

Shabir GA (2005) Institute of Validation Technology: step-by-step analytical methods validation and protocol in the quality system compliance industry. http://www.ivtnetwork.com/sites/default/files/StepbyStep%20Analytical%20Methods%20Valdation%20and%20Protocol%20in%20the%20Quality%20Sytem%20Compliance%20Industry.pdf Date of access: 3 Apr. 2019.

Shah Y, Khanna S, Jindal KC, Dighe VS (1992) Determination of rifampicin and isoniazid in pharmaceutical formulations by HPLC. *Drug development and industry pharmacy*, 18(14):1589-1596.

Snyder LR, Kirkland JJ, Glajch JL (1997). Completing the method: validation and transfer. *In* Snyder LR, Kirkland JJ, Glajch JL (*eds*.) Practical HPLC method development. 2nd ed. New York: John Wiley & Sons. p. 685-713.

Stancu C, Sima A (2001) Statins: mechanism of action. Journal of cellular and molecular medicine., 5(4):378-387.

Thompson PD, Panza G, Zaleski A, Taylor B (2016) Statin-Associated side effects. Journal of the american college of cardiology, 67:2395-2410.

USP see United States Pharmacopeia

United States Pharmacopeia. 2009. Validation of compendial methods. https://hmc.usp.org>c1225_1susp40.pdf Date of access: 26 Feb. 2019.

Wierzbicki AS, Poston R, Ferro A (2003) The lipid and non-lipid effects of statins. Pharmacology & therapeutics, 99:95-112.

244 **Table 1:** HPLC method validation parameters for the selected statins

	Atorvastatin	Fluvastatin	Lovastatin	Mevastatin	Pitavastatin	Pravastatin	Rosuvastatin	Simvastatin
Linearity								
Correlation coefficient R^2	0.9999	0.9999	0.9999	0.9999	1	0.9999	1	0.9999
Regression equation	y = 23.652x + 19.437	y = 23.499x + 19.437	y = 22.839 + 19.437	y = 22.839x + 19.437	y = 22.85x + 19.437	y = 23.499x + 19.437	y = 22.85x + 19.437	y = 23.652x + 19.437
Accuracy								
%Mean recovery	98.7	98.8	106.4	97.1	96.4	96.2	98.5	98.4
%RSD	0.7	1.3	6.3	1.0	2.2	2.2	1.0	1.2
Precision								
Intra-day precision (%RSD)	1.8	0.8	0.8	0.8	0.8	1.0	0.6	2.2
Inter-day precision between days (%RSD)	0.89	0.3	1.01	0.87	0.92	0.5	0.41	0.6
Sample stability over 24 h								
Mean %remaining	99.5	100.0	96.0	96.3	99.1	100.3	99.9	96.2
%RSD	0.31	0.08	2.95	1.69	0.84	0.25	0.09	2.24
System repeatability								
Retention time (%RSD)	0.14	5.45	7.90	0.09	0.17	0.14	4.61	7.07
Peak area (%RSD)	0.11	0.06	0.12	0.20	1.16	0.11	0.07	0.09
LOD and LOQ								
LOD (µg/ml)	0.14	0.14	0.15	0.13	0.15	0.14	0.13	0.13
LOQ (µg/ml)	0.55	0.55	0.62	0.52	0.58	0.54	0.53	0.52

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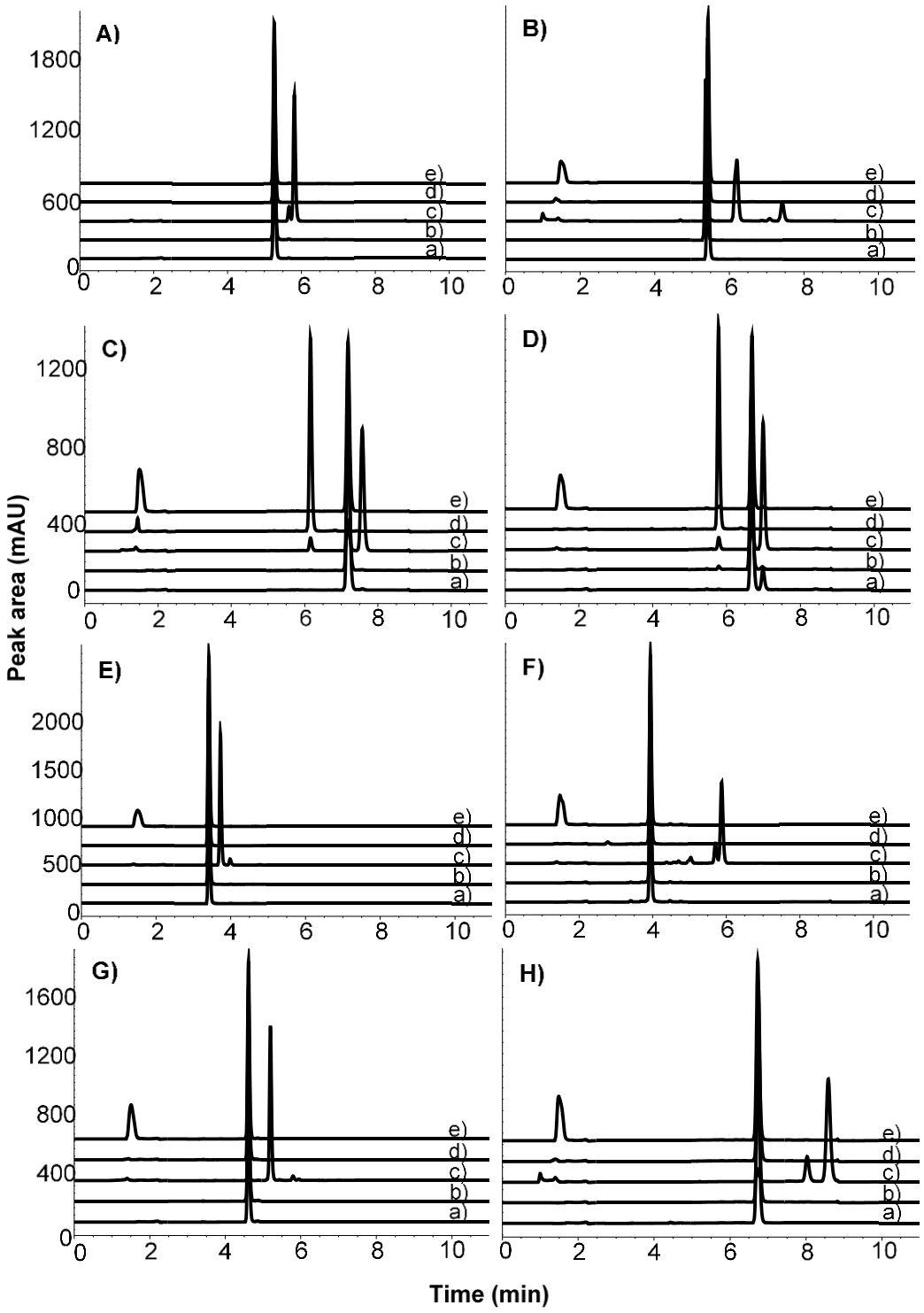

Fig. 1: HPLC chromatogram showing specificity data obtained for: A) atorvastatin, B) fluvastatin, C) lovastatin, D) mevastatin, E) pitavastatin, F) pravastatin, G) rosuvastatin and H) simvastatin; in addition: a) standard solution of each sample and samples stressed with 200 µl of b) H_2O, c) HCl, d) NaOH and e) H_2O_2

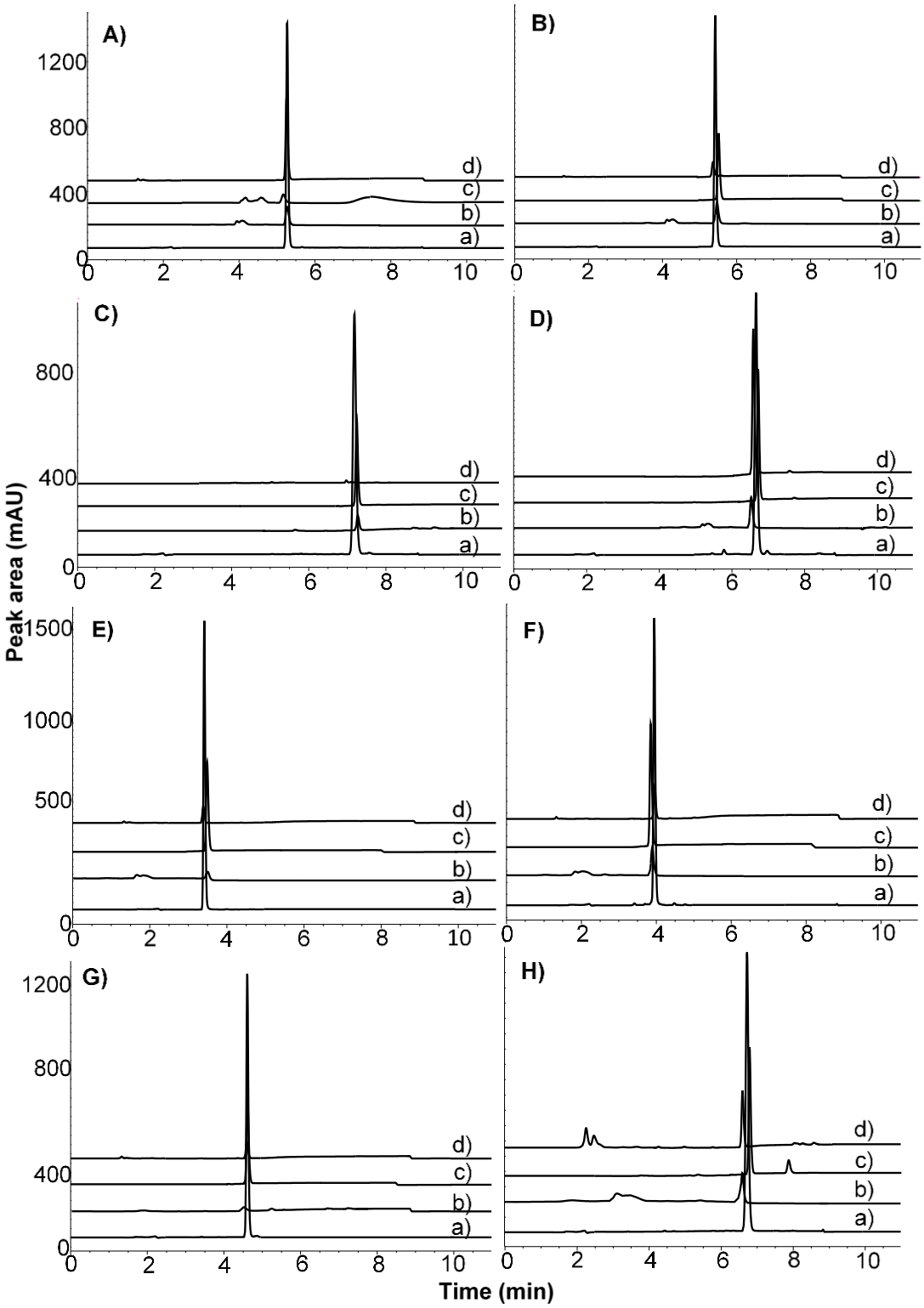

Fig .2: Chromatogram representation of: A) atorvastatin, B) fluvastatin, C) lovastatin, D) mevastatin, E) pitavastatin, F) pravastatin, G) rosuvastatin and H) simvastatin; in addition: a) standard solution, b) receptor phase extraction, c) tape-stripping (SCE) and d) skin samples (ED)

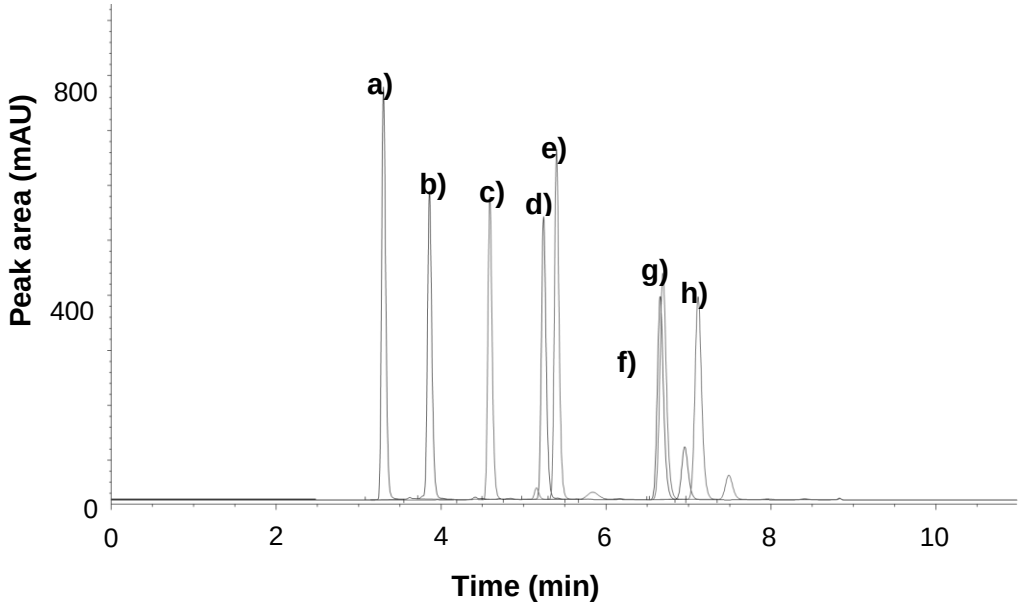

Fig. 3: Chromatogram representation of the simultaneous detection of the different statins: a) pitavastatin, b) pravastatin, c) rosuvastatin, d) atorvastatin, e) fluvastatin, f) mevastatin, g) simvastatin, h) lovastatin

CHAPTER 4

ARTICLE FOR THE PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

Chapter 4 is written (in article form) for publication in The International Journal of Pharmaceutics. The author's guide (listed in Appendix G) accepts both UK and US English. The authors have decided to write in UK English and the paragraphs of this chapter have been justified for the ease of reading.

Transdermal delivery of statins formulated in nano-emulsions and nano-emulgels

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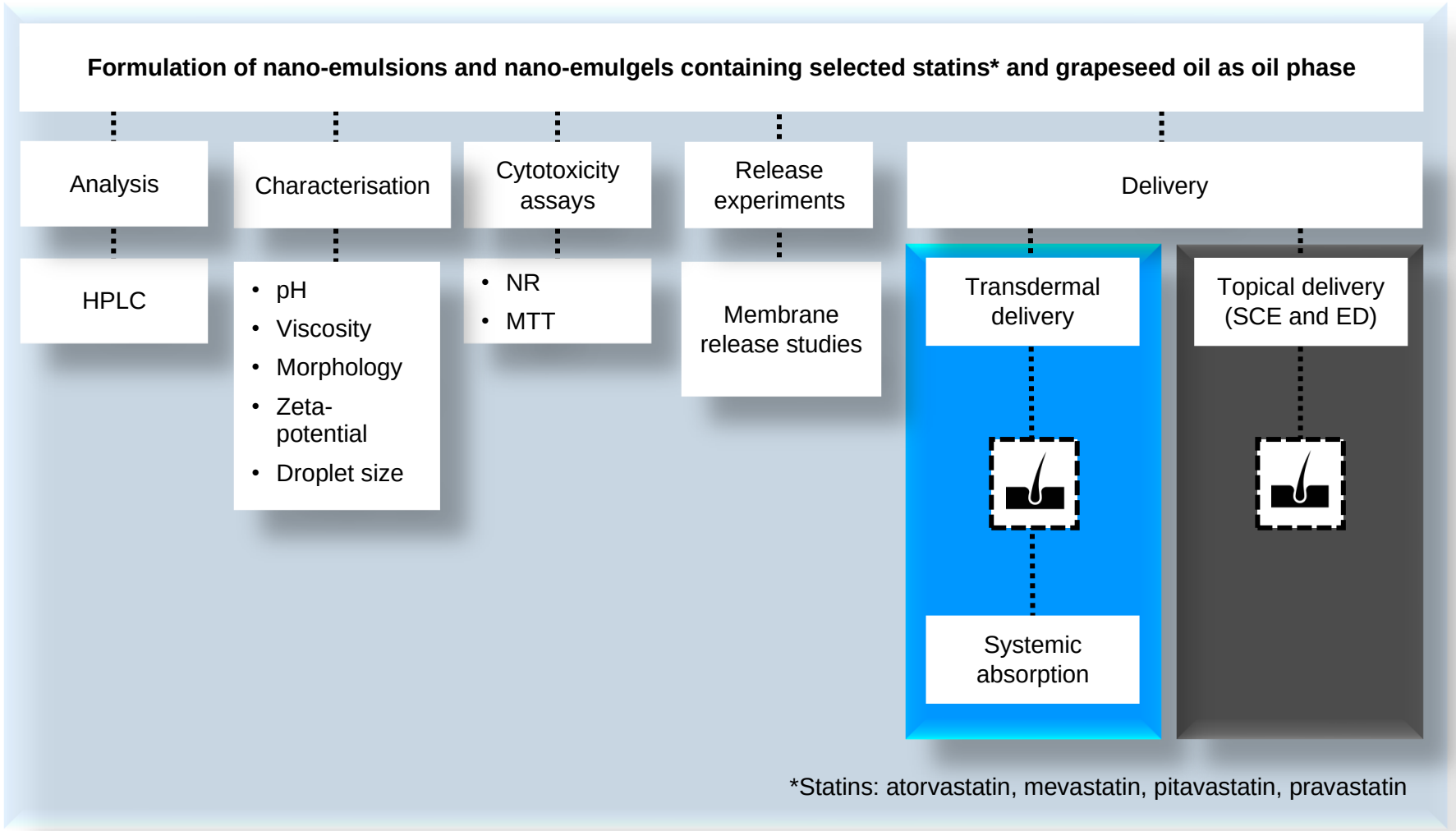
Abstract

Evidence suggests that statin therapy has significant mortality and morbidity benefit for both primary and secondary prevention of cardiovascular diseases. However, concern has been expressed regarding the adverse effects of orally administered statin use, e.g. gastro-intestinal side-effects and depleted bioavailability due to hepatic clearance, which can ultimately result in hepatotoxicity. Lipophilic and hydrophilic active pharmaceutical ingredients (APIs) can be enhanced by using nano-formulations. The purpose of this study was to formulate 2% statins (atorvastatin, mevastatin, pitavastatin and pravastatin) within nano-emulsions and nano-emulgels for transdermal delivery. This study also compared which statins sustained more favourable diffusion for transdermal delivery, and additionally, which vehicle proved most advantageous. Characterisation methods were performed on the nano-formulations (nano-emulsions and nano-emulgels) to ensure stability and cytotoxicity studies were conducted on premalignant immortalised human keratinocytes (HaCaT) to establish whether the HaCaT cell lines were affected by the statins. Membrane release studies indicated that the highest flux was obtained by the nano-emulsion and nano-emulgel containing pravastatin. From the *in vitro* skin diffusion studies, it was concluded that the nano-emulgel containing pitavastatin obtained the highest median amount per area diffused. Statistical analyses were performed to analyse the variances between the means of data obtained from the aforementioned studies.

Keywords: Statins, Grapeseed oil, Transdermal delivery, Formulation, Nano-emulsion, Nano-emulgel, cytotoxicity

Graphical abstract



1 Introduction

Globally, one baby is born with familial hypercholesterolaemia (FH) every minute (Wiegman *et al*., 2015). FH is a general genetic cause of premature coronary heart disease (CHD) in both adolescents and children. However, if diagnosed and treated early in childhood, individuals with FH can have normal life expectancy (Wiegman *et al*., 2015). Statins, also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, is the first-line treatment for the management of hypercholesterolaemia (Braamskamp *et al*., 2012). Since, inadequacies are associated with the oral administration of these active pharmaceutical ingredients (APIs), e.g. myalgia, gastrointestinal side-effects and increased risk of hepatotoxicity may occur (McKenney *et al*., 2007; Schierwagen *et al.,* 2017). An increased risk of statins side-effects can be expected with first-pass metabolism by cytochrome P450 (CYP450) enzymes that is responsible for the metabolism of all statins (excluding pravastatin) (Ramkumar *et al*., 2016). Food intake has a variable effect on statin absorption; since the bioavailability of atorvastatin and pravastatin is known to decrease with the consumption of food (Schachter, 2005). Thus, considering an alternative route of administration, namely transdermal delivery, can be suggested to reduce side-effects, as this administration route avoids hepatic first-pass metabolism (thus unaffected by CYP450), which may ultimately result in a lower dose being administered (N'Da, 2014).

In pharmaceutical sciences, transdermal delivery can be described as a transport process of an API from a dosage form to the target-site of action (Wiechers, 2008). The stratum corneum, has a complex structure, which functions as a barrier for APIs to permeate across the skin; a barrier not only applicable to APIs, but to chemical hazards within the environment as well; therefore, making the skin a protectant from external influences (Forslind *et al*., 1995). The physiochemical properties of the API intended for transdermal delivery should be evaluated to bypass the relatively impermeable barrier, since such candidates are required to meet certain criteria (Szunerits & Boukherroub, 2018). The statins (atorvastatin, mevastatin, pitavastatin and pravastatin) that were investigated during this study are non-compliant to several ideal

physiochemical requirements for transdermal delivery (Naik *et al*., 2000) considering the molecular mass <500 Da, log P of 1 to 3, aqueous solubility of >1 mg/ml and a melting point of <200°C (Naik *et al*., 2000). It is evident that limited statins possess these ideal properties, thus novel methods were proposed to increase the transdermal delivery of non-ideal APIs (Dodou, 2012). Hence, the statins were formulated within a carrier system to overcome the permeation barrier posed by human skin (Gabera *et al*., 2017).

During this study, nano-emulsions (**NEs**) and nano-emulgels (**NGs**) were used as carrier systems containing 2% (2 g per 100 ml) of the selected statins to investigate transdermal delivery. The statins that were used in the **NEs** and **NGs** were as follow: atorvastatin (**NE-ATO**; **NG-ATO**), mevastatin (**NE-MEV**; **NG-MEV**), pitavastatin (**NE-PIT**; **NG-PIT**) and pravastatin (**NE-PRA**; **NG-PRA**), respectively. Since, both **NEs** and **NGs** have the ability to avoid hepatic metabolism, enhance absorption, prolong half-lives and potentially decrease toxicity (Gabera *et al*., 2017). Additionally, to establish the most efficient formulation in this study, **NEs** were compared to the **NGs** (optimised **NE** containing gelling-agents). **NEs** are suggested to have a droplet size ranging from 20 to 200 nm (Ee *et al*., 2008). Nano-sized droplets of these systems can contribute to the effective systemic delivery of the selected API, due to easier penetration of the skin (Chaudhary *et al*., 2018). According to literature, gelling-agents (as additives) tend to reduce interfacial and surface tension more than **NEs**, which produces a more viscous formulation (Chellapa, 2015), which have been proved to enhance skin permeability (Chellapa, 2015, Eid *et al*., 2014). Conversely, a more effective delivery of **NEs** can be ascribed to the excipients utilised in the formula, like surfactants, co-surfactants and oils, which can ultimately change the lipid structure of the stratum corneum and formulation fluidity; subsequently, acting as penetration enhancers (Su *et al*., 2017). Grapeseed oil were utilised in this study with the dual function of serving as an oil phase and as penetration enhancer regarding the formulations. Penetration enhancers have the ability to further increase skin permeability (Kala & Juyal, 2018) by reversibly modifying the obstacles posed by the skin barrier (Boglarka *et al*., 2016; Kala & Juyal, 2018). Grapeseed oil contains

high content of polyunsaturated fatty acids, vitamin E and phenolic compounds (Fiori *et al.,* 2010), together with beneficial compounds, namely oleic acid (15.8%) and linoleic acid (69.6%) (AL-Bayati & Enaad, 2015). This natural oil is effective due to the C_{18}-fatty acids that are also found in the human skin, which can ultimately enhance skin permeability and allow the delivery of both lipophilic and hydrophilic APIs (Barry, 1983a; Čižinauskas *et al*., 2017) and decrease skin irritation (Büyüktimkin *et al*., 1997; Vermaak *et al*., 2011). Additionally, grapeseed oil is rich in proanthocyanidins, a unique compound that contains antioxidants that are 20 times greater than vitamin C and 50 times more efficient than vitamin E (AL-Bayati & Enaad, 2015). Furthermore, it is critical to establish whether a sufficient amount of the selected statin was delivered to exhibit a therapeutic effect, which will also be investigated.

2 Materials and Methods

2.1 Materials

Atorvastatin, mevastatin, pitavastatin and pravastatin were obtained from DB Fine (Johannesburg, RSA). Grapeseed oil (natural oil) was obtained from CJP Chemicals (Johannesburg, RSA), while Tween® 80 (hydrophilic surfactant), Span® 60 (lipophilic surfactant), Carbopol® Ultrez 20 (gelling agent) with analytical grade methanol were obtained from Sigma-Aldrich (Johannesburg, RSA). The phosphate buffer solution (PBS) contained compounds, e.g. sodium hydroxide (NaOH) and potassium dihydrogen orthophosphate (KH_2PO_4) that was obtained from Sigma-Aldrich (Johannesburg, RSA). Considering mobile phase preparation, HPLC grade orthophosphoric acid and acetonitrile were supplied by Sigma-Aldrich (Johannesburg, RSA) and utilised in combination with deionised high performance liquid chromatography (HPLC) grade water (Millipore, Milford, USA).

2.2 Methods

2.2.1 Analysis of atorvastatin, mevastatin, pitavastatin and pravastatin

An HPLC analytical method was developed and validated to determine the influence of the assay conditions and sample elements on the assay performance. In order to validate the HPLC method, an analytical instrument (HP1100 series HPLC equipped with a pump,

autosampler, ultraviolet (UV) detector and ChemStation Rev. A.10.03 data acquisition and analysis software or equivalent) was used. A Venusil XBP C_{18}(2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE) column was used and the UV detector was set at a wavelength of 240 nm, the flow rate at 1.0 ml/ml and default injection volume at 10 µl. The mobile phase contained acetonitrile (ACN)/Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand, RSA) with 0.1% orthophosphoric acid. Gradient elution was used by starting at 25% with ACN for 1.0 min, while increasing linearly to 90% for 3.0 min; consequently, it was held at 90% for 6.5 min and thereafter re-equilibration took place to start conditions (total runtime of 10.5 min). The retention time was ±4.166, 6.640, 3.386 and 3.853 min for atorvastatin, mevastatin, pitavastatin and pravastatin, respectively; methanol was utilised as a solvent during standard preparations.

Table 1:

Limit of detection (LOD) and lowest limit of quantification (LLOQ) of the statins

Controlled temperature of 25°C were kept for all analytical tests performed in this study.

2.2.1.1 Standard preparation

A standard solution was prepared by adding ±20 mg of the statin in a volumetric flask, which was diluted with methanol to 100 ml. A volume of 5 ml of the statin solution was diluted to 50 ml with methanol; this step was repeated to prepare a second dilution from the first dilution. The standard solution, with the additional two dilutions, were injected at various volumes (2.5, 5.0, 7.5, 10.0 µl) to obtain a standard curve.

2.2.2 Nano-emulsions and nano-emulgels

Eight formulas were developed, which contained the selected statins (2%). For each respective statin, an oil-in-water (o/w) **NE** and **NG** was formulated. The **NEs** were formulated by firstly dissolving the excipients in the respective phases (oil phase/water phase) and placing it on a hot plate with a magnetic stirrer (±40°C). Excipients, such as Tween® 80 were added within the water phase and Span® 60 together with the selected statins were added within the

oil phase (grapeseed oil). Once the excipients within the two phases were properly dissolved, the oil phase was added to the water phase (drop wise), which created an emulsion that was left to stir on the hotplate. However, ultrasonication (high energy-method) was used to finally obtain a **NE**. The **NE** was sonicated for 3 min with 1 min intervals with an ultrasonic probe. The **NGs** were formulated based on the **NE** with the addition of Carbopol® Ultrez 20 to the water phase. The time utilised to fully dissolve excipients within the two phases was adjusted to 10 min, which guaranteed proper dissolution of the gelling agent in the water phase. Thereafter, before adding the oil phase to the water phase, the water phase was sonicated for 3 min by using the ultrasonicator to free the water phase of any entrapped air due to the addition of the gelling agent (Carbopol® Ultrez 20). The water phase was then transferred into a suitable beaker and placed under an overhead mechanical stirrer (±850 rpm), while adding it (drop wise) to the oil phase. Thereafter, the formulation was left to stir for another 15 min. Finally, a formulation with a higher viscosity were obtained compared to the **NE**. However, ultrasonication (±3 min) could still be applied successfully to obtain nano-sized droplets. Once the formulation was completed, the pH of the formulations was adjusted. Adjustment of the pH is a critical step during the formulation of **NGs**; since, Carbopol® Ultrez 20 will only achieve optimum viscosity after neutralisation (Shin *et al*., 2000) and due to the low pH of these formulations before neutralisation of Carbopol® Ultrez 20 (Lubrizol, 2009). The adjustment of the pH also confirms a safe application to the skin (Nair *et al*., 2013).

Table 2:

Ingredients used during the formulation of the different **NEs** and **NGs**

NEs that contained the selected statins can be referred to as **NE-ATO** (**NE** with atorvastatin), **NE-MEV** (**NE** with mevastatin), **NE-PIT** (**NE** with pitavastatin) and **NE-PRA** (**NE** with pravastatin). The **NGs** are referred to as **NG-ATO** (**NG** with atorvastatin), **NG-MEV** (**NG** with mevastatin), **NG-PIT** (**NG** with pitavastatin) and **NG-PRA** (**NG** with pitavastatin).

2.2.3 Characterisation of selected statin formulations

Eight formulas were characterised by means of pH, droplet size, zeta-potential and viscosity. Additionally, transmission electron microscopy (TEM) was performed on the respective **NEs**.

2.2.3.1 TEM

Micrographs of the respective **NEs** were obtained by using a FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope (HRTEM) (Czech Republic, EU) with an Oxford INCA X-Sight EDS System. A dilution of the **NEs** was made with Milli-Q® water, where after a small volume of each of the dilutions was placed on a microscopic carbon-coated 300 mesh copper grid, via a micropipette and left to dry for 10-15 min (excess removed with filter paper). Finally, osmium tetroxide was used to stain samples.

2.2.3.2 pH

The pH of the **NEs** was measured with a Mettler Toledo® pH meter (Mettler Toledo, CU) by placing the Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) into the **NE** to acquire these pH values. Calibration of the apparatus was performed before initiating the triplicate measurements at 32°C.

2.2.3.3 Viscosity

The viscosity of the formulas was measured by utilising a Brookfield Viscometer (DV2T LV Ultra-Middleboro, Massachusetts, USA) linked to a water bath (±25°C). Once the ideal temperature was reached, formulas were placed into the water bath 45 min prior to viscosity testing. A cylindrical spindle (SC4-18) rotating at a speed of 200 rpm was utilised to measure the viscosity of the **NEs**. The viscosity of the **NGs** were measured by using T-bar spindles, rotating at a speed of 20 to 30 rpm. Viscosity readings were measured in centipoise (cP), in 10 sec intervals for 50 sec. Rheocalc T 1.2.19 software was utilised to gather multipoint data, to analyse and measure median viscosity values.

2.2.3.4 Droplet size

A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was utilised to determine the droplet size of the eight formulas. Three readings were taken from the eight

prepared samples, which were diluted separately with Milli-Q® water (1:100) and mixed thoroughly by rotating the flask while holding it. These dilutions were analysed by transferring 2 ml in a clear disposable zeta-cell (DTS1070 folded capillary cell), where after a median droplet size could be established.

2.2.3.5 Zeta-potential

A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was also utilised to measure the zeta-potential of eight formulas. However, triplicate measurements were initiated over a period of 300 sec between 3.5 to 65.5 rpm. Samples were then prepared by using the identical method as described in Section 2.2.3.5. Afterwards the samples were placed in a clear disposable zeta-cell (DTS1070 folded capillary cell) to calculate the median zeta-potential.

2.2.4 Diffusion experiments

Diffusion experiments consisted out of membrane release studies and *in vitro* skin diffusion, which were performed via the Franz cell method.

2.2.4.1 Membrane release studies

Eight different formulas were formulated and pre-heated in a water bath (32°C), while the receptor phase (PBS 7.4 pH and 10% denatured ethanol) was pre-heated in a second water bath (37°C) before membrane release studies could commence. A vertical Franz cell was utilised during this study that contained two compartments, e.g. the donor and receptor compartment. High vacuum grease, Dow Corning® was applied to each compartment and a magnetic stirrer was placed in the receptor compartment before positioning the synthetic membrane (polyvinylidene fluoride (PVDF), Pall® Life Sciences, Michigan, USA, with a pore size of 0.45 µm and 25 mm diameter) on the receptor compartment. Afterwards, the donor compartment was positioned on top of the receptor compartment and vacuum grease was used to seal the two connected compartments, to void leakage. Then a horseshoe clamp was used to tightly fasten the two compartments. The receptor compartment has a filling capacity of ±2 ml and a diffusion surface of 1.075 cm^2. Then only 2 ml PBS:ethanol (9:1 at pH 7.4) was

placed in the receptor compartment for each study (one specific statin formula per study). Before filling the donor compartment with the selected formula with ±1 ml, the receptor compartment was firstly visually inspected to ensure the presence of no air bubbles. Parafilm® together with a plastic cap were utilised to cover each of the donor compartments of the prepared Franz cells to avoid unnecessary contamination and the loss of constituents. The Franz cells were then positioned on a Franz cell stand and placed within the pre-heated water bath (37°C) (Grant Instruments, UK) on a magnetic stirring plate (Variomag, USA) to ensure adequate mixing. Every hour for the following 6 h, the receptor phase was extracted and refilled with PBS:ethanol (9:1 at pH 7.4; 37°C). Finally, the extracted PBS:ethanol (9:1 at pH 7.4) was placed in an HPLC vial to be analysed by means of HPLC.

Statistical analysis was performed on membrane release studies via ANOVA, Mann-Whitney U test and Tukey's HSD post-hoc tests. ANOVA was performed on the formula type and statins; the interaction was of the flux value where the statistical significance was $p<0.05$. One-way ANOVAs were performed to determine the effect of the selected statin on each of the **NEs** and **NGs**. As a result, statistically significant ($p<0.05$) effects were obtained during both ANOVAs, pairwise differences were established between the four respective statin means by using Tukey's HSD tests. The **NEs** means (**NE-ATO**, **NE-MEV**, **NE-PIT** and **NE-PRA**) and **NGs** (**NE-ATO**, **NE-MEV**, **NE-PIT** and **NE-PRA**) were statistically non-significant homogeneous groups on a 0.05 level. A non-parametric Mann-Whitney U test compared the data of the respective statins in the **NEs** and **NGs** according to p-values, which obtained statistically significant ($p<0.05$) effects.

2.2.4.2 Skin preparation

Caucasian skin (female) was obtained from abdominoplasty and used for the purpose of this study (ethical approval reference number: NWU 00111-17-A1-05). A Dermatome™ (Zimmer TDS, United Kingdom) was utilised to gather dermatomed skin samples of ±400 µm by pressing the Dermatome™ on the skin at an angle of ±45°. Afterwards the dermatomed skin samples were placed on Whatman® filter paper and enfolded within aluminium foil, which was

stored at -20°C until required for skin diffusion studies. However, the number of samples required were thawed and cut into circles before every respective study, in such a manner that each circle could be positioned between the receptor and donor compartment of each vertical Franz cell. Prior to sample fitting, visual examination was initiated to exclude skin samples with visible defects.

2.2.4.3 Skin diffusion

The same method (as explained in Section 2.2.4.1) was utilised for *in vitro* skin diffusion studies. The only difference between the two methods was that the PVDF synthetic membranes were substituted with dermatomed skin samples and positioned between the receptor and donor compartments (stratum corneum facing upwards). Thereafter, the receptor compartment was only extracted once; 12 h after the study commenced. These extracted samples were then placed in HPLC vials and analysed via HPLC to determine the amount of the selected API that diffused through the skin after 12 h (Baert *et al*., 2011).

Statistical analysis was carried out on *in vitro* skin diffusion studies via ANOVAs, Mann-Whitney U tests and Kruskal-Wallis tests. Statins effects ($p<0.01$) existed during both one-way ANOVAs; therefore, Kruskal-Wallis tests were followed to establish pairwise differences between the four statin means. During the analysis of the **NEs**, it was concluded that there were no significant differences between the mean values of (**NE-ATO** and **NE-MEV**); (**NE-PRA** and **NE-ATO**) and (**NE-PIT** and **NE-PRA**). However, the mean value was significantly different (on a 0.05 level of significance) between (**NE-PIT** and **NE-ATO**); (**NE-MEV** and **NE-PRA**) and on a 0.001 level of significance for (**NE-MEV** and **NE-PIT**). As for the **NGs**, it was observed that there were no significant differences between the mean values of (**NG-ATO** and **NG-MEV**); (**NG-PRA** and **NG-ATO**) and (**NG-PIT** and **NG-PRA**). However, the mean value was significantly different (on a 0.05 level of significance) between (**NG-MEV** and **NG-PRA**); (**NG-PIT** and **NG-ATO**) and on a 0.001 level of significance for (**NG-MEV** and **NG-PIT**).

2.2.5 Tape stripping

It is crucial to quantify the API in the skin regarding topical and transdermal research (Escobar Chávez *et al*., 2008). Tape stripping commenced directly after the 12 h diffusion studies were completed, and it can provide more specific data on whether topical delivery of the API within either the stratum corneum-epidermis (SCE) or epidermis-dermis (ED) occurred (OECD, 2004). Moreover, the Franz cell compartments were separated, and the skin was visually inspected prior to tape stripping. The skin samples were pinned to Parafilm® on a solid surface, any remaining formulation was gently dabbed off with the use of paper towel. In total, sixteen pieces were acquired by cutting 3M Scotch® Magic™ into strips that could efficiently cover the diffusion area. However, the first strip was discarded to avoid possible contamination. The remaining 15 strips, which contained the API and SCE, were placed in a polytop containing 5 ml of methanol, serving as the extraction solution. An additional polytop with 5 ml extraction solution was utilised for the remaining ED, which was cut into small pieces to increase the surface area before being placed in the polytop. These polytops were then stored in the fridge (±4°C) and left for ±8 h, thereafter, ±1.5 ml was extracted via a syringe from each of the polytops and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an HPLC vial for analyses (Pellett *et al*., 1997).

A three-way ANOVA was applied on the formula type, statin and skin layer (SCE/ED), which indicated a statistically significant difference ($p<0.05$). Additionally, one-way ANOVAs were performed to establish the effect of the statin for each of the four combinations of formula type and skin layer during tape stripping studies. Statistical significance was observed between the **NEs** between SCE/ED ($p=<0.001$), including the **NGs** between SCE/ED ($p=<0.001$). Tukey's HSD tests were performed to establish pairwise differences between the four statins means due to the significant differences regarding the three ANOVAs. Obtained results were as follow: the mean values of **NE-MEV** and **NE-PRA** together with **NG-ATO** and **NG-PRA** in the SCE were significantly different on a 0.05 level of significance. **NEs** and the ED obtained

no significant variance, nonetheless **NE-PRA** was only partly homogenous with **NE-MEV** and **NE-PIT** in the ED. Lastly, when comparing the **NGs** concerning the ED, a significant difference was observed between the mean values of **NG-ATO** and **NG-PRA**; however, **NG-MEV** differed significantly from the other formulas. Furthermore, Mann-Whitney U tests were performed to compare the type of formula (**NEs** and **NGs**) in terms of SCE and ED, separately. Significant differences were observed for all the statins (excluding mevastatin and SCE): atorvastatin (p=<0.001), pitavastatin (p=0.092) and pravastatin (p=0.020) when compared to SCE. The ED indicated: atorvastatin (p=<0.002), mevastatin (p=>0.001), pitavastatin (p=0.025) and pravastatin (p=0.001).

2.2.6 Data analysis

During the membrane release studies, the cumulative amount of respective statin per area (µg/cm^2) which had diffused through the membrane, was plotted against time (h) (Shakeel *et al*., 2007) to obtain the slope of the linear line (flux (µg/cm^{2}h)) (Ng *et al*., 2010). During skin diffusion studies, the median cumulative amount per area (µg/cm^2) of the selected statin that diffused through the skin was determined, together with median concentration (µg/ml) of the selected statin that diffused into the SCE and ED.

2.2.7 Statistical analysis

Descriptive analysis involves the determination of mean (which includes standard deviation) and median (middle score of distribution) values regarding the flux value obtained during membrane studies, and concentration values concerning skin diffusion (Sheskin, 2000). The use of analysis of variance (ANOVA) determined whether statistically significant effects occurred between the different factors; consequently, p-values were obtained. It can be considered that a p-value of equal or less than 0.05 is regarded to have a statistically significant effect (Concato & Hartigan, 2016). A statistically significant ($p<0.05$) interaction was associated with the type of formula (**NEs** and **NGs**) and the statins during membrane studies; hence, one-way ANOVAs were performed to establish the effect of statins for each **NE** and **NG**, which also presented statin effects ($p<0.05$). Thereafter, Tukey's honest

297 significance difference (HSD) tests were utilised to establish pairwise variances between the
298 four statin means. However, a non-parametric Mann-Whitney U test was used to compare
299 the data of the statins in the **NEs** and **NGs** according to p-values. The same methods were
300 implemented during skin diffusion studies. Since, the interaction between the type of formula,
301 statins and skin layer indicated statistical significance ($p<0.05$) during analysis of tape
302 stripping; one-way ANOVAs were initiated, to establish the effect of the statins within the
303 specific formula with regards to the selected skin layer (SCE/ED).

304 **3 Results and Discussion**

305 **3.1 Formulation of nano-emulsions and nano-emulgels**

306 From the eight formulas, each contained 2% API, which consisted of four **NEs** and four **NGs**,
307 therefore two formulas per statin. The **NEs** were homogenous, translucent and presented
308 with low viscosity. However, the **NGs** obtained a higher viscosity, which could provide a more
309 effortless application. The **NGs** were formulated in an attempt to overcome the main limitation
310 of the **NEs**, which is regarded as the rheological properties of these obtained dispersions
311 (Chellapa *et al*., 2015).

312 **3.2 Characterisation of semi-solid formulations**

313 Obtained data concerning the pH, viscosity, droplet size and zeta-potential analysis are
314 summarised in Table 3.

315 Table 3:

316 Summary of the characteristics (given in median values) performed with the **NEs** and the **NGs**

317

318 Two aspects need to be considered concerning the pH of a formulation intended for
319 transdermal delivery. Firstly, skin pH is proposed to range between 4 and 7 (Ng & Lau, 2015);
320 however, it can be suggested that formulations intended for topical application should retain
321 pH value within this range, as a pH of under 3 or above 9 can affect the integrity of skin (Naik
322 *et al*., 2000). Secondly, dissociation of weak acids or weak basis (molecules) is established
323 by pH; consequently, the degree of ionisation. Regarding transdermal delivery, the ionisation

of a compound is of relevance, as this goal can be achieved more efficiently when the API is a unionised form (Li *et al*., 2012), mainly due to the lipophilic barrier posed by human skin (Williams, 2003). However, transdermal and topical delivery can enable effective permeation and diffusion when the API is unionised (Li *et al*., 2012; Williams, 2003). At a pH of 5, atorvastatin (28.475%), pitavastatin (33.386%) and pravastatin (33.386%) displayed low unionised species, except for mevastatin (99.999%). The zeta-potential of the **NGs** and the **NEs** were compared and found that the **NGs** indicated higher zeta-potential values opposed to their respective **NEs**, indicating that the **NGs** were considered more stable than their respective **NEs**. Since the zeta-potential values were higher than -30 mV (Eid *et al*., 2014; Silva *et al*., 2012), it is evident that the formulas (**NGs** and **NEs**) were well within the required range for acceptable stability. Zeta-potential values were less negative after the addition of the selected statins, since the statins induced a stronger anionic surface charge, resulting in decreased negative surface charges (Sari *et al*., 2015).

Comparing the median droplet sizes of the **NEs** and the **NGs**, it was detected that the median droplet sizes of the **NGs** were larger opposed to **NEs**. Therefore, by the addition of Carbopol® Ultrez 20 to produce the **NGs**, the droplet sizes of the **NGs** increased. This phenomenon can be ascribed to the enhanced formation of cross-links in **NGs** (Eid *et al*., 2014). When the median polydispersity-index (PdI) of the **NGs** were compared to the median PdI of the **NEs**, it was detected that the **NEs** had insignificantly higher PdI values, which indicated that the **NEs** were more polydispersed (Gaumet *et al*., 2008) compared to their **NGs** that were more homogenous, except for **NE-PRA**, which indicated a lower PdI value than **NG-PRA**. This occurrence correlates with literature, suggesting that formulations containing Carbopol® Ultrez 20 do not have significant PdI value variations (Eid *et al*., 2014). The **NGs** presented with significantly larger viscosity readings compared to the **NEs**, which supported literature that combining an optimal **NE** with a gelling agent enhances the water phase's viscosity, as it deceases interfacial and surface tension even more than the **NEs** (Chellapa, 2015). However, it should be mentioned that the **NGs** pH levels were neutralised (Lubrizol, 2009) with NaOH

to between 6 and 7, which increased the viscosity drastically. TEM results are illustrated in Fig. 1 to visually illustrate the droplet size and the morphology of the **NEs**.

Fig.1: TEM micrographs of: a) **NE-ATO**, b) **NE-MEV**, c) **NE-PIT** and d) **NE-PRA**

3.3 Membrane diffusion experiments

Table 4:

Median flux (µg/cm^2.h), median amount per area diffused (µg/cm^2) and median concentrations (µg/ml) in the SCE and ED of **NEs** and **NGs** that contain grapeseed oil in comparison to those containing apricot kernel oil

Fig. 2: Box-plots that indicates the mean and median flux (µg/cm^2.h) for each of the respective statins in the **NEs** and the **NGs**

Flux data was gathered during the membrane release studies to determine the release of the statins from the different formulas (**NEs** and **NGs**). Subsequently, it can be suggested that the API was released from the vehicle, which was followed by diffusion through the PVDF synthetic membranes. From the membrane release data, it was revealed that the **NE-PRA** obtained the highest median flux, followed by **NG-PRA**, **NG-PIT**, **NE-PIT**, **NG-ATO**, **NE-ATO**, **NE-MEV** and lastly, **NG-MEV**. **NE-PRA** and **NE-MEV** showed higher median flux values than there **NGs**-counterpart, whereas **NG-PIT** and **NG-ATO** indicated higher median flux values opposed to their **NEs**-counterpart.

Focusing on the physicochemical properties of selected statins, it was detected that atorvastatin (log D: 1.00-1.25 (Fong, 2016); aqueous solubility: 0.19 mg/ml (Rodde *et al*., 2014)) and pitavastatin (log D: 1.50 (Fong, 2016); aqueous solubility: 0.500 mg/ml (Cayman chemical, 2014)) had ideal log D values (between 1 and 3 (Naik *et al*., 2000)), but presented with poor aqueous solubility values, while mevastatin (log D: 3.570 (Kim *et al*., 2011); aqueous solubility: 0.032 mg/ml (Drugbank, 2005c)) and pravastatin (log D: -0.47 (Fong, 2016);

aqueous solubility: 10.000 mg/ml (Cayman chemical, 2015)) were considered the most lipophilic and hydrophilic statins, respectively during this study. Therefore, the API, more than the type of formula e.g. **NEs** or **NGs**, may have significantly affected the release of the API from the formula. However, it can be mentioned that all the statins were effectively released from the nano-formulas (**NEs** and **NGs**) during the membrane release studies.

According to previous studies, **NEs** and **NGs** were also formulated containing 2% statins (atorvastatin, mevastatin, pitavastatin and pravastatin), although a different oil, e.g. apricot kernel oil was used (Marais, 2018; Maree, 2018). According to Table 4, the median flux values for the nano-formulas (**NEs** and **NGs**) containing grapeseed oil were significantly higher than those of the nano-formulas (**NEs** and **NGs**) containing apricot kernel oil (except for the **NE-MEV** and the **NG-PRA**). Thus, it can be considered that grapeseed oil used during this study influenced the API release more than apricot kernel oil from previous studies.

3.4 Skin diffusion experiment

Fig. 3: Box-plots indicating the mean and median amount per area diffused (µg/cm^2) for each of the selected statins in the **NEs** and the **NGs**

It is of importance to convey median values as the true concentration, which is unaffected by outliers, since the mean values can be affected by skewed distribution and ultimately lead to inaccurate estimation of the true concentration values (Dawson & Trapp, 2004). When the nano-formulas (**NEs** and **NGs**) were compared in terms of the median amount per area diffused (µg/cm^2), it was discovered that **NG-PIT** obtained the highest median amount per area diffused, followed by **NE-PIT**, **NG-PRA**, **NE-PRA**, **NG-ATO**, **NE-ATO**, **NG-MEV** and lastly, **NE-MEV**. After comparing the **NEs** with the **NGs**, it was noticed that the **NGs** had the higher median amount per area diffused values than the **NEs**, which supports literature, as high solubilisation capacity (characteristic of **NEs**) in conjunction with the enhanced skin adhesion of these semi-solids can cause skin permeation to increase (Eid *et al*., 2014). When comparing the statins, it was noted that pitavastatin attained the highest median amount per

area diffused (which may be attributed to the ideal log D required for transdermal diffusion (Naik *et al*., 2000; Subedi *et al*., 2010)), followed by pravastatin (very hydrophilic statin with high aqueous solubility (Naik *et al*. 2000)), atorvastatin (has an ideal log D needed for transdermal diffusion (Naik *et al*., 2000; Subedi *et al*., 2010), but has a very low aqueous solubility (Rodde *et al*., 2014) and high molecular weight) and lastly, mevastatin (could be ascribed to the extreme lipophilic nature of mevastatin compared to other statins, and possibly to the high affinity lipophilic APIs have for the stratum corneum, which may limit deeper penetration of such APIs (Schulz *et al*., 2017)). The fact that all the selected statins diffused through the skin, despite the limitations presented by the physiochemical properties of these APIs, could be attributed to the **NEs** as vehicles, as these dispersions has the capacity to dissolve both hydrophilic and lipophilic APIs and increase skin permeation (Elmataeeshy *et al*., 2018). It can also be mentioned that the excipients used in **NEs**, such as grapeseed oil or the surfactant, can ultimately enhance skin permeability, consequently assisting transdermal delivery (Aparna *et al*., 2015). Utilising **NGs** and **NEs** can facilitate transdermal delivery of molecules that would normally not penetrate the skin barrier (Gadkari *et al*., 2018).

When previous formulated **NEs** and **NGs** containing statins (2%) and apricot kernel oil (Marais, 2018; Maree, 2018) were compared to those formulated in the study with grapeseed oil, it was observed that the median amount per area diffused for the nano-formulas (**NEs** and **NGs**) containing grapeseed oil attained higher median amount per area diffused values than those of the nano-formulas (**NEs** and **NGs**) containing apricot kernel oil (except for the **NE-MEV** together with the **NG-MEV** and **NG-PRA**). Generally, it can be stated that the grapeseed oil improved the transdermal delivery of the API more than apricot kernel oil.

Table 5:

The concentration (µg/ml) of the selected statins within the respective formulas that diffused through the skin after 12 h, together with the plasma concentrations (µg/ml)

From Table 5, it is evident that the selected statins obtained higher concentrations within the receptor phase compared to the mean plasma concentrations after oral administration. However, this could not be determined for the formulas containing mevastatin, as not all mean plasma concentrations could be obtained from literature. Conclusively, it can be suggested that the atorvastatin, pitavastatin and pravastatin formulas may obtain significant therapeutic concentrations after initiating transdermal delivery.

3.5 Tape stripping

3.5.1 Stratum corneum-epidermis

Fig. 4: Box-plots indicating the mean and median concentration (µg/ml) of the **NEs** and the **NGs** for each of the selected statins present in the SCE

Obtained results concerning tape stripping are illustrated in Fig. 4 and illustrates that the **NEs** and **NGs** accumulated in the SCE, except **NG-MEV**. From the obtained results for median statin concentration within the SCE, it can be stated that **NE-ATO** obtained the highest median concentration, followed by **NE-PIT**, **NG-PIT**, **NE-PRA**, **NE-MEV**, **NG-PRA**, and lastly, **NG-ATO**. It can be observed that the **NGs** (**NG-PIT**, **NG-PRA** and **NG-ATO**) had lower median concentration values compared to their **NE**-counterpart. Literature proposed that **NGs** should obtain higher concentrations within SCE, since the addition of the gelling agent can enhance the affinity regarding the formulation for the stratum corneum (Zheng *et al*., 2016), this was not observed. Hence, in Table 3 the droplet sizes of the nano-formulas (**NEs** and **NGs**) are displayed, it is apparent that the **NGs** obtained larger droplet sizes than their respective **NEs**. Since the **NEs** have smaller droplets, they may have enhanced permeation (into the stratum corneum) due to smaller droplets having larger surface areas, which may have caused the APIs to the accumulate in this region (Arora, 2014).

After comparing the selected statins in regards to the nano-formulas (**NEs** and **NGs**), it was observed that the ranking of the statins were not the same for the different nano-formulas (as

458 seen during the membrane release and skin diffusion studies). Therefore, it can be concluded
459 that the type of formula, rather than the API had an influence on delivery into the SCE.
460 Comparing results of previous studies (**NEs** and **NGs** containing apricot kernel oil with statins
461 (2%)) performed by Maree (2018) and Marais (2018) with this study (**NEs** and **NGs** containing
462 grapeseed oil with statins (2%)), it was noticed that the median concentration values for the
463 nano-formulas (**NEs** and **NGs**) containing grapeseed oil accomplished higher values than
464 those of the nano-formulas (**NEs** and **NGs**) containing apricot kernel oil (except for the **NE-**
465 **MEV** together with the **NG-MEV**, **NG-ATO** and **NG-PRA**). Generally, it can be stated that the
466 grapeseed oil improved the delivery of the statins from the **NEs** more than the apricot kernel
467 oil or the **NGs** (see Table 4).

468 **3.5.2 Epidermis-dermis**

469

470 Fig. 5: Box-plots indicating the mean and median concentration (µg/ml) of the **NEs** and the
471 **NGs** for each of the selected statins present in the ED

472 Observed from Fig. 5, the median concentration obtained in the ED from highest to lowest
473 were as follows: **NE-ATO**, **NG-PIT**, **NE-PIT**, **NE-PRA**, **NG-MEV**, **NG-ATO**, **NG-PRA**, and
474 lastly, **NE-MEV**. **NE-ATO** and **NE-PRA** had higher median concentrations than their **NGs**-
475 counterpart, while **NG-PIT** and **NG-MEV** presented with higher median concentrations than
476 their **NEs**-counterpart.
477 The ranking of the statin delivery into the ED were not the same for the **NEs** when compared
478 to their respective **NGs**. Therefore, it can be concluded that neither the type of formula nor
479 the API had an influence on the delivery of the statins into the ED, yet all statins were
480 successfully delivered from the nano-formulas (**NEs** and **NGs**) into the ED.
481 Additionally, median concentration values in the ED were also compared with regards to
482 grapeseed oil and apricot kernel oil (for **NEs** and **NGs** containing 2% statin). As a result, the
483 median concentration for the nano-formulas (**NEs** and **NGs**) containing grapeseed oil obtained
484 higher median concentration values than those of the nano-formulas (**NEs** and **NGs**)

containing apricot kernel oil (except for the **NE-MEV** and **NG-PRA** as observed in Table 4. Finally, it can be concluded that the grapeseed oil enhanced the delivery of the statins into the ED more than apricot oil.

4 Conclusion

Through characterisation, an optimised formula for the **NEs** was selected. Afterwards **NGs** were formulated and characterised by using the optimised formula of the **NEs**. It was evident that all the formulas (**NEs** or **NGs**) were successful in releasing their respective statins during the membrane release studies. Hence, **NEs** and **NGs** followed the same sequence when releasing the statins.

It was observed when looking at the **NEs** that atorvastatin was the API with the highest topical (in both the SCE and ED) drug delivery, which followed by pitavastatin, pravastatin and lastly, mevastatin. However, this particular trend was not followed transdermally for the **NEs** with the exception for mevastatin, which had the lowest API delivery of all the **NEs**. Conclusively, **NE-MEV** had the smallest amount of API penetration through topical and transdermal delivery. This could possibly be due to the poor release of mevastatin from the formulation during the membrane release studies or due to the poor physicochemical properties (low aqueous solubility and high log D) of mevastatin (Drugbank, 2005c; Kim *et al.,* 2011; Naik *et al*., 2000). Literature has suggested that mevastatin, due to its high lipophilicity has the potential to convey a higher affinity for the stratum corneum (Förster *et al*., 2009), and should enable effortless penetration to this layer (Sugibayashi, 2017). However, this theory was not observed during this study and it can therefore be suggested that the lipophilic mevastatin rather had a greater affinity towards the grapeseed oil in the **NE** than the suggested lipophilic stratum corneum, which could have influenced the release of the API from the formulation, and successively decrease skin diffusion (Haque & Talukder, 2018).

Although atorvastatin (**NE-ATO**) had the best topical (SCE and ED) delivery of all the **NEs** and also possessed an ideal log D (Fong, 2016), its molecular weight (Al-Adl *et al*., 2017) and aqueous solubility (Rodde *et al.,* 2014) was very poor (Naik *et al*., 2000) and could therefore

hinder transdermal delivery. Nevertheless, it can be proposed that atorvastatin accumulated in the SCE and ED (compared to data obtained from diffusion studies) due to its undesirably low aqueous solubility, which delayed it from penetrating deeper into the hydrophilic regions of these skin layers.

It can be concluded that **NE-PIT** obtained the best transdermal delivery and the second highest topical (SCE and ED) delivery of all the aforementioned **NEs**, which may be due to the ideal log D (Fong, 2016) and moderate aqueous solubility (Cayman chemical, 2014) of pitavastatin.

It was detected that **NE-PRA** attained the second best transdermal delivery out of all the **NEs**, however when the topical (SCE and ED) data was analysed, it was perceived that pravastatin was not one of the top two statins to be delivered topically. Hence, this occurrence could be explained due to the effective release of pravastatin from the **NE** and the high aqueous solubility (Cayman chemical, 2015), which may have led to a high concentration gradient, therefore, acting as a driving force to enable the statin to reach systemic circulation (Chellapa, 2015).

Concerning **NGs**, it was evident that **NG-PIT** and **NG-ATO** had the best and third best topical (SCE and ED) delivery, respectively (the same trend was followed transdermally). Hence, from the topical and transdermal data, it was also observed that the **NG** promoted the delivery of pitavastatin not only into the systemic circulation (as observed with the **NEs**), but also into the skin layers, albeit pitavastatin having an ideal log D and less ideal aqueous solubility (Cayman chemical, 2014).

It was also noted that no **NG-MEV** was delivered in the SCE layers. Nonetheless, when data were observed, the **NG** promoted the delivery of mevastatin to the ED (and it appears that the **NG** targeted the ED for mevastatin, as mevastatin was once again the least systemically delivered of all the respective statins for the **NG**).

Pravastatin regarding **NGs** showed effective penetration for the SCE (second best delivery), but had the weakest delivery to the ED, followed by the second best transdermal delivery of

all the aforementioned statins. Thus, it seems that the **NG** sustained the hydrophilic pravastatin from passing through the lipophilic SCE and expressing a higher affinity for the hydrophilic region (Jankowski *et al*., 2017); more specifically the systemic circulation (more than the hydrophilic ED).

After analysing transdermal and topical data, it can be concluded that **NEs** and **NGs** enhanced statin delivery. The **NEs** showed higher statin concentrations in the SCE than their **NGs**-counterpart, while the **NGs** were more transdermally targeted at delivering the statins than their **NEs**-counterpart. **NGs** resulting with lower median concentrations in the SCE can be attributed to the addition of a gelling-agent (Carbopol® Ultrez 20). This gelling agent causes swelling of the **NE** to form the **NG** (which will release an API in a more controlled manner (Chellapa *et al*., 2015) and produce formulations with higher viscosity (Mahalingam *et al*., 2008; Mitsui, 1997), which ultimately enhances API permeation through the skin (Eid *et al*., 2014).

Overall, the **NEs** and **NGs** formulas containing grapeseed oil utilised during this study resulted in better transdermal delivery and topical (SCE and ED) of the statins than from the **NEs** and **NGs** containing apricot oil utilised during previous studies (Marais, 2018; Maree 2018). Grapeseed oil contains 15.8% oleic acid and 69.6% linoleic acid (AL-Bayati & Enaad, 2015) opposed to apricot oil contains 73.4% oleic acid and 31.7% linoleic acid (Alpaslan & Hayta, 2006). Hence, larger concentration of linoleic acid (C18:2 ω-6) (Rustan *et al*., 2001) in grapeseed oil is detected opposed to apricot kernel oil; however, apricot oil contains a larger concentration of oleic acid (C18:1 ω-9) (Rustan *et al*., 2001) compared to grapeseed oil. It has been proven that longer chain fatty acids increase percutaneous drug absorption, where unsaturated C_{18} chain fatty acids are also considered near to ideal (Cho *et al*., 2009; Williams & Barry, 2012). The higher linoleic concentrations may explain why grapeseed oil had better enhancing qualities, since it forms part of the basic skin structure and may have encountered less hindrance from the skin barrier (Hopkins, 2012; McCusker *et al.,* 2010; Suhonen *et al*., 1999). According to Choi *et al*. (2009), it was found that an increase in double bonds of these

essential fatty acids, such as linoleic acid (C18:2) can result in a significant decrease in skin permeation of a lipophilic compound (such as mevastatin). Mevastatin is highly lipophilic (Kim *et al*., 2011) as established; therefore, it can be proposed that formulations that contain more oleic acid (C18:1 ω-9) (mono-unsaturated fatty acid) like apricot oil will be more preferable for lipophilic APIs, like mevastatin when choosing an oil phase for the **NEs** and **NGs**.

Transdermal concentrations (obtained in study) and statin plasma concentrations after oral administration (found in literature) were compared, it was found that each formula (**NEs** and **NGs**) containing the statin presented with significantly higher concentrations in the receptor phase than the plasma concentrations after oral administration. Hence, transdermal delivery of statins may lead to therapeutic concentrations.

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Conflict of Interest

The authors declare no conflict of interest.

590 **References**

591 Al-Adl, S.M., Abdel-Aziz, L.M., Mohamed, M.A.M. 2017. Spectrophotometric determination
592 of atorvastatin calcium and rosuvastatin calcium in bulk and dosage form using P-
593 Dimethylaminobenzaldehyde. *Journal of applied pharmacy*, 9(1):1-7.

594 AL-Bayati, A.J. & Enaad, D.F. 2015. Histopathological study and surgery the effect of grape
595 seed oil on wound healing in rabbits. *International journal of science and research*, 4(6):959-
596 962.

597 Aparna, C., Srinivas, P. & Rao Patnaik, K.S.K. 2015. Enhanced transdermal permeability of
598 telmisartan by a novel nanoemulsion gel. *International journal of pharmaceutical sciences*,
599 7(4):335-342.

600 Arora, R., Aggarwal, G., Harikumar, S.L., Kaur, K. 2014. Nanoemulsion based hydrogel for
601 enhanced transdermal delivery of ketoprofen. *Advanced in pharmaceutics*, 2(1):1-12.

602 Barry, B.W. 1983. Dermatological formulations: percutaneous absorption. New York: Marcel
603 Dekker. pp. 480.

604 Baert, B., Vansteelandt, S. & De Spiegeleer, B. 2011. Ion mobility spectrometry as a high
605 throughput technique for *in vitro* transdermal Franz diffusion cell experiments of ibuprofen.
606 *Journal of pharmaceutical biomedicine*, 55:472-473.

607 Begur, M., Pai, V.K., Gowda, D.V., Srivastava, A., Raghundan, H.V., Shinde, C.G., Manusri
608 N. 2015. Enhanced permeability of Cyclosporine from a transdermally applied nanoemulgel.
609 *Der pharmacia sinica*, 6(2):69-79.

610 Boglarka, B., Gabor, V., Szilvia, B., Maria, B., Andras, K., Balint, S., Krisztina, T., Piroska, S.
611 & Erzsebet, C. 2016. Investigation of the efficacy of transdermal penetration enhancers
612 through the use of human skin and a skin mimic artificial membrane. *Journal of*
613 *pharmaceutical sciences*, 105:1134-1140.

614 Braamskamp, M.J., Wijburg, F.A. & Wiegman, A. 2012. Drug therapy of
615 hypercholesterolaemia in children and adolescents. *Drugs*, 72:759-72.

Burger, C. 2014. Formulation, *in vitro* release and transdermal diffusion of pravastatin by the implementation of the delivery gap principle. Potchefstroom: NWU. (Dissertation-MSc).

Büyüktimkin, N., Büyüktimkin, S. & Rytting, J.H. 1997. Chemical means of drug permeation enhancement. (*In* Ghosh, T.K., Pfister, W.R. & Yum, S.I.I., *ed*. Transdermal and topical drug delivery systems. Buffalo Grove: Interpharm Press. p. 357-475.)

Cayman chemical. 2014. Pitavastatin calcium salt (Product information 15414) https://www.caymanchem.com/pdfs/15414.pdf Date of access: 18 Jan. 2019.

Cayman chemical. 2015. Pravastatin (sodium salt) (Material safety data sheet). https://www.caymanchem.com/msdss/10010343m.pdf Date of access: 18 Feb. 2019.

Chaudhary, S., Verma, N.K., Panda, P., Singh, A.P. & Alam, G. 2018. Nanoemulsion based drug delivery-a review. *European journal of pharmaceutical and medical research*, 5(6):178-191.

Chellapa, P., Mohamed, A.T., Keleb, E.I., Elmahgoubi, A., Eid, A.M., Issa, Y.S. & Elmarzugi, N.A. 2015. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR journal of pharmacy*, 5:43-47.

Čižinauskas, V., Elie, N., Brunelle, A. & Briedis, V. 2017. Skin penetration enhancement by natural oils for dihydroquercetin delivery. *Molecules*, 22(9):1536.

Clarke, E.G.C., Moffat, A.C., Osselton, M.D. & Widdop, B. 2011. Clarke`s analysis of drugs and poisons in pharmaceuticals, body fluids and postmortem material. 4th *ed*. Pharmaceutical Press. p. 1947.

Concato, J. & Hartigan, J.A. 2016. P-values: from suggestion to superstition. *Journal of investigative medicine*, 64:1166-1171.

Dawson, B. & Trapp, R.G. 2004. Basic & clinical biostatistics. 4th *ed*. New York: McGraw-Hill. p. 30, 38-39.

DeGorter, M.K., Tirona, R.G., Schwarz, U.I., Choi, Y., Dresser, G.K., Suskin, N., Myers, K., Zou, G.Y., Iwuchukwu, O., Wei, W., Wilke, R.A., Hegele, R.A., Kim, R.B. 2013. Clinical and

642 pharmacogenetic predictors of circulating atorvastatin and rosuvastatin concentrations in
643 routine clinical care. *Circulation cardiovascular genetics*, 6:400-408.
644 Dodou, K. 2012. Developments in transdermal delivery open the pores to a new range of
645 712 drugs. *Pharmaceutical journal*, 288:144.
646 Drugbank. 2005. https://www.drugbank.ca/drugs/DB06693. Date of access: 20 May 2019.
647 Ee, S.L., Duan, X., Liew, J. & Nguyen, Q.D. 2008. Droplet size and stability of nano emulsions
648 produced by the temperature phase inversion method. *Chemical Engineering Journal*,
649 140:626-631.
650 Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization
651 and anti-inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of*
652 *nanomedicine & nanotechnology*, 5(2):1-10.
653 Elmataeeshy, M.A., Sokar, M.S., Bahey-El-Din, M. Shaker, D.S. 2018. Enhanced
654 transdermal permeability of terbinafine through novel nanoemulgel formulation; Development,
655 *in vitro* and *in vivo* characterization. *Future Journal of Pharmaceutical Sciences*, 4:18-28.
656 Escobar-Chávez, J.J., Rodríguez-Cruz, I.M., Domínguez-Delgado, C.L., Díaz-Torres, R.,
657 Revilla-Vázquez,A.L. & Aléncaster, N.C. 2012. Nanocarrier systems for transdermal drug
658 delivery. (*In* Sezer, A.D., *ed*. Recent advances in novel drug carrier systems. Intech, p201-
659 240.) https://www.intechopen.com/books/recent-advances-in-novel-drug-carrier-
660 systems/nanocarriersystems-for-transdermal-drug-delivery. Date of access: 4 Nov. 2019.
661 Fiori, L., Solana, M., Guella, G., Strim, C. & Tosi, P. 2010. Grape seed oil: Supercritical
662 extraction, chemical analysis and fractionation. 12th euro meet supercritical fluids, Graz
663 (Austria).
664 Fong, C. 2016. Statins in therapy: Cellular transport, side effects, drug-drug interactions and
665 cytotoxicity –the unrecognized role of lactones https://hal.archives-ouvertes.fr/hal-
666 01185910v2 Date of access: 12 Apr. 2019.
667 Forslind, B., Engstrom, S., Engblom, J. & Norlen, L. 1995. A novel approach to the

understanding of the human skin barrier function. *Journal of dermatological science* 14:117,118.

Gabera, M., Medhata, W., Hanya, M., Sahera, N., Fang, J., Elzoghby, A. 2017. Protein-lipid nanohybrids as emerging platforms for drug and gene delivery: Challenges and outcomes. *Journal of controlled release*, 254:75-91.

Gadkari, P.N., Patil, P.B. & Saudagar, R.B. 2018. A review on nanoemulsion based gel. *European journal of biomedical and pharmaceutical sciences*, 5:914-921.

Gaumet, M., Vargas, A., Gurny, R. & Delie, F. 2008. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, 69:1-9.

Haque, T. & Taluker, M.U. 2018. Chemical Enhancer: A simplistic way to modulate barrier function of the stratum corneum. *Advanced pharmaceutical bulletin*, 8(2):169-179.

Jankowski, A., Dya, R. & Sarecka-hujar, B. 2017. Dermal and transdermal delivery of active substances from semisolid bases. *Indian journal of pharmaceutical sciences*, 79(4):488-500.

Kala, S., Juyal, D. 2018. Recent developments on natural transdermal penetration enhancers. *International journal of pharmaceutical sciences and research*, 9(6):2190-2196.

Khandavilli, S. & Panchagnula, R. 2006. Studies of the skin permeation of lipophilic drugs: paclitaxel. *Pharmazie*, 6:471-473.

Kim, M.C., Ahn, Y., Jang, S.Y., Cho, K.H., Hwang, S.H., Lee, M.G., Ko, J.S., Park, K.H., Sim, D.S., Yoon, N.S., Yoon, H.J., Kim, K.H., Hong, T.J., Park, H.W., Kim, J.H., Jeong, M.H., Cho, J.G., Park, J.C. & Kang, J.C. 2011. Comparison of clinical outcomes of hydrophilic and lipophilic statins in patients with acute myocardial infarction. *The Korean journal of internal medicine*, 26:294-303.

Li, W., Zhou, J. & Xu, Y. 2015. Study of the *in vitro* cytotoxicity testing of medical devices (Review). *Biomedical reports*, 3:617-620.

Lubrizol. 2009. Viscosity of Carbopol® Ultrez 20 polymers in aqueous systems. http://www.lubrizol.com>lubrizol>Techicaldatasheet Date of access: 4 Jun. 2019.

Mahalingam, R., Li, X. & Jasti, B.R. 2008. Semisolid dosages: ointments, creams and gels. (*In* Gad, S.C., *ed*. Pharmaceutical manufacturing handbook: production and processes. New Jersey: John Wiley & Sons. p. 267-309).

Marais, S. 2018. The use of apricot oil emulsions for the transdermal delivery of selected statins. Potchefstroom: NWU. (Dissertation-MSc).

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

McKenney, J.M. Davidson, M.H. Jacobson, T.A. Guyton, J.R. 2006. National lipid association statin safety assessment task force. *American journal of cardiology*, 97(8A):89C-94C.

Mitsui, T. 1997. Polymers. (*In* Mitsui, T., *ed*. New cosmetic science. Netherlands: Elsevier. p. 138-140).

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

Nair, A., Jacob, S., Al-Dhubiab, B., Attimarad, M. & Harsha, S. 2013. Basic considerations in the dermatokinetics of topical formulations. *Brazilian journal of pharmaceutical sciences*, 49(3):423-434.

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

Ng, S-F., Rouse, J.J., Sanderson, D. & Eccleston, G. 2010a. A comparative study of transmembrane diffusion and permeation of ibuprofen across synthetic membranes using Franz diffusion cells. *Pharmaceutics*, 2:209-223.

OECD (Organisation for Economic Co-operation and Development). 2004. Guidance document for the conduct of skin absorption studies: OECD series on testing and assessment, No. 28. https://www.oecd-ilibrary.org/docserver/9789264078796en.pdf?expires=1541172508&id=id&accname=guest&checksum=796E77B02778DA73DADCC9E4C1EBB306 Date of access: 1 Nov. 2019.

721 Pellet, M.S., Roberts, M. & Hadgraft, J. 1997. Supersaturated solutions evaluated with an *in*
722 *vitro* stratum corneum tape-stripping technique. *International journal of pharmaceutics*,
723 151(1):91-98.
724 Pubchem. 2005a. https://pubchem.ncbi.nlm.nih.gov/compound/53232. Date of access: 5
725 Nov. 2019.
726 Ramkumar, A., Raghunath, A. & Raghunath, S. 2016. Statin therapy: review of safety and
727 potential side effects. *Acta cardiologica sinica*, 32:631-639.
728 Rodde, M.S., Divase, G.T., Devkar, T.B., Tekade, A.R. 2014. Solubility and bioavailability
729 enhancement of poorly aqueous soluble atorvastatin: *in vitro*, *ex vivo*, and *in vivo* studies.
730 *BioMed research international*, 3(2):1-11.
731 Sari, T.P., Mann, B., Kumar, R., Singh, R.R.B., Sharma, R., Bhardwaj, M. & Athira, S., 2015.
732 Preparation and characterization of nanoemulsion encapsulating curcumin. *Food*
733 *Hydrocolloids*, 43:540-546.
734 Schachter, M. 2005. Chemical, pharmacokinetic and pharmacodynamic properties of statins:
735 an update. *Fundamental & clinical pharmacology*, 19(1):117-125.
736 Schierwagen, R., Trebicka, J., Uschner, F.E., Magdaleno, F., Klein, S. 2017. Rationale for
737 the use of statins in liver disease. *American journal of physiology gastrointestinal and liver*
738 *physiology*, 312:G407-G412.
739 Schulz, R., Yamamoto, K., Klossek, A., Flesh, R., Hönzke, S., Rancan, F., Vogt, A., Blume
740 Peytavi, U., Hedtrich, S., Schäfer-Korting, M., Rühl, E. Netz, R. 2017. Data-based modeling
741 of drug penetration relates human skin barrier function to the interplay of diffusivity and free
742 energy profiles. Proceedings of the national academy of sciences of the united states of
743 america, 114(14):3631-3636.
744 Serajuddin, A.T.M. 2007. Salt formation to improve drug solubility. *Advanced drug delivery*
745 *reviews*, 59:603.

746 Shin, S., Kim, Y. & Oh, I. 2000. Mucoadhesive and physicochemical characterization of
747 carbopol-poloxamer gels containing triamcinolone acetonide. *Drug development and*
748 *industrial pharmacy*, 26(3):307-312.
749 Silva, H.D., Cerqueira, M.A., Vicente, A.A. 2012. Nanoemulsions for food applications:
750 development and characterization. *Food and bioprocess technology*, 5:854-867
751 Su, R., Fan, W., Yu, Q., Dong, X., Qi, J., Zhu, Q., Zhoa, W., Wu, W., Chen, Z., Li, Y. & Lu, Y.
752 2017. Size-dependent penetration of nanoemulsions into epidermis and hair follicles:
753 implications for transdermal delivery and immunization. *Oncotarget*, 8(24):38214-38226.
754 Subedi, R.K., Oh, S.Y., Chun, M. & Choi, H. 2010. Recent advantages in transdermal drug
755 delivery. *Archives of pharmacal research*, 33:339-351.
756 Sugibayashi, K. 2017. Skin permeation and disposition of therapeutic and cosmeceutical
757 compounds: Springer. p. 428.
758 Szunerits, S. & Boukherroub, R. 2018. Heat: a highly efficient skin enhancer for transdermal
759 drug delivery. *Frontiers in bioengineering and biotechnology*, 6:1-13.
760 Therapeutic goods administration. 2013 Australian public assessment report for pitavastatin.
761 https://www.tga.gov.au/sites/default/files/auspar-pitavastatin-130902.docx. Date of access:
762 15 Feb. 2019.
763 Vermaak, I., Kamatou, G.P.P., Komane-Mofokeng, B., Viljoen, A.M. & Beckett, K. 2011.
764 African seed oils of commercial importance: cosmetic application. *South African journal of*
765 *botany*, 77:920-933.
766 Wiechers, J.W. 2008. The influence of emollients on skin penetration from emulsions. (*In*
767 Wiechers, J.W., *ed*. Science and applications of skin delivery systems. Carol Stream, IL:
768 Allured. p. 94, 95.)
769 Williams, A.C. 2003. Structure and function of human skin. (*In* Williams, A.C., *ed*.
770 Transdermal and topical drug delivery: from theory to clinical practice. London:
771 Pharmaceutical Press. p. 1-26).

772 Wiegman, A., Gidding, S.S., Watts, G.F., Chapman, M.J., Ginsberg, H.N., Cuchel, M., Ose,
773 L., Averna, M., Boileau, C., Borén, J. & Bruckert, E., 2015. Familial hypercholesterolaemia
774 in children and adolescents: gaining decades of life by optimizing detection and treatment.
775 *European heart journal*, 36(36):2425-2437.
776 Yoshida, M., Oliveira, M., Gomes, E., Mussel, W., Castro, W. & Soares, C. 2011. Thermal
777 characterization of lovastatin in pharmaceutical formulations. *Journal of thermal analysis and*
778 *calorimetry*, 106(3):657-664.
779 Zheng, Y., Ouyang, W-Q., Wei, Y-P., Syed, S.F., Hao, C-S., Wang, B-Z. & Shang, Y-H. 2016.
780 Effects of Carbopol® 934 proportion on nanoemulsion gel for topical and transdermal drug
781 delivery: a skin permeation study. *International journal of nanomedicine*, 11:5971-5987.
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Tables

Table 1:

Limit of detection (LOD) and lowest limit of quantification (LLOQ) of the statins

Statins	Atorvastatin	Mevastatin	Pitavastatin	Pravastatin
LOD (µg/ml)	3.47	2.80	6.15	3.47
LLOQ (µg/ml)	14.87	13.50	22.37	13.75

789 Table 2:

790 Ingredients used during the formulation of the different **NEs** and **NGs**

	NE	%m/m	NG	%m/m
Phase A	Statin	2.0%	Statin	2.0%
	Grapeseed oil	6.0%	Grapeseed oil	6.0%
	Span® 60	6.0%	Span® 60	6.0%
Phase B	Milli-Q® water	80.0%	Milli-Q® water	80.0%
	Tween® 80	6.0%	Tween® 80	6.0%
			Carbopol® Ultrez 20	0.46%

791

792

793 Table 3:

794 Summary of the characteristics (given in median values) performed with the **NEs** and the **NGs**

	pH	Zeta-potential (mV)	Droplet size (nm)	Viscosity (cP)
NE-ATO	6.13	-35.00	145.52	3.96
NG-ATO	5.45	-57.10	146.15	2322.21
NE-MEV	6.30	-39.50	144.30	3.14
NG-MEV	6.17	-49.80	183.14	1270.12
NE-PIT	6.33	-35.20	149.60	4.08
NG-PIT	5.06	-56.40	200.98	1638.03
NE-PRA	6.41	-40.30	148.21	3.74
NG-PRA	5.10	-43.10	233.07	10987.03

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796

797 Table 4:

798 Median flux (µg/cm^2.h), median amount per area diffused (µg/cm^2) and median concentrations

799 (µg/ml) in the SCE and ED of **NEs** and **NGs** that contain grapeseed oil in comparison to those

800 containing apricot kernel oil

	NE-ATO	NG-ATO	NE-MEV	NG-MEV	NE-PIT	NG-PIT	NE-PRA	NG-PRA
Median flux (µg/cm^2.h)								
Grapeseed oil	116.98	158.52	11.64	6.11	197.07	259.64	911.23	385.38
Apricot oil	102.98[a]	101.20[a]	18.97[b]	4.87[b]	150.39[a]	107.34[a]	93.40[a]	393.35[a]
Median amount per area diffused (µg/cm^2)								
Grapeseed oil	0.18	0.32	0.14	0.15	1.10	9.75	0.33	0.80
Apricot oil	0.05[a]	0.07[a]	0.48[b]	0.85[b]	0.39[a]	0.72[a]	0.28[a]	1.14[a]
SCE median concentration (µg/ml)								
Grapeseed oil	8.01	0.56	0.92	-	5.67	2.72	1.26	0.82
Apricot oil	7.09[a]	0.72[a]	1.87[b]	1.42[b]	1.67[a]	1.22[a]	1.05[a]	1.51[a]
ED median concentration (µg/ml)								
Grapeseed oil	32.72	5.728	0.77	11.76	18.68	19.16	14.37	1.44
Apricot oil	24.55[a]	3.82[a]	1.34[b]	3.43[b]	6.48[a]	9.77[a]	2.47[a]	1.84[a]

801 a) Maree, 2018, b) Marais, 2018

802

803 Table 5:

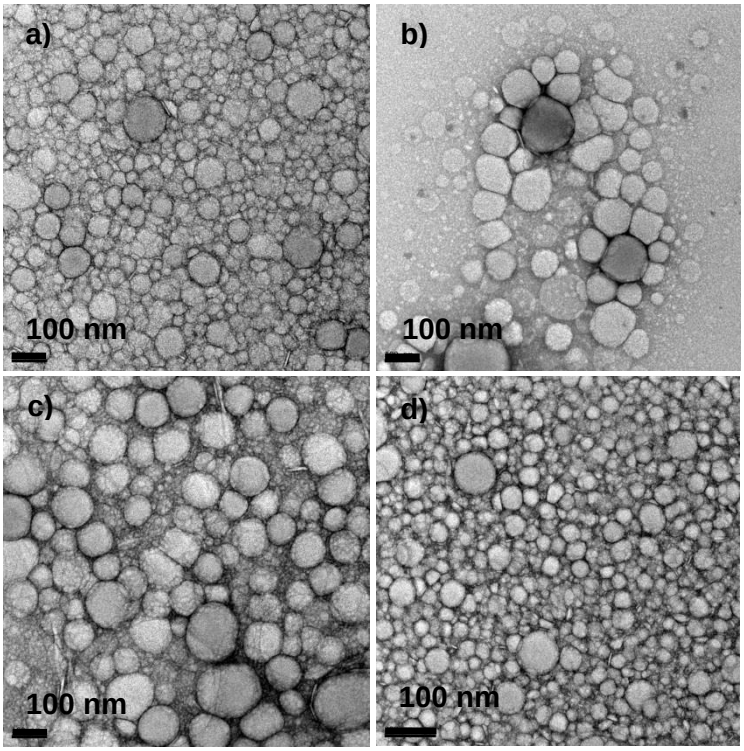
804 The concentration (µg/ml) of the selected statins within the respective formulas that diffused
805 through the skin after 12 h, together with the plasma concentrations (µg/ml)

	Concentration (µg/ml)
Atorvastatin plasma concentration (oral)	0.0009[a]
NE-ATO	0.1790
NG-ATO	0.9930
Pitavastatin plasma concentration (oral)	0.3000[b]
NE-PIT	1.2180
NG-PIT	12.1540
Pravastatin plasma concentration (oral)	0.0641[c]
NE-PRA	0.3510
NG-PRA	0.9150

806 a) DeGorter *et al*., 2013, b) Routes, 2004, c) Therapeutic goods administration, 2013, d)
807 Clarke *et al*., 2011

808

809 **Figures**



810

811 Fig. 1: TEM micrographs of: a) **NE-ATO**, b) **NE-MEV**, c) **NE-PIT** and d) **NE-PRA**

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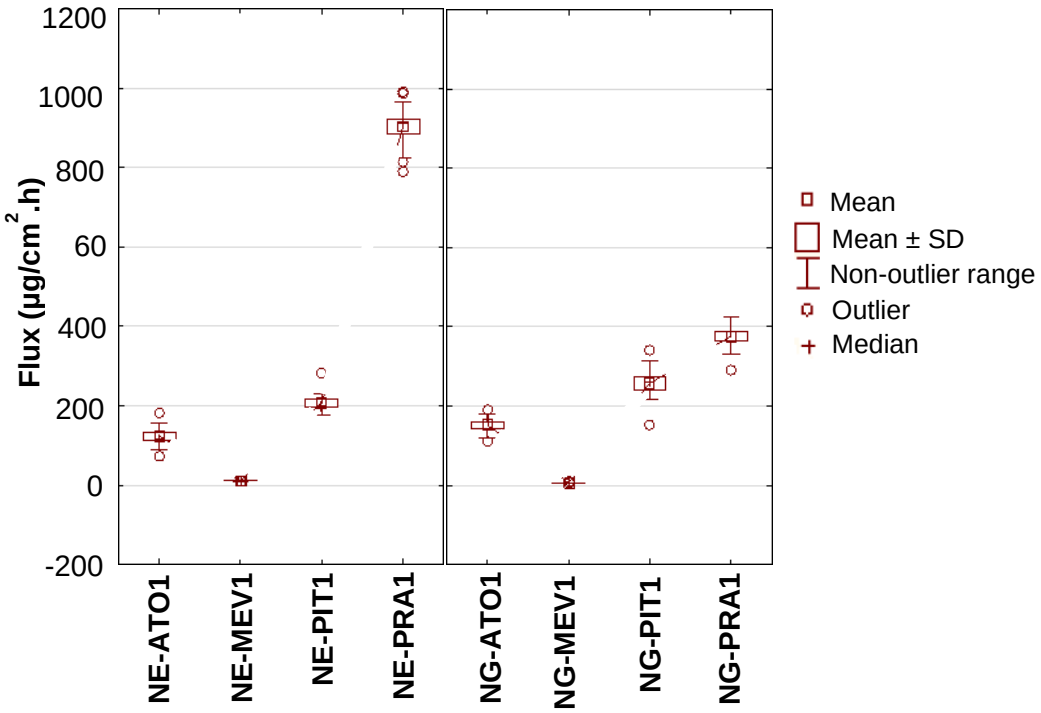

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814 Fig. 2: Box-plots indicating the mean and median flux (µg/cm^2.h) for each of the selected

815 statins in the **NEs** and the **NGs**

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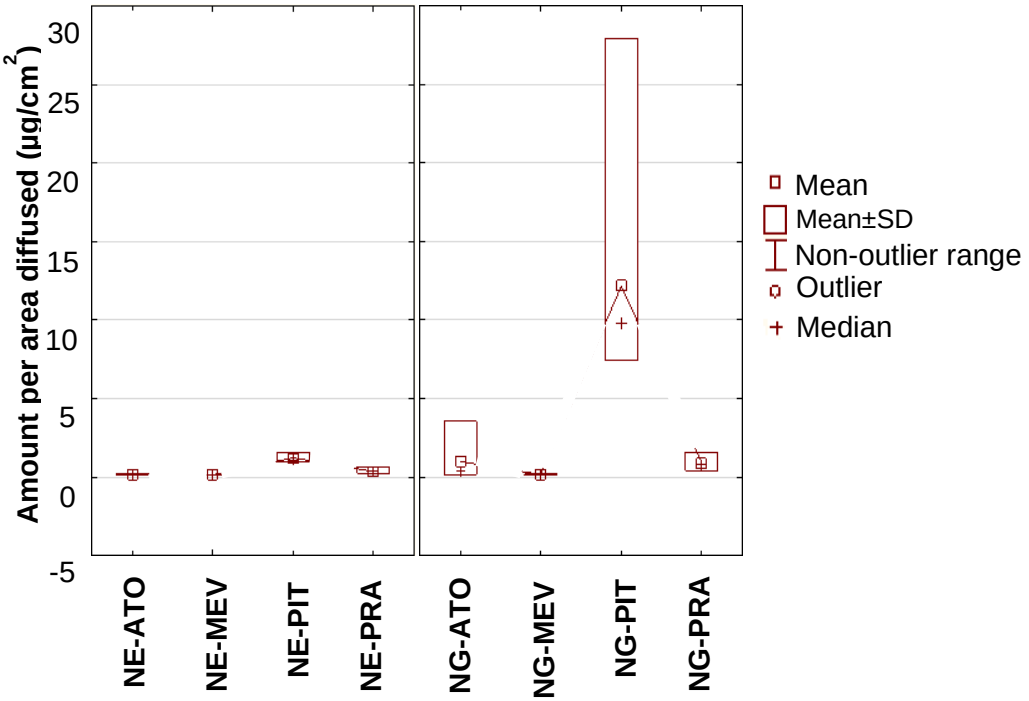
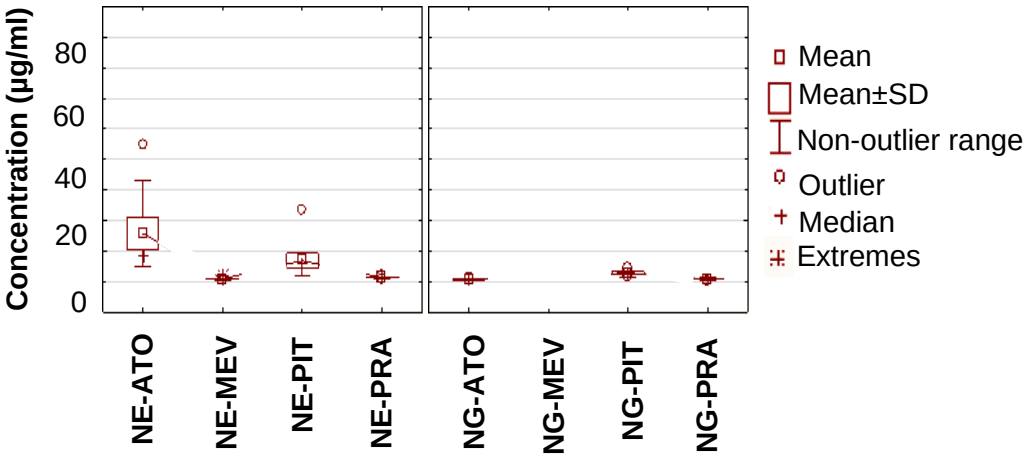


817

818 Fig. 3: Box-plots indicating the mean and median amount per area diffused (µg/cm^2) for each

819 of the selected statins in the **NEs** and the **NGs**

820

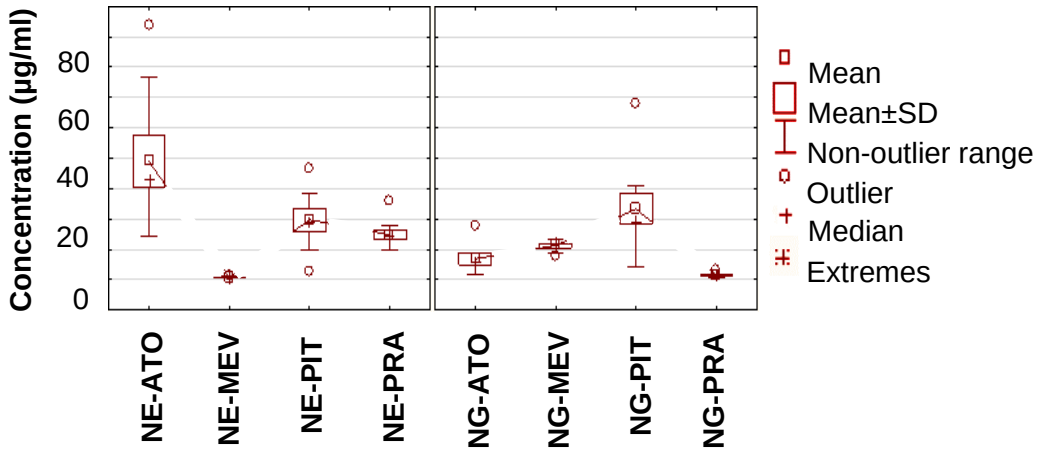


821

822 Fig. 4: Box-plots indicating the mean and median concentration (µg/ml) of the **NEs** and the

823 **NGs** for each of the selected statins present in the SCE

824



825

826 Fig. 5: Box-plots indicating the mean and median concentration (µg/ml) of the **NEs** and the

827 **NGs** for each of the selected statins present in the ED

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

Hypercholesterolaemia elevates cholesterol levels, increases the risk of atherosclerotic CVD and affects roughly 1 in 250 individuals globally (Nordestgaard *et al*., 2013; Onwe *et al*., 2015; Watts *et al*., 2015). FH is associated with very high levels of LDL, due to gene mutations causing abnormally low uptake of LDL within the liver (Mytilinaiou *et al*., 2018). Hence, accumulation of LDL in the plasma occurs, increasing the risk of premature CVD (Krogh *et al*., 2018). Currently, oral administered statins (HMG-CoA reductase inhibitors) are the first-line medical therapy for hyperlipidaemia (Zodda *et al*., 2018.). Statin induced therapy reduces cholesterol levels in the liver and raises the LDL-receptors in the liver and in turn promotes the clearance of LDL-cholesterol circulating in the blood (Bays, 2005; Fong, 2016; Hennessy *et al*., 2016). However, the oral route of administration presents with obstacles such as gastro-intestinal side-effects and low bioavailability due to first-pass metabolism (CYP450 liver enzymes) (Sahebzamani *et al*., 2014; Zodda *et al*., 2018). Potential side-effects can lead to poor patient compliance or even discontinuation of therapy (Mancini *et al.,* 2013). The tendency to cause hepatotoxicity, myalgia, as well as gastrointestinal effects varies between the different statins (Mancini *et al.,* 2013). Statins have been linked to rare instances of clinically apparent acute liver injury. Nevertheless, the most reports have been with atorvastatin and the fewest with pravastatin and pitavastatin (Björnsson, 2017). The onset latency varies considerably and can be more than six months or even several years after starting (Karahalil *et al*., 2017). Most cases are hepatocellular, but cholestatic hepatitis is also well described for most statins (Karahalil *et al*., 2017). The monitoring of hepatotoxicity (to prevent serious liver disease) by means of ALT tests has been unsuccessful; mainly because of its poor predictive value (Tolman, 2002). Therefore, by exploring other routes of administration, the adverse effects experienced with regards to the liver might be avoided. The transdermal route offered a more convenient and patient compliant route of administration, since first-pass metabolism is avoided when utilising the skin for administration (Geethu *et al*., 2014).

The aim of this study was to investigate the transdermal delivery of the selected statins, namely atorvastatin, mevastatin, pitavastatin and pravastatin, after incorporation into both a **NE** and a **NG**, respectively. Both the **NEs** and the **NGs** consisted of grapeseed oil in the oil phase. Furthermore, the potential toxicity of the **NEs** were determined with and without the API.

The specific objectives were to:

- Develop and validate a method of analysis, i.e. HPLC to determine the concentrations of the selected statins.

- Formulate both **NEs** and **NGs** containing the selected statins separately together with natural oil, e.g. grapeseed oil, as the oil phase.
- Characterise both **NEs** and **NGs** with respect to the zeta-potential, viscosity, droplet size, pH, visual examination, entrapment efficacy and morphology.
- Determine the release of the selected statins from the **NEs** and **NGs** by using membrane release studies.
- Use Franz cell skin diffusion studies and tape stripping to determine the transdermal and topical delivery of selected statins from the **NEs** and **NGs**, respectively.
- Evaluate the cytotoxic effects of each selected statin (separately) and in combination with the **NE** using *in vitro* cell cultures (HaCaT) via NR- and MTT-assays.

An HPLC analytical method was developed and validated and the method proved to be reliable, accurate and reproducible to analyse the selected statins (atorvastatin, mevastatin, pitavastatin and pravastatin).

Pre-formulation of the **NEs** were performed to determine the appropriate surfactant-surfactant ratio in which 2% statins (separately) could be formulated. The most stable ratio was Tween® 80:Span® 60 (3:3), also known as optimised **NE1**, which was the **NE** that performed the best after the physical and chemical characterisation of the competing **NEs**. These optimised **NE1s** were used to formulate the **NG1s** by adding Carbopol® Ultrez 20 (gelling agent) to the **NE1**, each containing the selected statin. The **NG1s** were also characterised. The visual inspection established that the **NE1s** had a smooth, milk-like appearance, while the **NG1s** presented as thick, white and shiny. There was no sedimentation or visible oil droplets present in either the **NE1s** or **NG1s**. After the pH of the **NG1s** and **NE1s** were adjusted, these nano-formulas presented with pH values between 4 and 8, which is acceptable for topical administration, since the skin (pH 5) can tolerate pH values of 3 – 9 (Barry, 2002). Zeta-potential values confirmed the stability of the **NE1s** and **NG1s**, since it is suggested that formulations with a zeta-potential of more than 30 mV or less than – 30 mV can remain stable over time, and both nano-formulas had values within the ideal range (Eid *et al*., 2014; Silva *et al*., 2012). Both the **NE1s** and **NGs** abided to the required parameters and had droplet sizes < 500 nm (Nalini *et al*., 2017) and PdI values of < 0.5 (Elmataeeshy *et al*., 2018). It was found that **NE1s** were more polydispersed (Gaumet *et al*., 2008) opposed to their **NG1s** which were more homogenous (excluding **NE-PRA1**) when the PdI values were inspected. Viscosity measures (cP) between the **NE1s** and the **NG1s** differed significantly after the addition of the gelling agent in **NE1s**; therefore, the **NG1s** presented with substantially higher viscosity measurements opposed to **NE1s**.

During the membrane release studies, it was revealed that the **NE1** and **NG1** containing pravastatin presented with the highest flux, followed by pitavastatin, atorvastatin and mevastatin for both the **NEs** and **NGs**. It was also detected that all the statins were successfully released from the different nano-formulas during the release studies.

Skin diffusion studies revealed that the amount per area of the statins that diffused through skin for the **NG1s** were higher than those of the respective **NE1s**. The **NE1** and **NG1** containing pitavastatin (log D of 1.50 (Fong, 2016)) presented with the highest amount per area diffused, followed by pravastatin, atorvastatin and lastly, mevastatin that presented with an undesirable aqueous solubility of 0.032 mg/ml (Drugbank, 2005c) and log D of 3.570 (Kim *et al*., 2011) for both the **NEs** and **NGs**. After the diffusion studies were completed, the concentration that diffused transdermally were compared to plasma concentrations after oral administration. It was observed that the **NG1s** and **NE1s** obtained higher statin concentrations transdermally when compared to those obtained after oral administration (except for mevastatin, as no data were available in the literature), which could hold promise for transdermal statin therapy in the future, especially regarding compliance for instance in the case of children on statin therapy (Primosch *et al*., 2001).

The **NE1** containing atorvastatin and the **NG1** containing pitavastatin displayed the highest median concentration in the SCE and the ED for the **NE1s** and **NG1s**, respectively. It was also observed that SCE delivery of the statins from the **NE1s** were higher than those of the respective **NG1s**, which can be explained by the physicochemical properties.

It was found that the **NE1s** and **NG1s** with grapeseed oil (used during this study), generally, resulted in better topical and transdermal delivery of the selected statins, than the **NEs** and **NGs** with apricot oil utilised during previous studies (Marais, 2018; Maree, 2018). However, this was not true for atorvastatin, mevastatin and pravastatin within **NGs**, which obtained better results for apricot oil in the SCE, as well as for mevastatin (in the **NEs**) and pravastatin (in the **NGs**) within the apricot oil formulas attained higher topical (SCE and ED) and transdermal delivery than the grapeseed oil formulas.

Lastly, *in vitro* cytotoxicity studies were conducted on the **NE1s**, placebo of the **NE1**, and the statins stock solutions (statin alone, containing no **NE1s** excipients). These cytotoxicity studies were performed on HaCaT cells as a representative of the *in vitro* keratinocyte model. The methods used included the methylthiazol tetrazolium-assay, assessing mitochondrial activity of cells, and the neutral red-assay, assessing the lysosomal activity of cells. The concentration values to which these compounds inhibited 50% of the cell growth (IC_{50}) for the methylthiazol tetrazolium-assay results of statins stock solutions indicated that mevastatin presented more cytostatic to the HaCaT cells compared to atorvastatin, pitavastatin and pravastatin. Neutral red-

assay results for IC_{50} values of statins stock solutions confirmed that mevastatin presented more cytostatic towards HaCaT cells than atorvastatin, pitavastatin and pravastatin.

The methylthiazol tetrazolium- and neutral red-assays were greatly affected by the lipophilic formulations (Stockert *et al*., 2012); therefore, lipophilic statins, dispersions (**NE1s** and placebo of the **NE1**) and lipophilic excipients could interfere with data and counter accurate results, especially in higher concentrations. In conclusion, the statins were non-toxic toward the HaCaT cells at concentrations used during the transdermal studies.

Looking to the future, it is likely that first-generation patch technology can be used for delivery of small molecule drugs with the right set of properties, especially those drugs that are currently administered orally, e.g. statins. Further *in vitro* studies can be investigated, e.g. three dimensional (3D) cell cultures, which mimic functions seen in human tissues significantly better than cells grown using classical two dimensional (2D) culture systems (Wrzesinski *et al*., 2015) as seen in toxicity studies. Literature has compared 2D and 3D cell culture models regarding the various cellular morphology and metabolism, indicating a better representation of *in vivo* conditions of the latter cell culture environment (Bonnier *et al*., 2005).

References

Barry, B. 2002. Transdermal drug delivery. (*In* Aulton, M.E., *ed*. Pharmaceutics: the science of dosage form design. 2nd ed. London: Churchill Livingstone. p. 499-533.

Bays, H. 2005. Stain safety: An overview and assessment of the data. *The American journal of cardiology*, 97:7C-26C.

Björnsson, E.S.J.L.I. 2017. Hepatotoxicity of statins and other lipid-lowering agents. *Liver international*, 37(2):173-178.

Bonnier, F., Keating, M.E., Wrobel, T.P., Majzner, K., Baranska, M., Garcia-Munoz, A., Blanco, A. & Byrne, H.J. 2015. Cell viability assessment using the Alamar blue assay: a comparison of 2D and 3D cell culture models. *Toxicology in vitro*, 29(1):124-131.

Buchanan, B., Meng, A., Poulin, M-M., Zuccolo, J., Azike, C.G., Gabriele, J. & Baranowski, C. 2018. Comparative pharmacokinetics and safety assessment of transdermal berberine and dihydroberberine. *Plos one*, 13(3):1-20.

Drugbank. 2005. https://www.drugbank.ca/drugs/DB06693. Date of access: 18 May 2019.

Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti-inflammatory activity of swietenia macrophylla nanoemulgel. *Journal of nanomedicine and nanotechnology,* 5(2):1-10.

Elmataeeshy, M.E., Sokar, M.S., Bahey-El-Din, M.B. & Shaker, D.S. 2018. Enhanced transdermal permeability of terbinafine through novel nanoemulgel formulation. *Future journal of pharmaceutical sciences*, 4(1):18-28.

Fong, C. 2016. Statins in therapy: Understanding their hydrophilicity, lipophilicity, binding to 3-hydroxy-3-methylglutaryl-CoA reductase, ability to cross the blood brain barrier and metabolic stability based on electrostatic molecular orbital studies. *European journal of medicinal chemistry*, 85:661-674.

Gaumet, M., Vargas, A., Gurny, R. & Delie, F. 2008. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *European journal of pharmaceutics and biopharmaceutics*, 69:1-9.

Geethu, V.S., Manikandan, P., Nethaji, R., Surenderan, N.S. & Babu, G. 2014. A review on: transdermal drug delivery systems. *Indo American journal of pharmaceutical research*, 4:1809-1824.

Hennessy, E., Adams, C., Reen, F.J., O"Gara, F. 2016. Statins inhibit bacterial growth and virulence. *Antimicrobial agents and chemotherapy*, 5(2):1-44.

Ita, K. 2016. Transdermal delivery of vaccines–Recent progress and critical issues. *Biomedicine & pharmacotherapy*, 83:1080-1088.

Karahalil, B., Hare, E., Koç, G., Uslu, İ., Şentürk, K., Özkan, Y.J.A.o.I.H. & Toxicology. 2017. Hepatotoxicity associated with statins. *Global journal of pharmacology*, 68(4):254-260.

Kim, M.C., Ahn, Y., Jang, S.Y., Cho, K.H., Hwang, S.H., Lee, M.G., Ko, J.S., Park, K.H., Sim, D.S., Yoon, N.S., Yoon, H.J., Kim, K.H., Hong, T.J., Park, H.W., Kim, J.H., Jeong, M.H., Cho, J.G., Park, J.C. & Kang, J.C. 2011. Comparison of clinical outcomes of hydrophilic and lipophilic statins in patients with acute myocardial infarction. *The Korean journal of internal medicine*, 26:294-303.

Krogh, H.W., Mundal, L., Holven, K.B. & Retterstol, K. 2018. Patients with familial hypercholesterolaemia are characterized by presence of cardiovascular disease at the time of death. *European heart journal*, 37:1398-1405.

Mancini, G.B.J., R.A., Tashakkor, A.Y., Baker, S., Bergeron, J., Fitchett, D., Frohlich, J., Genest, J., Gupta, M., Hegele, R.A., Ng, D.S., Pearson, G.J., & Pope, J. 2013. Diagnosis, prevention and management of statin adverse effects and intolerance: Canadian working group consensus update. *Canadian journal of cardiology*, 29:1553-1568.

Marais, S. 2018. The use of apricot oil emulsions for the transdermal delivery of selected statins. Potchefstroom: NWU. (Dissertation-MSc).

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

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

Mytilinaiou, M., Kyrou, I., Khan, M., Grammatopoulos, D.K. & Randeva, H.S. 2018. Familial hypercholesterolemia: new horizons for diagnosis and effective management. *Frontiers in pharmacology*, 9:1-29.

Nalini, T., Kumari, V.S. & Basha S.K. 2017. Novel nanosystems for herbal drug delivery. *World journal of pharmacy and pharmaceutical sciences*, 6(5):1447-1463.

Nordestgaard, B.G., Chapman, M.J., Humphries, S.E., Ginsberg, H.N., Masana, L., Descamps, O.S., Wilkund, O., Hegele, R.A., Raal, F.J., Defesche, J.C., Wiegman, A., Santos, R.D., Watts, G.F., Parhofer, K.G., Hovingh, Kovanen, P.T., Boileau, C., Averna, M., Borén, J., Bruckert, E., Catapano, A.L., Kuivenhoven, J.A., Pajukanta, P., Ray, K., Stalenhoef, A.F.H., Stroes, E., Taskinen, M. & Tybjaerg-Hansen, A. 2013. Familial hypercholesterolaemia is underdiagnosed and undertreated in the general population: guidence for clinicians to prevent coronary heart disease. *European heart journal*, 34:3478-3490.

Onwe, P.E., Folawiyo, M.A., Okike, P.I., Balogun, M.E., Umahi, G., Besong, E.E., Okorocha, A.E., Afoke, A.O. 2015. Lipid profile and the growing concern on lipid related diseases. *Journal of pharmacy and biological sciences*, 10(5):22-27.

Primosch, R.E. & Bender, F., 2001. Factors associated with administration route when using midazolam for pediatric conscious sedation. *ASDC journal of dentistry for children*, *68*(4):233-8.

Sahebzamani, F.M., Munro, C.L., Marroquin, O.C., Diamond, D.M., Keller, E., Kip, K.E. 2014. Examination of the FDA warning for statins and cognitive dysfunction. *Journal of pharmacovigilance*, 2(4):1-9.

Silva, H.D., Cerqueira, M.A., Vicente, A.A. 2012. Nanoemulsions for food applications: development and characterization. *Food and bioprocess technology*, 5:854-867

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

Tolman, K.G. 2002. The liver and lovastatin. *The American journal of cardiology*, 89:1374-1380.

Watts, G.F., Shaw, J.E., Pang, J., Magliano, D.J., Jennings, G.L.R. & Cariington, M.J. 2015. Prevalence and treatment of familial hypercholesterolaemia in Australian communities. *International journal of cardiology*, 185:69-71.

Wrzesinski, K. & J Fey, S. 2015. From 2D to 3D-a new dimension for modelling the effect of natural products on human tissue. *Current pharmaceutical design*, 21(38):5605-5616.

Zodda, D., Giammona, R., Schifilliti, S. 2018. Treatment strategy for dyslipidaemia in cardiovascular disease prevention: focus on old and new drugs. *Pharmacy*, 6(10):1-16.

APPENDIX A

VALIDATION OF THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETECTION OF ATORVASTATIN, MEVASTATIN, PITAVASTATIN AND PRAVASTATIN

A.1 Validation

Method validation can be defined as the procedure of presenting recorded confirmation to ensure the method performs as intended (Shabir, 2003). Method validation is a requirement in quality criteria and most guidelines regarding laboratories (Huber, 2007). The results of a validated method offer confirmation for accuracy, creditability and reliability (Huber, 2007) and therefore good analytical practice (Huber, 2007).

A.2 Chromatographic conditions

An HPLC analytical method was developed and validated during this study to establish the influence of the assay conditions and sample elements on the assay performance. When developing and HPLC method, the acceptance criteria should be determined. To validate the HPLC method used, an analytical instrument (HP1100 series HPLC equipped with a pump, autosampler, ultraviolet (UV) detector and ChemStation Rev. A.10.03 data acquisition and analysis software or equivalent) was utilised. Specific chromatographic conditions were taken into account as shown in Table A.1.

Table A.1: Chromatographic conditions for HPLC analytical method

Column	Venusil XBP C18(2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE)
Flow rate	1.0 ml/min
Injection volume	10 µl
Detection wavelength	UV at 240 nm
Retention time pitavastatin	± 3.386 min
Retention time pravastatin	± 3.853 min
Retention time atorvastatin	± 4.166 min
Retention time mevastatin	± 6.640 min
Stop time	10.500 min
Solvent	Methanol
Mobile phase	Acetonitrile/water with 0.1% orthophosphoric acid

In this study, the mobile phase contained acetonitrile (ACN)/Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand, RSA) with 0.1% orthophosphoric acid, which started at 25% of ACN for 1.0 min, this was linearly increased to 90% for 3.0 min; subsequently, it was held at 90% for 6.5 min and then re-equilibrated to start conditions.

A.3 Method validation guidelines

Laboratory testing is necessary for analytical method validation and the obtained results should meet the requirements for the stated analytical parameters (USP, 2009). Therefore, method validation necessitates extensive laboratory validation tests that have been classified by national and universal boards (different groups) (Huber, 2007; Paithankar, 2013). Required parameters are presented in correlation coefficient (R^2), relative standard deviation (RSD) or standard deviation (SD).

The United States Pharmacopeia (USP), the International Conference for Harmonization (ICH) and the overall requirements for the competence of testing and calibration laboratories (ISO/IEC 17025) was combined. These parameters are summarised in Table A.2 (Huber, 2007).

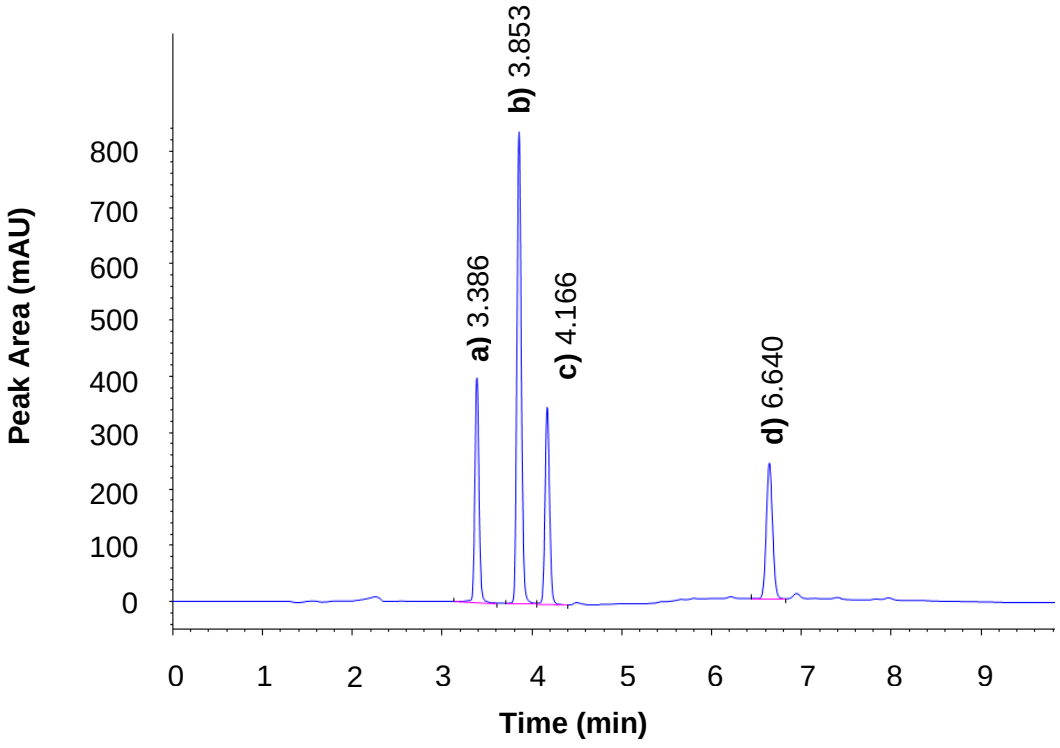


Figure A.1: HPLC chromatogram displaying standard solution peaks of statins, in addition: a) pitavastatin, b) pravastatin, c) atorvastatin and d) mevastatin

Table A.2: Summary of analytical method validation guidelines and the results for atorvastatin, mevastatin, pitavastatin and pravastatin

Accuracy					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
Recovery (90.0 – 110.0%)		98.7%	97.1%	96.4%	96.2%
Linearity					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
$R^2 \geq 0.999$		$R^2 = 1.000$	$R^2 = 0.999$	$R^2 = 1.000$	$R^2 = 0.999$
LOD (µg/ml)					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
%RSD < 15.00%		5.69	5.83	1.56	3.19
LLOQ (µg/ml)					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
%RSD < 20.00%		0.32	1.86	0.61	0.36
Precision (intra-day)					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
%RSD ≤ 5.00%		1.80	0.80	0.80	1.00
Precision (inter-day)					
Day 1	%RSD ≤ 5.00	1.80	0.80	0.80	1.00
Day 2	%RSD ≤ 5.00	0.60	0.78	0.90	0.20
Day 3	%RSD ≤ 5.00	0.28	1.04	1.06	0.30
Robustness (Section A.3.4)					
System repeatability (Peak area)					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
%RSD < 2.00%		0.11	0.20	1.16	0.11
System repeatability (Retention time (min))					
%RSD < 2.00%		0.14	0.09	0.17	0.14
System stability					
Ideal parameter		**Atorvastatin**	**Mevastatin**	**Pitavastatin**	**Pravastatin**
%RSD < 2.00%		0.31	1.69	0.84	0.25

A.3.1 Linearity

Linearity can be defined as the capability of the analytical procedure to obtain results that correspond with the amount of analyte present in the given sample (ICH, 2005; Paithankar, 2013). Testing for linearity can be performed by diluting the stock solution (Huber, 2007).

Linearity is determined by preparing six standards with concentrations between supposed concentration ranges (APVMA, 2004). The linear relationship is determined by visual examination of the analytical response plot compared to analyte concentration (µg/ml). The

y-unit signifies the peak area of the analyte (statin), whereas the x-unit signifies the concentration analyte (statin) present in the sample and c is the y-intercept (Snyder *et al*., 1997). To determine the R^2 for each analyte, the slope (m) should display a straight regression line (Snyder *et al*., 1997). It is an indication of a strong relationship between the response and the analyte concentration, when the value of the coefficient exceeds 0.98 (Paithankar, 2013; Snyder *et al*., 1997). The linearity of the data is displayed through the linear regression equation (Equation A.1) (Johnson & Buskirk, 1998, Paithankar, 2013; Snyder *et al*., 1997).

$$y = mx + c \qquad \textbf{Equation A.1}$$

A.3.1.1 Linearity method

To determine the linearity of the selected statins (atorvastatin, mevastatin, pitavastatin and pravastatin), approximately 20 mg of each selected statin was weighed and transferred into a 100 ml volumetric flask. The statins were dissolved and filled to volume with methanol to prepare a 100 ml stock solution (Standard 1). Then, 5 ml of the stock solution was diluted to 50 ml with methanol (Standard 2). Precisely 5 ml of Standard 2 was diluted to 50 ml with methanol (Standard 3). Finally, the standards were transferred into HPLC vials to be analysed. The vials were inserted into the HPLC and injected in duplicate with injection volumes of 2.5, 5.0, 7.5 and 10.0 µl to establish the linear regression.

Table A.3: The standard linearity results of atorvastatin

Concentration (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)
0.2	3.966	5.533	4.7
0.5	10.852	10.984	10.9
1.0	20.477	20.459	20.5
1.5	29.527	30.004	29.8
2.0	39.887	39.652	39.8
5.0	118.263	118.680	118.5
10.0	236.463	235.828	236.1
14.9	352.300	352.248	352.3
19.9	469.281	469.007	469.1
49.8	1133.096	1127.797	1130.4
99.7	2256.647	2254.138	2255.4
149.5	3362.931	3362.168	3362.5
199.3	4476.859	4473.297	4475.1
		R^2	1.000
		y-intercept	5.835
		Slope	22.309

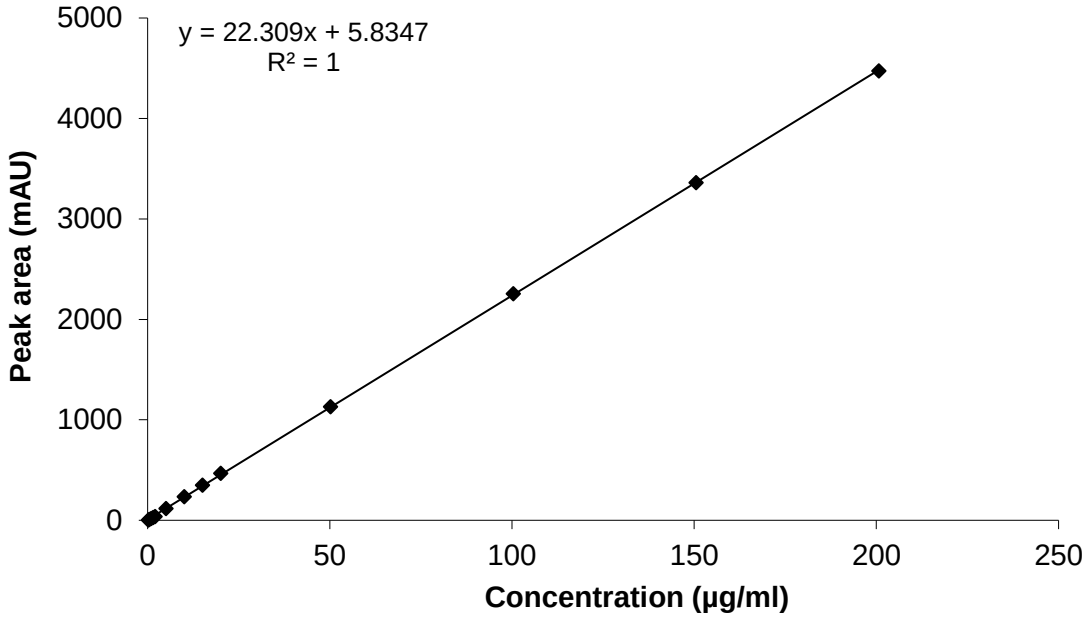


Figure A.2: The standard linear regression curve for atorvastatin

The slope displayed a straight regression line (APVMA, 2004; Snyder *et al*., 1997) (Figure A.2). Atorvastatin's standard solution linearity displayed $R^2 = 1$, which indicated a strong relationship between analyte concentration and response (Paithankar, 2013).

Table A.4: The standard linearity results of mevastatin

Concentration (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)
0.2	4.681	5.514	5.1
0.5	11.661	11.024	11.3
1.0	23.946	27.215	25.6
1.5	34.353	35.857	35.1
2.1	47.591	46.406	47.0
5.2	150.125	141.198	145.7
10.3	292.188	273.873	283.0
15.5	432.515	408.639	420.6
20.6	565.773	565.248	565.5
51.6	1321.813	1310.654	1316.2
103.2	2616.268	2623.764	2620.0
154.8	3892.815	3904.051	3898.4
206.4	5169.170	5160.158	5164.7
		R^2	0.999
		y-intercept	13.859
		Slope	25.707

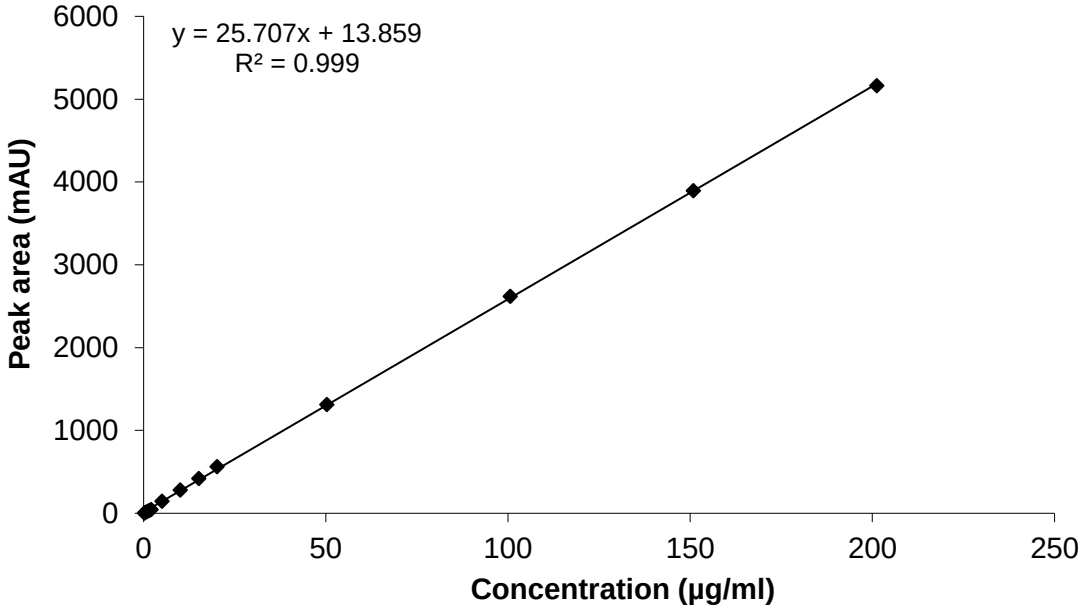


Figure A.3: The standard linear regression curve for mevastatin

The slope displayed a straight regression line (APVMA, 2004; Snyder *et al*., 1997) (Figure A.3). Mevastatin's standard solution linearity obtained was $R^2 = 0.9999$, which indicated a strong relationship between analyte concentration and response (Paithankar, 2013).

Table A.5: The standard linearity results of pitavastatin

Concentration (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)
0.2	6.944	8.469	7.7
0.5	12.802	13.234	13.0
1.0	22.686	22.795	22.7
1.5	35.202	36.951	36.1
2.1	46.400	47.474	46.9
5.2	130.559	129.619	130.1
10.3	253.687	252.719	253.2
15.5	373.014	373.924	373.5
20.6	495.151	497.012	496.1
51.6	1176.454	1173.177	1174.8
103.2	2314.660	2308.341	2311.5
154.7	3410.149	3413.399	3411.8
206.3	4484.740	4491.304	4488.0
		R^2	1.000
		y-intercept	19.455
		Slope	22.47

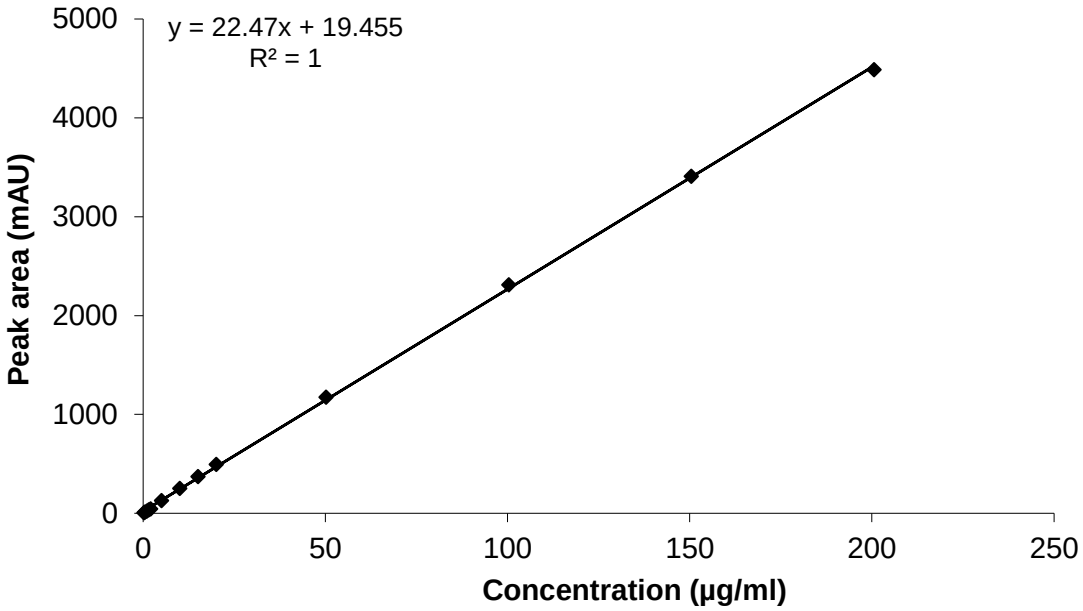


Figure A.4: The standard linear regression curve for pitavastatin

The slope displayed a straight regression line (APVMA, 2004; Snyder *et al*., 1997) (Figure A.4). Pitavastatin's standard solution linearity obtained was $R^2 = 1$, which indicated a strong relationship between analyte concentration and response (Paithankar, 2013).

Table A.6: The standard linearity results of pravastatin

Concentration (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)
0.2	10.529	11.827	11.2
0.5	25.058	24.848	25.0
1.0	46.538	46.012	46.3
1.5	67.310	68.106	67.7
2.0	89.372	93.077	91.2
5.0	259.194	259.081	259.1
10.0	516.395	515.630	516.0
15.0	769.907	768.677	769.3
20.1	1023.578	1025.595	1024.6
50.2	2455.085	2445.125	2450.1
100.3	4854.212	4844.085	4849.1
150.5	7170.594	7166.169	7168.4
200.6	9430.678	9428.001	9429.3
		R^2	0.999
		y-intercept	30.249
		Slope	46.105

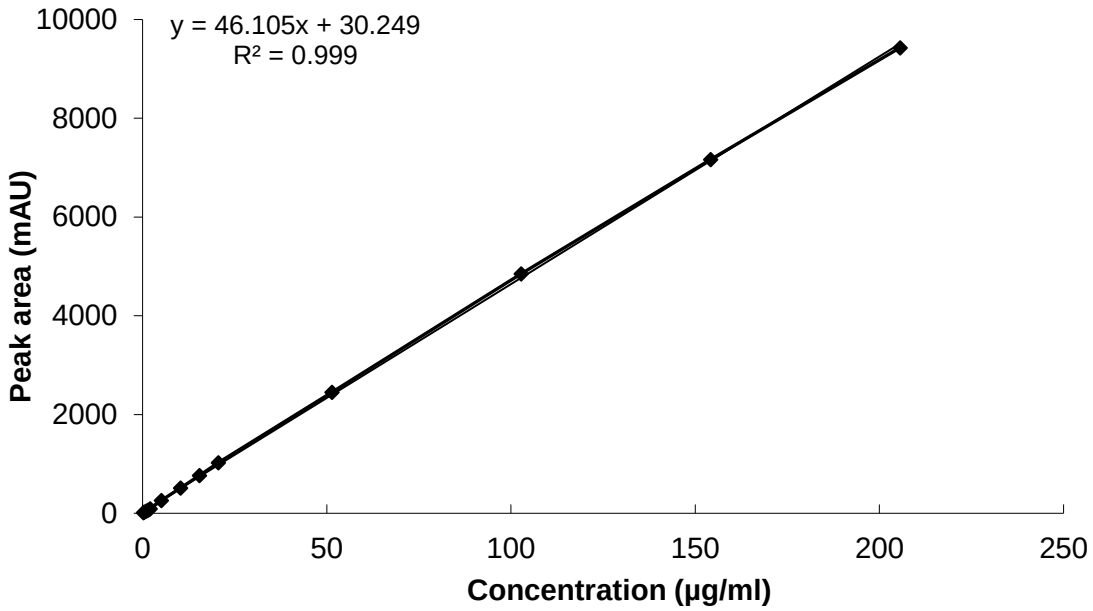


Figure A.5: The standard linear regression curve for pravastatin

The slope displayed a straight regression line (APVMA, 2004; Snyder *et al*., 1997) (Figure A.5). Pravastatin's standard solution linearity obtained was $R^2 = 0.999$, which indicated a strong relationship between analyte concentration and response (Paithankar, 2013).

A.3.2 Accuracy

Accuracy can be recognised as the degree to which the accepted reference value of analyte in the sample compares to the actual measured value (APVMA, 2004; ICH, 2005, Paithankar, 2013). A sample that contains pure solvent together with a specified weighed concentration is accepted as a valid reference value (Huber, 2007; Paithankar, 2013) and therefore applicable to this study. It is important that the samples be identical to each other, for this method is highly influenced by the preparation of samples (Huber, 2007; Paithankar, 2013).

Accuracy methods may differ from particular assay values, therefore nine samples are recommended with three diverse concentrations to cover the full calibration curve in the allocated range (APVMA, 2004; Bansal & DeStefano, 2007; ICH, 2005; Snyder *et al*., 1997). In this study, three concentrations were used in triplicate. Accuracy (%recovery) can be described as the relationship between the perceived results and the results expected (APVMA, 2004; Paithankar, 2013). Expected recovery is mainly influenced by the analyte concentration, the method in which the sample was prepared and the placebo (APVMA, 2004). After sample injection, the %recovery should be within 98 to 102%. This percentage is regarded as a ratio between obtained and estimated results, which acts as an indicator of the methods accuracy (APVMA, 2004).

Table A.7: Mean recovery (%) ranges (APVMA, 2004)

Ideal recovery mean	Active (%)
98 – 102%	≥ 10.0
90 – 110%	≥ 1.0
80 – 120%	0.1 – 1.0
75 – 125%	< 0.1

The spiked placebo recovery method was used in this study. For accuracy analysis, a placebo nano-emulsion was prepared as follows; each statin required nine volumetric flasks:

- Three flasks contain 3.0 ml spike solution and 0.6 ml placebo nano-emulsion.
- Three flasks contain 4.0 ml spiked solution and 0.8 ml placebo nano-emulsion.
- Three flasks contain 5.0 ml spike solution and 1.0 ml placebo nano-emulsion.

The above solutions were diluted (up to 100 ml) with methanol and mixed exhaustively in an ultrasonic bath after which 1 ml of each flask were transferred into HPLC vials for analysis. The standard spike solution was prepared by weighing ± 20 mg of each API (statin) in individual 100 ml volumetric flasks, which was diluted with methanol up to 100 ml. After the selected statin dissolved, a small amount of the spike solution was transferred to an HPLC vial to be injected. The obtained results were investigated and compared to the expected results.

The prepared samples produced calculate concentrations of 160, 200 and 240 µg/ml of each statin. Samples were transferred to vials and analysed against a standard solution of 20 mg/100 ml. Every sample was injected (in duplicate) with an injection volume of 10 µl.

Table A.8: Placebo nano-emulsion formula

Phase 1		Phase 2	
Amount	**Substance**	**Amount**	**Substance**
40.000 ml	Water	4.350 ml	Grapeseed oil
2.804 ml	Tween® 80	3 g	Span® 60

Table A.9: Accuracy results of atorvastatin

Concentration spiked (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)	Recovery (µg/ml)	Recovery (%)
61.3	1382.0	1387.2	1384.6	59.6	97.2
61.3	1399.0	1401.0	1400.0	60.3	98.3
61.3	1399.9	1402.4	1401.2	60.4	98.4
81.8	1873.1	1883.1	1878.1	81.4	99.5
81.8	1882.1	1855.3	1868.7	80.9	99.0
81.8	1891.7	1867.1	1879.4	81.4	99.6
102.2	2317.1	2315.3	2316.2	100.7	98.5
102.2	2326.6	2321.4	2324.0	101.0	98.8
102.2	2320.5	2323.1	2321.8	100.9	98.7

Table A.10: Statistical analysis of atorvastatin

Statistical analysis	
Mean recovery (%)	98.7
SD	0.7
%RSD	0.7

Atorvastatin was within ideal standard limits (ranged between 90 – 110%), since the mean recovery (%) was 98.7% (APVMA, 2004).

Table A.11: Accuracy results of mevastatin

Concentration spiked (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)	Recovery (µg/ml)	Recovery (%)
60.2	1444.2	1431.3	1437.7	58.2	96.7
60.2	1429.7	1422.8	1426.2	57.7	95.8
60.2	1418.9	1419.8	1419.3	57.4	95.3
80.2	1911.8	1921.3	1916.6	79.3	98.8
80.2	1900.8	1878.9	1889.8	78.1	97.3
80.2	1906.8	1884.7	1895.7	78.4	97.7
100.3	2329.2	2323.6	2326.4	97.3	97.0
100.3	2345.2	2348.6	2346.9	98.2	97.9
100.3	2327.8	2332.4	2330.1	97.5	97.2

Table A.12: Statistical analysis of mevastatin

Statistical analysis	
Mean recovery (%)	97.1
SD	1.0
%RSD	1.0

Mevastatin was within ideal standard limits (ranged between 90 – 110%), since the mean recovery (%) was 97.1% (APVMA, 2004).

Table A.13: Accuracy results of pitavastatin

Concentration spiked (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)	Recovery (µg/ml)	Recovery (%)
60.7	1967.1	1975.0	1971.0	56.2	92.7
60.7	1998.4	1998.0	1998.2	57.1	94.1
60.7	1994.3	1993.2	1993.8	57.0	93.9
80.9	2635.8	2647.6	2641.7	78.4	96.9
80.9	2657.5	2623.5	2640.5	78.3	96.8
80.9	2686.2	2654.5	2670.4	79.3	98.0
101.1	3272.4	3266.1	3269.3	99.1	98.0
101.1	3295.5	3289.6	3292.5	99.8	98.7
101.1	3282.1	3276.8	3279.5	99.4	98.3

Table A.14: Statistical analysis of pitavastatin

Statistical analysis	
Mean recovery (%)	96.4
SD	2.1
%RSD	2.2

Pitavastatin was within ideal standard limits (ranged between 90 – 110%), since the mean recovery (%) was 96.4% (APVMA, 2004).

Table A.15: Accuracy results of pravastatin

Concentration spiked (µg/ml)	Area 1 (mAU)	Area 2 (mAU)	Mean area (mAU)	Recovery (µg/ml)	Recovery (%)
62.7	1374.5	1381.1	1377.8	58.0	92.4
62.7	1392.2	1393.6	1392.9	58.7	93.6
62.7	1393.4	1394.6	1394.0	58.7	93.7
83.6	1860.9	1869.1	1865.0	81.5	97.5
83.6	1869.5	1841.2	1855.3	81.0	96.9
83.6	1883.5	1852.6	1868.0	81.6	97.6
104.5	2294.1	2291.1	2292.6	102.1	97.7
104.5	2303.4	2299.1	2301.3	102.6	98.1
104.5	2296.5	2295.0	2295.7	102.3	97.9

Table A.16: Statistical analysis of pitavastatin

Statistical analysis	
Mean recovery (%)	96.2
SD	2.1
%RSD	2.2

Pravastatin was within ideal standard limits (ranged between 90 – 110%), since the mean recovery (%) was 96.2% (APVMA, 2004).

A.3.3 Precision

Precision can be described as the degree of variation (standard deviation) regarding attained data from various measurements under specific conditions with the use of a main sample (APVMA, 2004; ICH, 2005; Paithankar, 2013; Snyder *et al*., 1997). Precision for this analytical method was examined based on three principles, e.g. repeatability (intra-day precision), intermediate precision (inter-day precision) and reproducibility (Graham, 2011; Huber, 2007; ICH, 2005; Paithankar, 2013; USP, 2011). Reproducibility will be explained under ruggedness; however, repeatability (intra-day precision) and intermediate precision (inter-day precision) will be determined and explained here. To establish precision, a minimum of five replicate samples are needed (APVMA, 2004). When analysed, the %RSD value is determined (APVMA, 2004; Paithankar, 2013).

Nine samples were prepared with three different concentrations for each nano-emulsion containing the different statins separately. Approximately 0.6, 0.8 and 1.0 g were weighed and diluted with methanol in a 100 ml volumetric flasks. The dilutions were done in triplicate and analysed by HPLC. Each sample was injected in duplicate within 24 h (ICH, 2005).

Table A.17: Suggested levels of precision (APVMA, 2004)

Precision (%RSD)	Constituent in sample
≤ 2%	> 10.0%
≤ 5%	1.0 up to 10.0%
≤ 10%	0.1 up to 1.0%
≤ 20%	< 0.1%

A.3.3.1 Precision (intra-day)

Repeatability can be described as intra-assay precision of the method while under similar operating conditions during a short duration (ICH, 2005) in the same laboratory (Huber, 2007). As mentioned before, a minimum of nine samples were analysed in order to measure repeatability, where three diverse sample concentrations were injected in triplicate and analysed on the HPLC within 24 h (ICH, 2005). This preparation method is similar to the accuracy preparation method.

Table A.18: Repeatability (intra-day precision) results of atorvastatin (Day 1)

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
617.7	3262.11	3298.70	3280.40	123.9	103.2
612.3	3305.90	3295.50	3300.70	125.8	104.8
610.2	3269.64	3267.80	3268.70	125.0	104.1
805.8	4364.65	4361.90	4363.30	169.3	105.8
817.8	4333.99	4268.90	4301.40	164.4	102.8
825.0	4326.64	4323.30	4325.00	163.9	102.4
1032.1	5223.65	5240.70	5232.20	198.6	99.3
1026.8	5407.62	5413.80	5410.70	206.6	103.3
1009.5	5379.66	5377.80	5378.70	208.8	104.4
				Mean	103.20
				SD	1.80
				%RSD	1.80

Atorvastatin met the criteria for intra-day precision (%RSD equal or less than 2.00%), which was 1.80% (APVMA, 2004; Snyder *et al*., 1997).

Table A.19: Repeatability (intra-day precision) results of mevastatin (Day 1)

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
616.80	3595.30	3647.90	3621.60	121.9	101.5
624.10	3666.20	3695.20	3680.70	122.5	102.1
641.50	3784.30	3816.00	3800.10	123.2	102.7
812.50	4822.90	4792.30	4807.60	165.7	103.5
823.50	4878.00	4870.50	4874.30	165.8	103.6
825.10	4806.30	4760.40	4783.40	162.3	101.4
1032.50	5956.10	6020.10	5988.10	204.4	102.2
1006.90	5834.80	5741.20	5788.00	202.4	101.2
1023.80	5938.10	5874.00	5906.00	203.2	101.6
				Mean	102.3
				SD	0.9
				%RSD	0.80

Mevastatin met the criteria for intra-day precision (%RSD equal or less than 2.00%), which was 0.80% (APVMA, 2004; Snyder *et al*., 1997).

Table A.20: Repeatability (intra-day precision) results of pitavastatin (Day 1)

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
623.10	4717.9	4744.4	4731.1	146.4	122.0
623.50	4776.4	4732.2	4754.3	147.0	122.5
630.40	4780.1	4809.7	4794.9	146.7	122.3
833.30	6317.2	6206.4	6261.8	195.9	122.4
804.30	6069.2	5961.1	6015.1	194.6	121.6
837.30	6245.2	6240.2	6242.7	194.3	121.5
1040.60	7591.7	7611.1	7601.4	239.9	119.9
1028.90	7508.2	7500.4	7504.3	239.4	119.7
1001.80	7294.6	7319.1	7306.8	239.1	119.6
				Mean	121.50
				SD	1.00
				%RSD	0.80

Pitavastatin met the criteria for intra-day precision (%RSD equal or less than 2.00%), which was 0.80% (APVMA, 2004; Snyder *et al*., 1997).

Table A.21: Repeatability (intra-day precision) results of pravastatin (Day 1)

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Mean	µg/ml	%
613.80	2756.3	2758.7	2757.5	118.5	98.7
619.30	2783.3	2787.8	2785.5	118.7	98.9
610.60	2724.5	2725.4	2725.0	117.6	98.0
805.10	3631.7	3565.4	3641.9	161.1	100.7
820.80	3641.9	3720.4	3681.1	159.8	99.9
823.50	3724.6	3735.2	3729.9	161.4	100.9
1001.40	4443.3	4386.7	4415.0	197.6	98.8
1036.70	4626.1	4628.6	4627.4	200.3	100.2
1009.40	4385.4	4449.2	4417.3	196.1	98.1
				Mean	99.50
				SD	1.00
				%RSD	1.00

Pravastatin met the criteria for intra-day precision (%RSD equal or less than 2.00%), which was 1.00% (APVMA, 2004; Paithankar, 2013; Snyder *et al*., 1997).

From Tables A.18 – A.21, it can be concluded that all samples tested fell within the acceptable %RSD limits of ≤ 2.0%:

- Atorvastatin: 1.8%
- Mevastatin: 0.8%
- Pitavastatin: 0.8%
- Pravastatin: 1.0%

A.3.3.2 Precision (inter-day)

Intermediate precision can be defined as the degree of comparison between multiple measurements utilising identical methods under similar conditions (ICH, 2005), performed in the same laboratory over a few consecutive days (Huber, 2007). Reproducibility of the analytical methods was achieved via analysis of three homogenous samples with known concentrations on three consecutive days. The acceptable criteria for intermediate precision should be equal to or less than 3.00% (Rafael *et al*., 2007).

In this study, a concentration of 0.8 g was utilised in triplicate and diluted with 100 ml methanol. The first day, the results of repeatability was used. The second day, the samples were prepared as follow: three flasks with 4.0 ml (16 mg) spiked solution and 0.8 g placebo nano-emulsion were

diluted with 100.0 ml methanol for each statin. These samples were adequately dissolved in an ultrasonic bath. Approximately 1.0 ml of each flask was transferred to HPLC vials to be analysed (in duplicate) via HPLC. A standard solution was prepared by dissolving approximately 20 mg of each API (statin) in 100.0 ml methanol. On the third day, the following samples were prepared: three volumetric flasks contained 4.0 ml (916 mg) of the spike solution and 0.8 g nano emulsion, which was diluted with 100.0 ml methanol for each statin. Samples were adequately dissolved in an ultrasonic bath. Approximately 1.0 ml of each flask was extracted and transferred to HPLC vials to be analysed via HPLC. For inter-day precision the same method was used as for intra-day precision; however, only one concentration was chosen for this validation; the intermediate amount of 0.8 g of each of the selected statins was diluted with 100.0 ml methanol (in triplicate), placed into HPLC vials for analysis and injected in duplicate volumes of 2.5 µl, 5.0 µl, 7.5 µl and 10.0 µl.

Table A.22: Inter-day precision of atorvastatin

Day	Concentration spiked (µg/ml)	Peak area			Recovery		Mean	SD	%RSD
		1	2	Average	(µg/ml)	%			
1	805.8	4364.7	4361.9	4363.3	169.3	105.8	103.2	1.8	1.80
	817.8	4334.0	4268.9	4301.4	164.4	102.8			
	825.0	4326.6	4323.3	4325.0	163.9	102.4			
2	811.0	4487.8	4377.0	4432.4	177.5	111.0	110.0	0.7	0.60
	836.8	4510.4	4506.0	4508.2	175.1	109.4			
	812.9	4384.5	4401.4	4393.0	175.5	109.7			
3	825.4	4458.9	4445.6	4452.0	175.3	109.5	109.3	0.3	0.28
	812.0	4359.0	4355.6	4357.0	174.2	108.9			
	819.5	4413.9	4429.2	4422.0	175.3	109.5			

The %RSD for atorvastatin was determined as 1.80% (Day 1), 0.60% (Day 2) and 0.28% (Day 3), which was smaller than the 3% recommended level of precision (APVMA, 2004; Rafael *et al*., 2007).

Table A.23: Inter-day precision of mevastatin

Day	Concentration spiked (µg/ml)	Peak area			Recovery		Mean	SD	%RSD
		1	2	Average	(µg/ml)	%			
1	812.5	4822.9	4792.3	4807.6	165.7	103.5	102.3	0.9	0.80
	823.5	4878.0	4870.5	4874.3	165.8	103.6			
	825.1	4806.3	4760.4	4783.4	162.3	101.4			
2	805.0	4729.7	4748.3	4739.0	159.9	99.9	100.9	0.8	0.78
	821.7	4854.4	4902.8	4879.0	161.4	100.9			
	810.9	4850.1	4871.7	4861.0	162.9	101.8			
3	829.6	5255.7	5189.4	5223.0	171.6	107.2	107.2	1.1	1.04
	818.2	5249.7	5176.4	5213.0	173.6	108.5			
	820.6	5105.3	5096.8	5101.0	169.3	105.8			

The %RSD for mevastatin was determined as 0.80% (Day 1), 0.78% (Day 2) and 1.04% (Day 3), which was smaller than the 3% recommended level of precision (APVMA, 2004; Rafael *et al*., 2007).

Table A.24: Inter-day precision of pitavastatin

Day	Concentration spiked (µg/ml)	Peak area			Recovery		Mean	SD	%RSD
		1	2	Average	(µg/ml)	%			
1	833.3	6317.2	6206.4	6261.8	195.9	122.4	121.5	1.0	0.80
	804.3	6069.2	5961.1	6015.1	194.6	121.6			
	837.3	6245.2	6240.2	6242.7	194.3	121.5			
2	802.8	3885.4	3901.0	3893	148.7	92.9	92.7	0.8	0.90
	801.4	3925.1	3899.4	3912	149.7	93.6			
	822.2	3968.2	3886.8	3927	146.5	91.6			
3	823.8	3847.1	3813.0	3830	142.5	89.1	90.1	1.0	1.06
	832.4	3971.0	3959.1	3965	146.1	91.3			
	825.8	3866.9	3869.5	3868	143.6	89.7			

The %RSD for pitavastatin was determined as 0.80% (Day 1), 0.90% (Day 2) and 1.06% (Day 3), which was smaller than the 3% recommended level of precision (APVMA, 2004; Rafael *et al*., 2007).

Table A.25: Inter-day precision of pravastatin

Day	Concentration spiked (µg/ml)	Peak area			Recovery		Mean	SD	%RSD
		1	2	Average	(µg/ml)	%			
1	805.1	3631.7	3565.4	3641.9	161.1	100.7	99.5	1.0	1.00
	820.8	3641.9	3720.4	3681.1	159.8	99.9			
	823.5	3724.6	3735.2	3729.9	161.4	100.9			
2	813.4	5325.5	5259.0	5292.0	175.5	109.7	109.4	0.2	0.20
	829.6	5379.8	5382.8	5381.0	175.1	109.4			
	817.9	5291.9	5299.9	5296.0	174.7	109.2			
3	825.0	3685.4	3708.5	3697.0	161.6	101.0	100.7	0.3	0.30
	822.1	3672.4	3649.6	3661.0	160.5	100.3			
	803.2	3600.9	3601.6	3601.0	161.5	100.9			

The %RSD for pravastatin was determined as 1.0% (Day 1), 0.20% (Day 2) and 0.30% (Day 3), which was smaller than the 3% recommended level of precision (APVMA, 2004; Rafael *et al*., 2007).

A.3.4 Robustness

Robustness can be described as the constancy of an analytical measurement after methodical alterations of specific parameters (ICH, 2005; Naseef *et al*., 2018; Paithankar, 2013). This indicates the reliability during normal usage (ICH, 2005; Paithankar, 2013). Attained measurements indicate whether retention time peaks of samples on chromatograms were affected by the variations of parameters (Paithankar, 2013; Snyders *et al*., 1997). The data obtained by this experiment will establish if revalidation of a method is required due to possible altered parameters (Huber, 2007; Paithankar, 2013). Ruggedness is defined as the level to which results are reproducible under specific diverse standard test conditions (USP, 2011), e.g. variation of laboratories, operators, reagent batches, assay temperatures and days (Huber, 2007). Ruggedness will not be performed in this study as there is access to one only laboratory with HPLC equipment. Characteristic alterations with the utilisation of HPLC includes: variations of columns, alteration in flow rate, alteration in temperature, gradient alteration, wavelength alteration, alteration of the pH in mobile phase (ICH, 2005).

This study determined robustness through the alternation of the gradient, flow rate and wavelength. A standard solution was formulated, which contained ± 20 mg of the respective statins in separate 100 ml volumetric flasks. Samples were diluted with methanol as solvent (100 ml). The statins were dissolved in flasks and placed in an ultrasonic bath (Elma Transonic EL540). Approximately 1 ml of each statin flask was extracted and transferred to HPLC

vials and analysed. These four standard solutions were analysed under normal chromatographic conditions (Tables A.26 – A.29, see Sample 1) and diverse chromatographic conditions (Tables A.26 – A.29, see Samples 2 and 3) to establish if the alterations would affect the sample and retention time. The standard dilution of respective statins was injected in triplicate at diverse parameters. The first injection (25% acetonitrile, 1.0 ml flow rate, and 240 nm wavelength) was done at normal parameters, which was utilised as a reference. The second injection operated at 20% acetonitrile, 1.2 ml flow rate and 244 nm wavelength. The third injection operated at 30% acetonitrile, 0.8 ml flow rate and 244 nm wavelength. Figure A.6 displays the chromatogram for the three injections with diverse parameters for the selected statins.

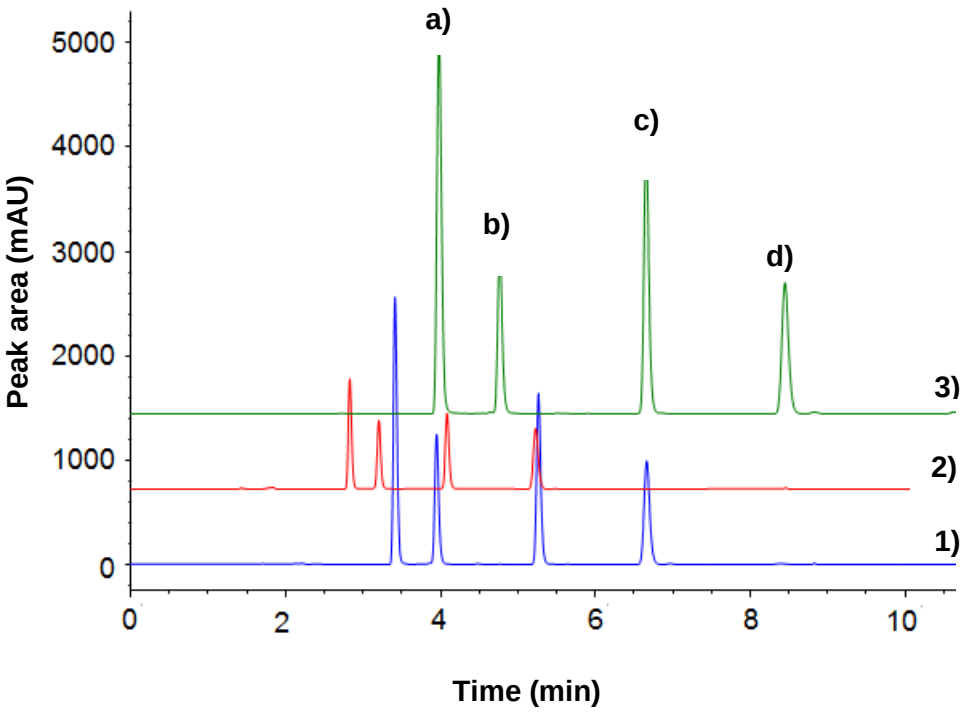


Figure A.6: This HPLC chromatogram illustrates robustness data of a standard solution injected at altered test parameters, e.g. a) pitavastatin, b) pravastatin, c) atorvastatin and d) mevastatin, while 1) represents normal conditions operating at 1.0 ml/min flow rate, 240 nm wavelength and 25% acetonitrile; 2) diverse conditions operating at 1.2 ml/min flow rate, 236 nm wavelength and 20% acetonitrile, and 3) diverse conditions operating at 0.8 ml/min flow rate, 244 nm wavelength and 30% acetonitrile

Table A.26: Robustness pre- and post-alternation data for atorvastatin

Sample	HPLC parameters					
	%ACN mobile phase	Injection volume (µl)	Flow rate (ml/min)	Wavelength (nm)	Peak area(mAU)	Retention time (min)
1	25	10	1.0	240	5309.32	5.27
2	20	5	1.2	236	2484.61	4.08
3	30	15	0.8	244	11831.50	6.66

Table A.27: Robustness pre- and post-alternation data for mevastatin

Sample	HPLC parameters					
	%ACN mobile phase	Injection volume (µl)	Flow rate (ml/min)	Wavelength (nm)	Peak area (mAU)	Retention time (min)
1	25	10	1.0	240	4454.28	6.69
2	20	5	1.2	236	2595.81	5.23
3	30	15	0.8	244	7610.57	8.45

Table A.28: Robustness pre- and post-alternation data for pitavastatin

Sample	HPLC parameters					
	%ACN mobile phase	Injection volume (µl)	Flow rate (ml/min)	Wavelength (nm)	Peak area (mAU)	Retention time (min)
1	25	10	1.0	240	6618.18	3.41
2	20	5	1.2	236	1054.74	2.84
3	30	15	0.8	244	15696.30	6.66

Table A.29: Robustness pre- and post-alternation data for pravastatin

Sample	HPLC parameters					
	%ACN mobile phase	Injection volume (µl)	Flow rate (ml/min)	Wavelength (nm)	Peak area (mAU)	Retention time (min)
1	25	10	1.0	240	4472.68	3.95
2	20	5	1.2	236	653.43	3.27
3	30	15	0.8	244	6438.67	4.76

Tables A.26 – A.29 indicate peak areas and retention times for respective statins. The peak area and retention time values of the second and third injection was minimal in comparison to the first injection. Therefore, minimal variation of peak areas is indicated. The shifts in peak retention times for each of the respective statins were as follow:

- Atorvastatin: 5.27 – 6.66 min

- Mevastatin: 6.69 – 8.45 min
- Pitavastatin: 3.414 – 6.66 min
- Pravastatin: 3.95 – 4.76 min

A.3.5 System stability

System stability determines if the validation method utilised can accurately perceive degradation of the compounds (Huber, 2007). Therefore, this method quantifies the biasness of the experiment, which is performed on stock solutions that contains a specific concentration of the chemical compound in an appropriate solvent measured in hourly intervals (Huber, 2007).

This study utilised ± 10 mg (100 µg/ml) of the respective statin sample, which was prepared and diluted with methanol (100 ml). Approximately 1 ml was extracted and transferred to HPLC vials to be analysed. The sample was injected in 1 h time intervals up to 24 h. A solution with a degradation value higher than 2%, signifies an unstable sample; therefore, not to be utilised from the time of degradation (Paithankar, 2013).

Table A.30: Sample stability data for atorvastatin

Time (h)	Peak area	%Remaining
0	2415.8	100.0
1	2411.3	99.8
2	2395.2	99.1
3	2391.4	99.0
4	2401.1	99.4
5	2399.8	99.3
6	2401.8	99.4
7	2393.0	99.1
8	2395.2	99.1
9	2397.7	99.2
10	2395.2	99.1
11	2400.1	99.3
12	2402.3	99.4
13	2405.5	99.6
14	2400.0	99.3
15	2404.7	99.5
16	2402.0	99.4
17	2410.8	99.8
18	2409.0	99.7
19	2406.4	99.6
20	2412.4	99.9
21	2410.6	99.8
22	2412.2	99.9
23	2417.3	100.1
24	2414.8	100.0
Mean	2404.2	99.5
SD	7.40	0.31
%RSD	0.31	0.31

The %RSD for atorvastatin was 0.31%, which is considered stable and acceptable as the degradation value was less than 2.00% (Paithankar, 2013).

Table A.31: Sample stability data for mevastatin

Time (h)	Peak area	%Remaining
0	2672.0	100.0
1	2656.2	99.4
2	2646.2	99.0
3	2628.3	98.4
4	2624.0	98.2
5	2613.5	97.8
6	2602.9	97.4
7	2602.4	97.4
8	2577.7	96.5
9	2569.0	96.1
10	2555.0	95.6
11	2548.6	95.4
12	2548.5	95.4
13	2532.0	94.8
14	2534.5	94.9
15	2534.6	94.9
16	2548.8	95.4
17	2548.4	95.4
18	2534.0	94.8
19	2534.5	94.9
20	2534.6	94.9
21	2548.9	95.4
22	2548.5	95.4
23	2534.0	94.8
24	2534.9	94.9
Mean	2572.5	96.3
SD	43.42	1.63
%RSD	1.69	1.69

The %RSD for mevastatin was 1.69%, which is considered stable and acceptable as the degradation value was less than 2.00% (Paithankar, 2013).

Table A.32: Sample stability data for pitavastatin

Time (h)	Peak area	%Remaining
0	3346.6	100.0
1	3348.1	100.0
2	3342.7	99.9
3	3278.9	98.0
4	3352.9	100.2
5	3292.6	98.4
6	3318.7	99.2
7	3337.3	99.7
8	3327.9	99.4
9	3323.3	99.3
10	3345.4	100.0
11	3315.0	99.1
12	3348.2	100.0
13	3335.4	99.7
14	3295.2	98.5
15	3343.8	99.9
16	3324.0	99.3
17	3285.8	98.2
18	3318.5	99.2
19	3316.8	99.1
20	3335.7	99.7
21	3295.9	98.5
22	3278.6	98.0
23	3260.0	97.4
24	3259.6	97.4
Mean	3317.1	99.1
SD	27.98	0.84
%RSD	0.84	0.84

The %RSD for pitavastatin was 0.84%, which is considered stable and acceptable as the degradation value was less than 2% (Paithankar, 2013).

Table A.33: Sample stability data for pravastatin

Time (h)	Peak area	%Remaining
0	2389.9	100.0
1	2386.5	99.9
2	2388.9	100.0
3	2386.1	99.8
4	2393.7	100.2
5	2393.4	100.1
6	2393.6	100.2
7	2389.1	100.0
8	2392.1	100.1
9	2395.2	100.2
10	2393.6	100.2
11	2397.6	100.3
12	2399.6	100.4
13	2402.3	100.5
14	2397.2	100.3
15	2400.5	100.4
16	2397.5	100.3
17	2404.0	100.6
18	2399.8	100.4
19	2400.2	100.4
20	2405.0	100.6
21	2402.9	100.5
22	2403.0	100.5
23	2406.1	100.7
24	2405.1	100.6
Mean	2396.9	100.3
SD	5.93	0.25
%RSD	0.25	0.25

The %RSD for pravastatin was 0.25%, which is considered stable and acceptable as the degradation value was less than 2.00% (Paithankar, 2013).

A.3.6 System repeatability

System repeatability was determined to evaluate the repeatability of peak areas and retention times of samples under the same conditions. To establish the repeatability of the system, respective statin (atorvastatin, mevastatin, pitavastatin and pravastatin) samples were prepared. Approximately 20 mg was weighed and transferred into a 100 ml volumetric flask, which was

diluted with methanol (100 ml). Approximately 1 ml of each selected statin solution were extracted and transferred in marked HPLC vials. These samples were then injected into the HPLC (seven consecutive times). The %RSD should be less than 2% for both peak area and retention times for the method to be acceptable and reliable (Cowley, 2012; Paithankar, 2013).

Table A.34: System repeatability concerning atorvastatin

Injection	Peak area (mAU)	Retention time (min)
1	2378.5	3.953
2	2373.9	3.948
3	2374.2	3.940
4	2375.0	3.938
5	2376.0	3.936
6	2378.2	3.940
7	2381.9	3.946
Mean	2376.8	3.943
SD	2.68	0.006
%RSD	0.11	0.144

Atorvastatin system repeatability was determined as 0.11% (peak area) and 0.144% (retention time), which meets the criteria, since the %RSD was less than 2.00% (for both the peak area and retention time) (Cowley, 2012; Paithankar, 2013).

Table A.35: System repeatability concerning mevastatin

Injection	Peak area (mAU)	Retention time (min)
1	2699.7	6.567
2	2694.8	6.582
3	2692.6	6.570
4	2688.8	6.568
5	2687.8	6.560
6	2681.7	6.572
7	2686.2	6.571
Mean	2690.2	6.570
SD	5.51	0.006
%RSD	0.20	0.093

Mevastatin system repeatability was determined as 0.20% (peak area) and 0.093% (retention time), which meets the criteria, since the %RSD was less than 2.00% (for both the peak area and retention time) (Cowley, 2012; Paithankar, 2013).

Table A.36: System repeatability concerning pitavastatin

Injection	Peak area (mAU)	Retention time (min)
1	3248.5	3.422
2	3300.4	3.421
3	3214.2	3.406
4	3282.1	3.412
5	3226.9	3.407
6	3303.9	3.415
7	3319.8	3.417
Mean	3270.8	3.414
SD	38.04	0.006
%RSD	1.16	0.171

Pitavastatin system repeatability was determined as 1.16% (peak area) and 0.171% (retention time), which meets the criteria, since the %RSD was less than 2.00% (for both the peak area and retention time) (Cowley, 2012; Paithankar, 2013).

Table A.37: System repeatability concerning pravastatin

Injection	Peak area (mAU)	Retention time (min)
1	2378.5	3.953
2	2373.9	3.948
3	2374.2	3.940
4	2375.0	3.938
5	2376.0	3.936
6	2378.2	3.940
7	2381.9	3.946
Mean	2376.8	3.943
SD	2.68	0.006
%RSD	0.11	0.144

Pravastatin system repeatability was determined as 0.11% (peak area) and 0.144% (retention time), which meets the criteria, since the %RSD was less than 2.00% (for both the peak area and retention time) (Cowley, 2012; Paithankar, 2013).

A.3.7 Specificity

Specificity of an analytical method can be described as the ability of the method to identify an analyte accurately in the occurrence of degradants, the matrix or impurities. To accurately establish specificity, tests are performed to authenticate the reaction of the peak (tested

compound) in the presence of impurities (Huber, 2007; ICH 2005). Therefore, no peak interference with the retention time of the API should be observed (Snyders *et al*., 1997).

The specificity was determined via the preparation of six samples as follow:

1. A placebo vial was equipped by adding methanol to an HPLC vial.
2. A standard solution was prepared by adding approximately 20 mg of each respective statin in a volumetric flask (100 ml). Approximately 1 ml was placed in the HPLC vial.
3. Four samples were prepared by using the standard solution. Approximately 1 ml were placed in four separate test tubes. The standard solution was diluted by adding 200 µl of water (Milli-Q® water), 2 M sodium hydroxide (NaOH), 2 M hydrochloric acid (HCl) and 2 M hydrogen peroxide (H_2O_2) in individual test tubes, which were mixed thoroughly via vortexing.
4. Approximately 1 ml of the stationary test tubes were transferred into a HPLC vial.
5. Samples were injected (duplicate) in the HPLC with a 10 min run time.

Table A.38: Specificity data for atorvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
a) H_2O	4551.6	4555.7	4553.7	5.262
b) HCl	3779.8	3781.0	3780.4	5.808
c) NaOH	4600.1	4597.6	4598.9	5.267
d) H_2O_2	4622.8	4618.9	4620.8	5.260

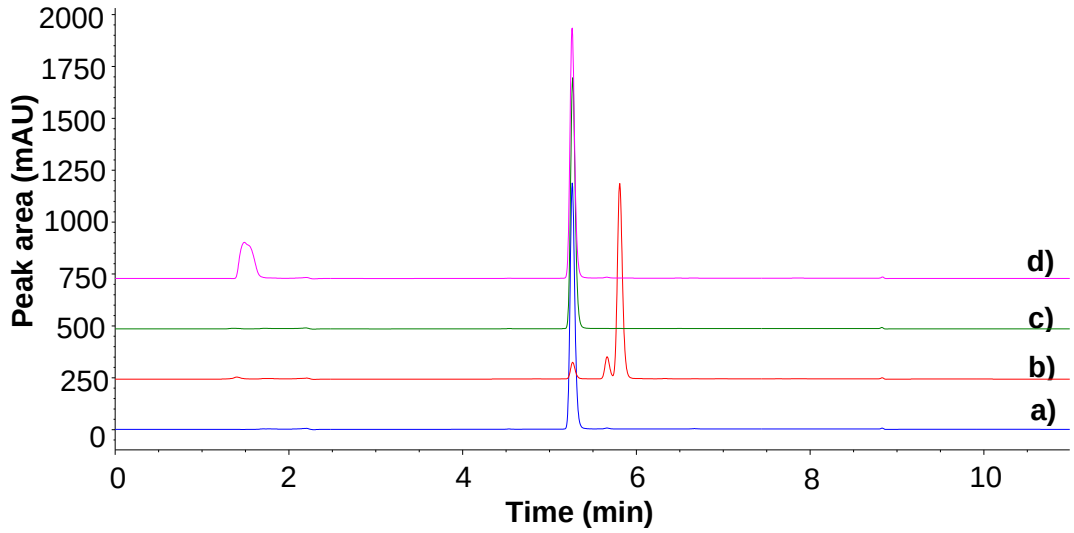


Figure A.7: HPLC chromatogram displaying specificity data obtained for atorvastatin with 200 µl of: a) H_2O, b) HCl, c) NaOH and d) H_2O_2

Table A.39: Specificity data for mevastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
a) H_2O	4226.5	4220.9	4223.7	6.664
b) HCl	3428.9	3436.7	3432.8	6.974
c) NaOH	4361.4	4366.3	4363.9	5.786
d) H_2O_2	4316.6	4314.4	4315.5	6.691

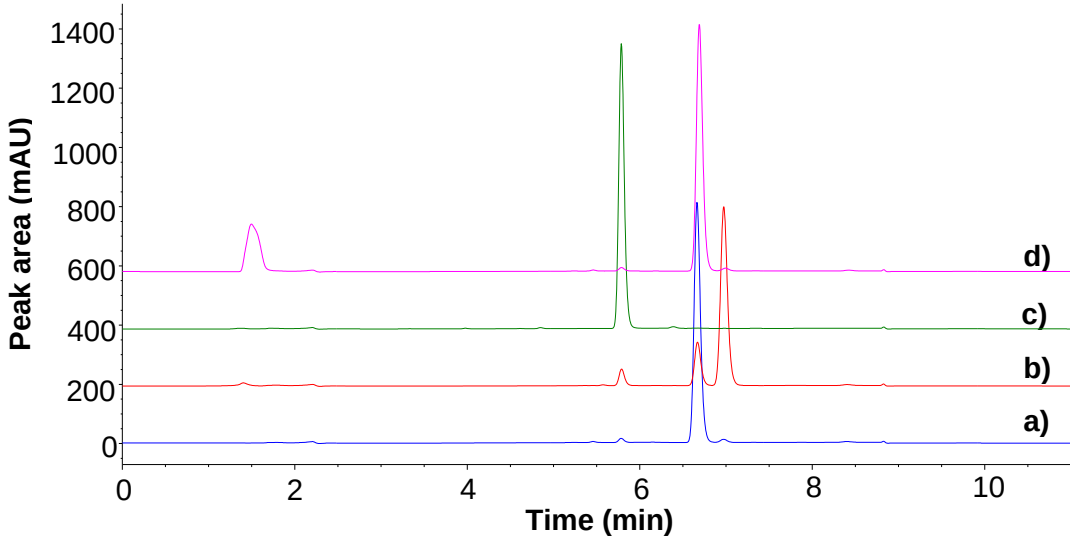


Figure A.8: HPLC chromatogram displaying specificity data obtained for mevastatin with 200 µl of: a) H_2O, b) HCl, c) NaOH and d) H_2O_2

Table A.40: Specificity data for pitavastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
a) H_2O	5683.1	5702.5	5692.8	3.414
b) HCl	4561.7	4616.3	4589.0	3.730
c) NaOH	5733.8	5681.1	5707.4	3.412
d) H_2O_2	5834.4	5871.9	5853.2	3.410

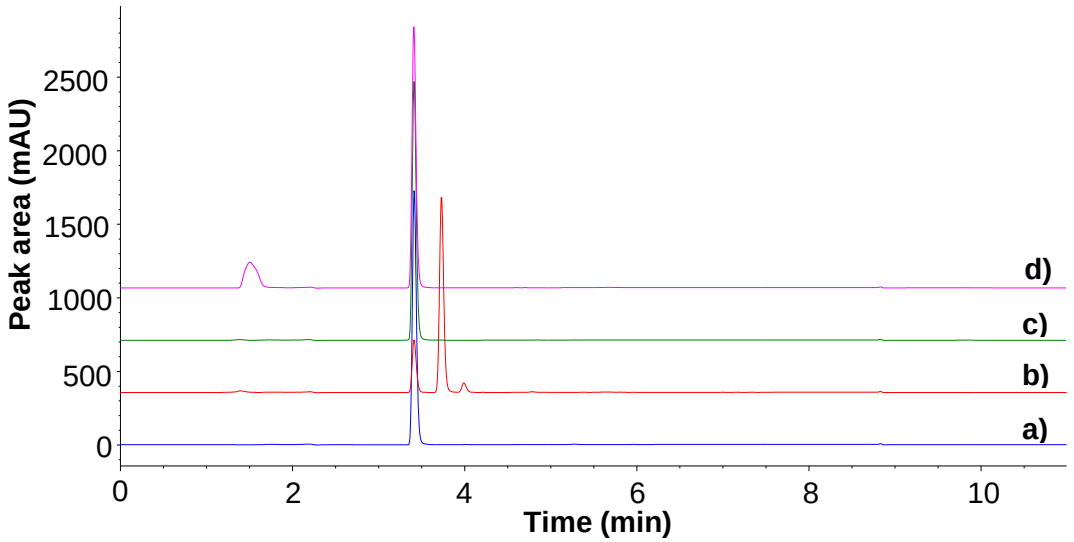


Figure A.9: HPLC chromatogram displaying specificity data obtained for pitavastatin with 200 µl of: a) H_2O, b) HCl, c) NaOH and d) H_2O_2

Table A.41: Specificity data for pravastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
a) H_2O	3830.0	3825.8	3827.9	3.940
b) HCl	2400.3	2398.5	2399.4	5.878
c) NaOH	3816.5	3810.9	3813.7	3.940
d) H_2O_2	3922.1	3923.1	3922.6	3.943

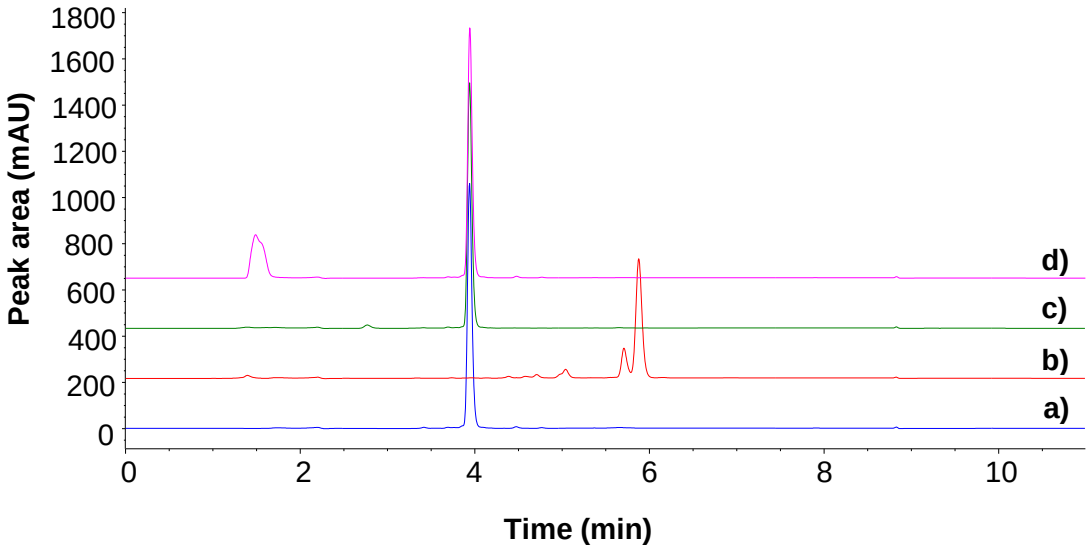


Figure A.10: HPLC chromatogram displaying specificity data obtained for pravastatin with 200 µl of: a) H_2O, b) HCl, c) NaOH and d) H_2O_2

A.3.8 Limit of detection and limit of quantitation

Limit of detection (LOD) can be described, as the lowest concentration of an analyte in a sample that can be detected; however, not quantified (APVMA, 2004; Snyder *et al*., 1997). Lowest limit of detection (LLOD) refers to the lowest amount of an analyte in a sample, which cannot be quantified, but can possibly be detected (ICH, 2005). The LOD is determined by analysing samples with documented analyte concentrations and calculated analyte (lowest level), which can be identified under certain analytical conditions (APVMA, 2004, Snyder *et al*., 1997). The peak response of the lowest calibration standard that links with the analyte is measured six to ten times (APVMA, 2004). The acceptable criterion for LOD is a maximum %RSD of 15% (FDA, 2001; Rathmann *et al*., 2015).

Limit of quantitation (LOQ) can be described as the lowest detectable amount of analyte in a sample under certain conditions (value can be determined) (APVMA, 2004). Lower limit of quantitation (LLOQ) refers to the lowest amount of analyte in a sample that can be quantitatively determined with suitable accuracy and precision under specific conditions (APVMA, 2004; Rozet *et al*., 2011; Wolfinger *et al*., 2018). LOQ is utilised as a guideline for analytical procedures, particularly for detecting impurities, degradation of products and active substances in low quantities (APVMA, 2004). The solution sample should be injected six to ten times (APVMA, 2004). The acceptable criterion for LOQ is maximum %RSD of 20% (FDA, 2001; González *et al*., 2014, Rathmann *et al*., 2015).

A solution was prepared that contained 5 mg of the respective statin in a 100 ml volumetric flask, which was diluted with methanol (100 ml). Approximately 1 ml of this solution was transferred in a separate 100 ml volumetric flask via a micropipette and diluted with methanol (100 ml). Then ± 1 ml of the diluted solution was extracted and transferred in a HPLC vial for analysis. The solution was injected six times in the HPLC at altered injection volumes, e.g. 2.5, 5.0, 7.5 and 10.0 µl.

Table A.42: LOD and LLOQ results of atorvastatin

LOD/LLOQ	LOD	LLOQ
Injection volume	2.5	10.0
Concentration (µg/ml)	0.138	0.550
Peak area	3.5	14.9
	3.6	14.8
	3.7	14.9
	3.2	14.9
	3.6	14.8
	3.2	14.9
Mean	3.47	14.87
SD	0.200	0.057
RSD%	5.69	0.32

The %RSD of atorvastatin regarding LOD (5.69%) and LLOQ (0.32%) were below 15 and 20%, respectively; thus, the criteria was met (FDA, 2001; González *et al*., 2014; Rathmann *et al*., 2015).

Table A.43: LOD and LLOQ results of mevastatin

LOD/LLOQ	LOD	LLOQ
Injection volume	2.5	10.0
Concentration (µg/ml)	0.130	0.520
Peak area	2.8	13.6
	3.0	13.7
	3.0	13.2
	2.6	13.7
	2.8	13.1
	2.6	13.7
Mean	2.80	13.50
SD	0.160	0.250
RSD%	5.83	1.86

The %RSD of mevastatin regarding LOD (5.83%) and LLOQ (1.86%) were below 15 and 20%, respectively; thus, the criteria was met (FDA, 2001; González *et al*., 2014; Rathmann *et al*., 2015).

Table A.44: LOD and LLOQ results of pitavastatin

LOD/LLOQ	LOD	LLOQ
Injection volume	2.5	10.0
Concentration (µg/ml)	0.145	0.580
Peak area	6.2	22.4
	6.1	22.4
	6.3	22.1
	6.0	22.5
	6.1	22.5
	6.2	22.3
Mean	6.15	22.37
SD	0.100	0.140
RSD%	1.56	0.61

The %RSD of pitavastatin regarding LOD (1.56%) and LLOQ (0.61%) were below 15 and 20%, respectively; thus, the criteria was met (FDA, 2001; González *et al*., 2014; Rathmann *et al*., 2015).

Table A.45: LOD and LLOQ results of pravastatin

LOD/LLOQ	LOD	LLOQ
Injection volume	2.5	10.0
Concentration (µg/ml)	0.135	0.540
Peak area	3.5	13.8
	3.4	13.8
	3.7	13.7
	3.4	13.7
	3.4	13.7
	3.4	13.8
Mean	3.47	13.75
SD	0.110	0.050
RSD%	3.19	0.36

The %RSD of pravastatin regarding LOD (3.19%) and LLOQ (0.36%) were below 15 and 20%, respectively; thus, the criteria was met (FDA, 2001; González *et al*., 2014; Rathmann *et al*., 2015).

A.3.9 Conclusion

The HPLC method for the respective statins, e.g. atorvastatin, mevastatin, pitavastatin and pravastatin, was successfully validated. This method proved to be precise, reliable and constant. The method will be able to quantify the respective statins in both the nano-emulsions and nano-emulgels. The obtained results proposed verification for reliability, accuracy and credibility; therefore, it is appropriate to analyse and detect the respective statins in the formulations. The acceptance criteria were thus met by this method. As a result, this HPLC method will also be utilised and applied for the testing of *in vitro* skin diffusion studies to quantify the respective statins.

References

APVMA see Australian Pesticides and Veterinary Medicines Authority.

Australian pesticides and veterinary medicines authority. 2004. Guidelines for the validation of analytical methods for active constituents, agricultural and veterinary chemical products. http://apvma.gov.au/sites/default/files/docs/guideline-69-analytical-methods.pdf Date of access: 12 Jan. 2019.

Bansal, S. & DeStefano, A. 2007. Key elements of bioanalytical method validation for small molecules. American association of pharmaceutical scientists. *The AAPS journal,* 9(1):109-144.

Cowley, A. 2012. The effect of selected natural oils on the permeation of flurbiprofen through human skin. Potchefstroom: NWU. (Dissertation - MSc).

FDA see Food and Drug Administration.

Food and drug administration. 2001. Guidance for Industry: bioanalytical method validation. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf Date of access: 16 Jan. 2019.

González, O., Blanko, M.E., Iriart, G., Bartolomé, L., Maguregui, M.I. & Alonso, R.M. 2014. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *Journal of chromatography A*, 1353:10-27.

Graham, P. 2011. Quality control in the pharmaceutical industry. (In Moffat, A.C., Osselton, M.D. & Widdop, B., eds. Clarke's analysis of drugs and poisons. London: Pharmaceutical Press.).

Huber, L. 2007. Validation of Analytical Methods. Agilent technologies: *USA*. 1-76p.

ICH see International Conference of Harmonisation. 2005. Validation of analytical procedures: text and methodology Q2 (R1). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf Date of access: 11 Feb. 2019.

Johnson, J.D. & Van Buskirk, G.E. 1998. Analytical method validation. *Journal of validation technology*, 22:1-5.

Naseef, H., Moqadi, R. & Qurt, M. 2018. Development and validation of an HPLC method for determination of antidiabetic drug alogliptin benzoate in bulk and tablets. *Journal of analytical methods in chemistry*, 2(3):12-16.

Paithankar, H.V. 2013. HPLC method validation for pharmaceuticals. *International journal of universal pharmacy and bio sciences*, 2(4):229–240.

Rafael, J.A., Jabor, J.R., Casagrande, R., Georgetti,, S.R., De Fátima Borin, M., Fonseca, M.J.V. 2007. Validation of HPLC, DPPH and nitrosation methods for mesalamine determination in pharmaceutical dosage forms. *Brazillian journal of pharmaceutical sciences*, 43(1):97-103.

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

Rozet, E., Marini, R.D., Ziemons, E., Boulanger, B. & Hubert, Ph. 2011. Advances in validation, risk and uncertainty assessment of bioanalytical methods. *Journal of pharmaceutical and biomedical analysis*, 55(4):848:858.

Shabir, G.A. 2003. Validation of high-performance liquid chromatography methods for pharmaceutical analysis Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. *Journal of chromatography*, 987(1-2):57-66.

Snyder, L.R., Kirkland, J.J. & Glajch, J.L. 1997a. Quantitation (Including trace analysis). (*In* Snyder, L.R., Kirkland, J.J. & Glajch, J.L., *eds*. Practical HPLC method development. 2nd *ed*. New York: John Wiley & Sons. p. 643-684.

Snyder, L.R., Kirkland, J.J. & Glajch, J.L 1997b. Completing the method: validation and transfer. (*In* Snyder, L.R., Kirkland, J.J. & Glajch, J.L*., eds*. Practical HPLC method development. 2nd *ed*. New York: John Wiley & Sons. p. 685-713).

Swartz, M.E. & Krull, I.S. 2012. *Handbook of analytical validation*. Boca Raton: CRC Press. USP see United States Pharmacopeia

United States Pharmacopeia. 2009. Validation of compendial methods. https://hmc.usp.org>c1225_1susp40.pdf Date of access: 20 Feb. 2019.

USP see United States Pharmacopeia.

United States Pharmacopeia. 2011. Validation of compendial procedures. http://www.drugfuture.com/pharmacopoeia/usp35/pdf/08770882%20[1225]%20validation%20of%20compendial%20procedures.pdf Date of access: 11 Feb. 2019.

Wolfinger, R.D., Beedanagari, S., Boitier, E., Chen, T., Couttet, P., Ellinger-Ziegelbauer, H., Guillemain, G., Mariet, C., Mouritzen, P., O'Lone, R., Pine, P.S., Sharapova, T., Yan, J., Yuen, P.S. & Thompson, K.L. 2018. Two approaches for estimating the lower limit of quantitation (LLOQ) of microRNA levels assayed as exploratory biomarkers by RT-qPCR. *BMC Biotechnology*, 18:1-9.

APPENDIX B

CHARACTERISATION OF O/W NANO-EMULSIONS CONTAINING RESPECTIVE STATINS AND GRAPESEED OIL

B.1 Introduction

Transdermal preparations refer to dermally applied formulas that contain both the API, as well as penetration enhancing compounds, e.g. grapeseed oil (Wilbur, 2017); these are specifically developed to improve drug delivery beyond the layers of the skin, resulting in adequate systemic absorption (Wilbur, 2017). During this study the focus was on the transdermal route, as it may apply to four statins, namely atorvastatin, mevastatin, pravastatin, and pitavastatin, used for the medical treatment of hypercholesterolaemia. Not all statins have the ideal physicochemical properties for transdermal delivery and therefore would have to be included in a drug delivery vehicle, i.e. nano-emulsions (**NEs**) and/or nano-emulgels (**NGs**). **NEs** were formulated with the use of grapeseed oil and will be discussed further in this Chapter. Effective release of the API can influence drug flux for transdermal or topical delivery (Barry, 2002). However, selection of the correct vehicle for delivery is critical to bring the API effectively in contact with the skin (Weiss, 2014). Furthermore, the selection of an acceptable non-irritating delivery system (**NEs** and **NGs**), compatible with the intended API, is important (Weiss, 2011). Lipophilic drugs (statins) can potentially be delivered transdermally by using o/w **NEs** as delivery system (Chime *et al*., 2014).

Nano-emulsions have become a widely applied formulation for the delivery of pharmaceutical ingredients (Özgün, 2013). The small droplet size presents some useful properties such as robust stability, high surface area per unit volume, visually pleasing transparent appearance, and adjustable rheology (Gupta *et al*., 2016). Nano-emulsions are kinetically stable over long periods of time and relatively insensitive to chemical and physical changes (Gupta *et al*., 2016). This formulation provides rapid penetration of APIs through the skin due to the large surface area of droplets (Sharma *et al*., 2010). **NEs** are considered to act as penetration enhancers themselves due to the excipients used (Reza, 2011). The droplet size of **NEs** ranges between 100 and 500 nm (Nalini *et al*., 2017) and has a limited size distribution (Kumar, 2014). To obtain nano-scale size droplets, emulsification methods are necessary during the formulation process (low- or high energy) (Abolmaali *et al*., 2011; Tadros *et al*., 2004). Therefore, a high energy emulsification method was followed.

Emulsification methods include, jet dispersion, microfluidisation (Kumar, 2014), sonication, high pressure homogenisation and high amplitude ultrasonication (Ochoa *et al*., 2016). **NEs** were emulsified by using an ultrasonicator (Model UP200St, Hielscher Ultrasonics, Teltow, DE). The ultrasonicator creates vibrations, once the probe is inserted in the formulation to produce

cavitation (Kumar, 2014). **NEs** entail two immiscible liquid phases, e.g. oil phase and water phase, which is combined to produce one phase in general (Kumar, 2014). Three types of **NEs** exist, namely w/o (water is dispersed in oil), oil-in-water (o/w; oil droplets in water) and bicontinuous (oil droplets and water are dispersed and surrounded by a continuous system) nano-emulsions (Kumar, 2014).

B.2 Excipients utilised to formulate a nano-emulsion

This study utilised specific ingredients from suppliers to initiate the formulation of the **NEs** as seen in Table B.1.

Table B.1: Ingredients, batch numbers, suppliers and functions utilised to formulate **NEs**

Ingredients	Batch numbers	Suppliers	Function
Atorvastatin	20170518	DB Fine chemicals	API
Mevastatin	USMV-1703006	DB Fine chemicals	API
Pitavastatin	AFM-170502	DB Fine chemicals	API
Pravastatin	0651001	DB Fine chemicals	API
Span® 60	5361721034	Sigma & Aldrich	Lipophilic surfactant/emulsifier
Tween® 80	BCBV7863	Sigma & Aldrich	Hydrophilic surfactant/emulsifier
Grapeseed oil	0712E040876	CJP Chemicals	Oil phase (natural oil) and penetration enhancer
Milli-Q® water	Direct Pure UP	In-Lab	Water phase/solvent

B.2.1 The oil phase

The specific oil used in a **NE** is of essential importance for it can alter skin barrier obstacles, specifically the stratum corneum (Baibhav *et al*., 2011), which can ultimately increase API permeation and absorption (Alexander *et al*., 2012). To achieve an optimised o/w **NE**, a suitable oil phase can be used to obtain maximum API solubility (Kaur *et al*., 2017), which assists the process of emulsification (Debnath *et al*., 2011; Reddy *et al*., 2013; Setya *et al*., 2014).

Natural oils containing both antioxidants and fatty acids are frequently used as penetration enhancers to indorse skin permeation (Fang *et al*., 2004) and are generally used in topical pharmaceutical preparations (Cizinauskas *et al*., 2017). Regarding skin structure and permeability enhancement relationship, unsaturated fatty acids are more effective in increasing the permeability of the skin barrier compared to saturated fats (Čižinauskas *et al*., 2017). Grapeseed oil (natural oil) was used as the main constituent in the oil phase.

Grape seeds are waste products of wineries and are often referred as important agricultural and industrial waste (Dos Santos Freitas *et al.*, 2008) with the potentials to be utilised in

pharmaceutical and cosmetic applications (Bail *et al.*, 2008; Sotiropoulou *et al.*, 2012). Grapeseed oil is safe to use in pharmaceutical, cosmetic and food products (Kaya *et al*., 2008). This oil is exceptionally rich in tannins and oligomeric proanthocyanidins (thousand times richer opposed to other oils) (Aparicio *et al.*, 1999) and that is the reason why it has high stability and resistance to oxidation reactions (Sotiropoulou *et al.*, 2012). Grapeseed oil is also exceptionally high in polyunsaturated fatty acids, vitamin E and phenolic compounds (Fiori *et al*., 2010). Further, this oil comprehends essential unsaturated fatty acids, such as α-linolenic acid (ω-3) and γ-linolenic acid (ω-6) at approximately 61 – 73%, which cannot be synthesised by the human body (Hussein *et al.*, 2015).

B.2.2 The water phase

In this study, o/w **NEs** were formulated, where the water phase formed the greatest amount of liquid. An o/w **NE** can include water or alcohols (Hyma *et al*., 2014) and functions as a solvent (Silva *et al*., 2012). Milli-Q® water was used in the study as the solvent in the water phase.

B.2.3 Surfactants

A surfactant acts as an interfacial film between the different phases of a formulation to stabilise the system it is applied to (Kumar, 2014), by reducing surface tension, enhancing emulsification (Baibhav *et al*., 2011; Hyma *et al*., 2014), easing droplet splitting (decreased particle size) (Huyn, 2012) and improving **NE** stability (Setya *et al*., 2014). The coalescence of newly formed drops is prevented by these surface-active agents (Chime *et al*., 2014; Reddy *et al*., 2013; Setya *et al*., 2014; Tadros *et al*., 2004). The ideal HLB value is between 8 and 19 (Eid *et al*., 2014; Setya *et al*., 2014). For the purpose of this study, Span® 60 (sorbitan monostearate) and Tween® 80 (polysorbate 80) were used.

The HLB value for Span® 60 is 4.7 and needs to be added in the oil phase, which is known as an oil soluble surfactant (Reddy *et al*., 2013; Zhang, 2009). Span® 60 is a non-ionic surfactant that functions as a wetting agent, which increases emulsification (Zhang, 2009) and stability (Baibhav *et al*., 2011; Zhao *et al*., 2013). Span® 60 can modify physical characteristics of formulations by decreasing the interfacial surface tension, which can ultimately increase formulation viscosity (Zhao *et al*., 2013).

Tween® 80 is a broad non-ionic water soluble surfactant (Koocheki *et al*., 2011; Reddy *et al*., 2013) that was initially developed by combining both polyethoxylated sorbitan and oleic acid, which holds an HLB value of 15 (Zhang, 2009). Water-soluble surfactants lessen the amount of energy required to create a **NE** and alter formulation stability (Chime *et al*., 2014). Tween® 80 can also decrease the interfacial tension (Koocheki *et al*., 2011) and acts as an emulsifier, by

increasing melting endurance and providing smooth textures in formulations (Huyn, 2012). Tween® 80 can be distinguished as a thick, yellowish liquid, which can be used in cosmetic products (Huyn, 2012). Consequently, Tween® 80 increases the speed of surfactant absorption by emulsion droplets, which ultimately reduces droplet sizes and enhances viscosity (Koocheki *et al*., 2011).

Surfactants are generally used in various transport systems, e.g. **NEs**, ointments and creams. Span® 60 and Tween® 80 also act as p-glycoprotein inhibitors to support API absorption, besides their wetting, solubilising, permeability enhancing and emulsification properties (Kumarn *et al*., 2011). However, the solubility of the API in an oil formulation remains critical after considering the advantages of surfactants (Debnath *et al*., 2011; Reddy *et al*., 2013; Setya *et al*., 2014). Thus, oil and water solubility of the respective statins in the grapeseed oil had to be established before formulation was initiated.

B.3 Solubility of the selected statins in grapeseed oil

B.3.1 Sample preparation

Exactly 5 ml of grapeseed oil was transferred into 13 individual marked test tubes. Three test tubes were used for each selected statin (atorvastatin, mevastatin, pitavastatin and pravastatin) and the extra test tube only contained grapeseed oil. The oil-containing test tubes was oversaturated with the selected statin and placed into an Elma Transsonic EL540 ultrasonic bath to be dissolved completely. The 13 test tubes were placed in a water bath (32 °C) for 24 h.

Test tubes were removed after 24 h and centrifuged for 15 min at 45 000 rpm. A volume of 1 ml of the supernatant of each test tube was placed in individual volumetric flasks and weighed. The volumetric flasks were then diluted up to 25 ml with tetrahydrofuran (THF) by using a micropipette and mixed adequately until dissolved. A volume of 1 ml THF solution was extracted from each flask containing the grapeseed oil with the respective statin and transferred into distinct marked HPLC vials and analysed by HPLC.

B.3.2 Standard and placebo preparation

A standard solution was prepared for each selected statin, which contained approximately 5 mg of each statin dissolved in 25 ml THF. Then 1 ml of each standard solution were injected into four individual marked HPLC vials and analysed by HPLC.

Exactly 1 ml from the extra test tube that only contained grapeseed oil was weighed and then diluted with THF to a final volume of 25 ml (without any API). Then 1 ml of the placebo was transferred into a marked HPLC vial and analysed by HPLC.

B.3.3 Measured oil solubility values

After HPLC analysis was completed, the concentration of each statin in the oil was established and analysed as follows:

- Atorvastatin: 11.5 mg/ml
- Mevastatin: 120.12 mg/ml
- Pitavastatin: 68.46 mg/ml
- Pravastatin: 0.57 mg/ml

The results obtained during this experiment may be related to what is proposed in the literature, which states that the higher solubility of atorvastatin, mevastatin and pitavastatin compared to pravastatin in grapeseed oil can be due to the higher lipophilicity of the three compounds (Kim *et al*., 2011).

B.4 Formulation goal

The objective was to determine one optimised formula in this study, in which all four statins presented to be stable and complied with the necessitated physical property parameters, which in turn reduced variables, enhanced credibility and created an opportunity for comparability. Before the formulation procedure was initiated, literature and documented studies concerning **NEs**, surfactant ratios, etc. were researched. Thereafter the formulation process was implemented to successfully formulate an optimised nano-emulsion.

B.5 Formulation of nano-emulsions

Table B.2: Statin formula codes

Statin	Formula	API Codes
Atorvastatin (ATO)	**NE1**	**NE-ATO1**
	NE2	**NE-ATO2**
	NE3	**NE-ATO3**
Mevastatin (MEV)	**NE1**	**NE-MEV1**
	NE2	**NE-MEV2**
	NE3	**NE-MEV3**
Pitavastatin (PIT)	**NE1**	**NE-PIT1**
	NE2	**NE-PIT2**
	NE3	**NE-PIT3**
Pravastatin (PRA)	**NE1**	**NE-PRA1**
	NE2	**NE-PRA2**
	NE3	**NE-PRA3**

Various surfactant ratios were used to identify which **NE** formula was the most stable and adequate concerning dissolution. The following three ratios (a, b, c) were studied:

a. **NE1** = Tween® 80:Span® 60 (3:3)

b. **NE2** = Tween® 80:Span® 60 (3:2)

c. **NE3** = Tween® 80:Span® 60 (2:3)

These **NEs** were then formulated and the statins included as illustrated in Figure B.1, which followed allocation of codes, as presented in Table B.2.

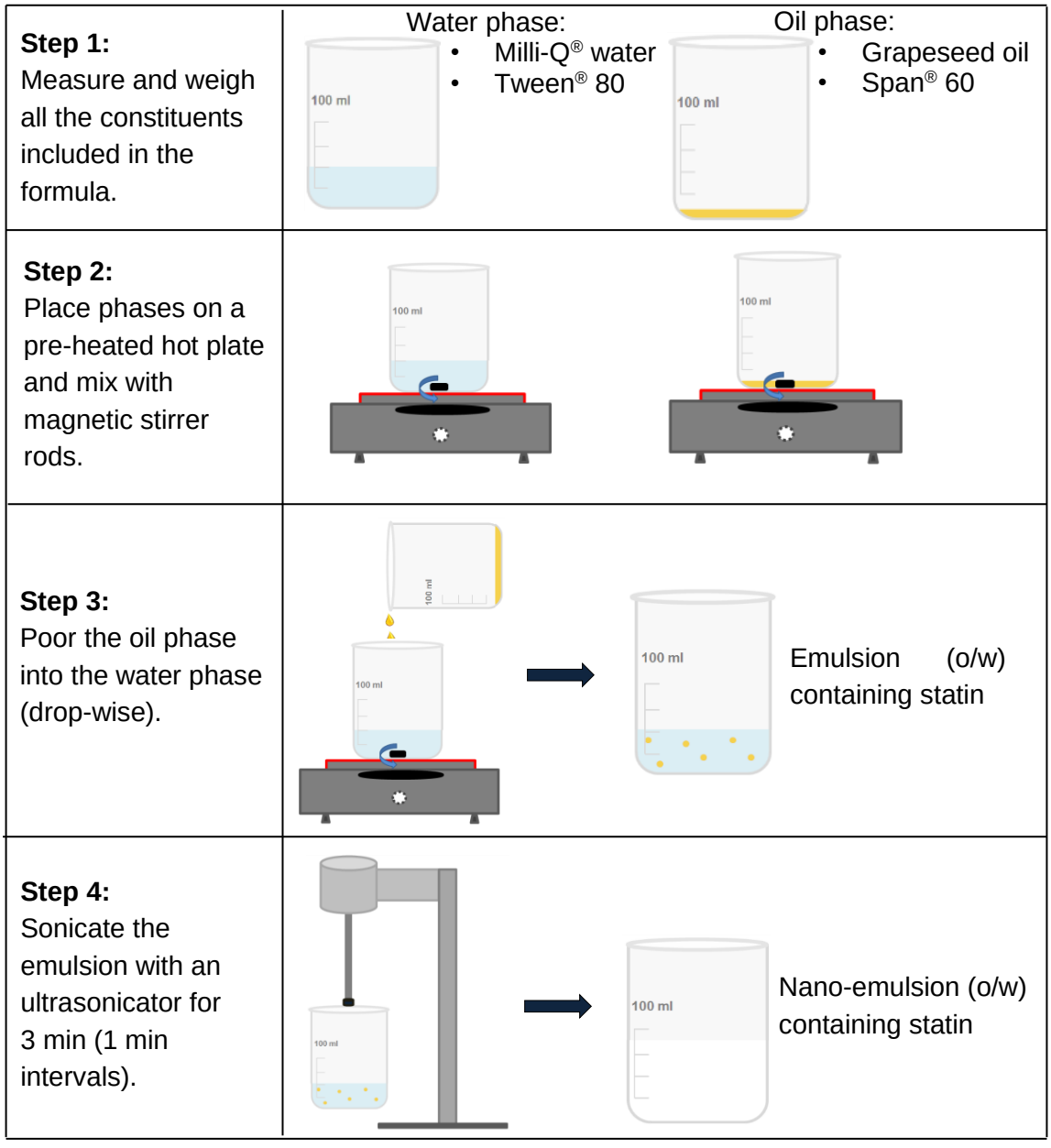


Figure B.1: Method followed to prepare o/w nano-emulsions (for each respective statin)

Table B.3: Nano-emulsion formulas

	Phase	Constituent	Quantity
NE1	**Water**	Milli-Q® water	40.000 ml
		Tween® 80	2.804 ml
	Oil	Statin	1 g
		Span® 60	3 g
		Grapeseed oil	4.350 ml
NE2	**Water**	Milli-Q® water	40.000 ml
		Tween® 80	2.791 ml
	Oil	Statin	1 g
		Span® 60	2 g
		Grapeseed oil	5.459 ml
NE3	**Water**	Milli-Q® water	40.000 ml
		Tween® 80	1.860 ml
	Oil	Statin	1 g
		Span® 60	3 g
		Grapeseed oil	4.367 ml

B.6 Methods utilised in characterisation of nano-emulsions

There are different methods to analyse and characterise nano-emulsions (Nejadmansouria *et al*., 2016). In this study the following characteristics were analysed on **NE1**, **NE2** and **NE3** (Kumar 2014; Nejadmansouria *et al*., 2016):

- Visual inspection
- pH
- Zeta-potential measurement (surface potential)
- Droplet size and distribution
- Viscosity
- Morphological analysis
- Entrapment efficiency (%EE)

B.6.1 Visual inspection

Once **NE** formulation were completed, a Nikon® D3500 DSLR camera was used to capture images (Figure B.2) of the different formula dispersions. The visual inspection concluded that there was no presence of flocculation or sedimentation in the **NEs**. The **NEs** has a whitish-milky

appearance (absence of visible oil droplets); therefore, it can be suggested that oil droplets were efficiently dispersed within the water phase of each **NE**. With visual examination, **NEs** presents watery, and as a result low viscosity values may occur (Thakur *et al*., 2012)

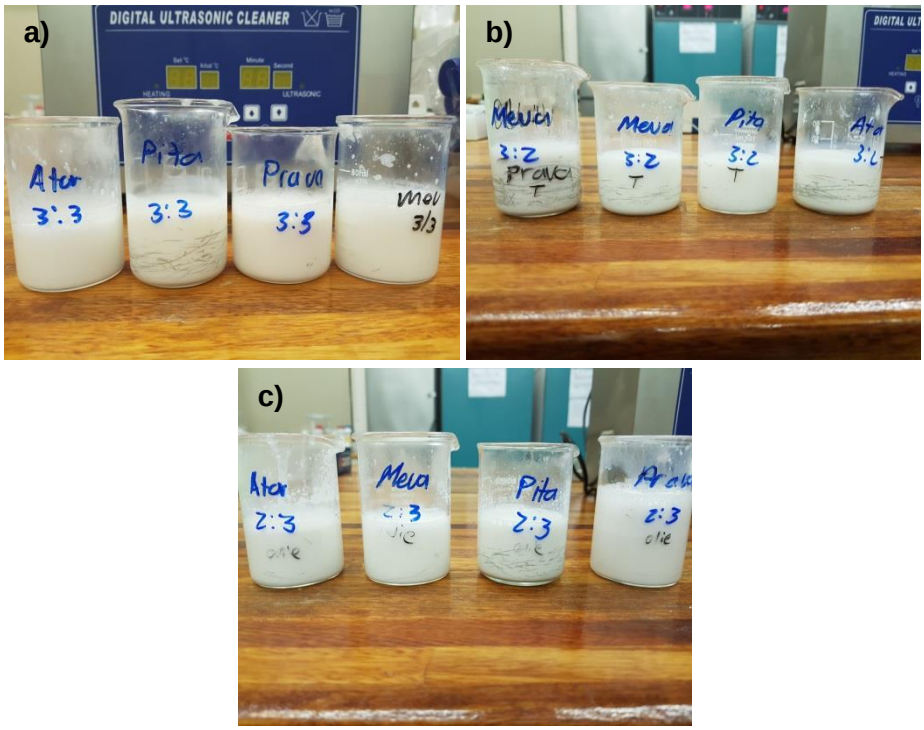


Figure B.2: Photos illustrating: a) **NE1**, b) **NE2** and c) **NE3** containing statins

B.6.2 pH

Measuring the pH of topical formulations is necessary to ensure that the dispersions are safe to use on the skin and non-irritating (Basera *et al*., 2015). The pH measurement establishes a value by the quantity of hydrogen ions [H^+] and hydroxide ions [OH^-] in the formulation and can be categorised as acidic or basic (Hach Company, 2010.). The quantity H^+ ions opposed to OH^- ions will express the pH value, and a dispersion is seen as stable when these amounts remain constant (Hach Company, 2010). The skin normally has a pH value of ± 5 (Ng & Lau, 2015; Williams, 2013) and according to Barry (2002), human skin can sustain pH values of 3 – 9, which will serve as a parameter in this study. The pH of the **NEs** was measured with a Mettler Toledo® pH meter (Mettler Toledo, CU), by placing the Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) into the **NE** to determine the pH value.

Table B.4: Measured pH values for **NE1**, **NE2** and **NE3** containing statins

Formulations	pH			
	1	2	3	Average
NE-ATO1	6.12	6.13	6.14	6.13 ± 0.008
NE-ATO2	6.43	6.44	6.44	6.44 ± 0.005
NE-ATO3	6.58	6.58	6.57	6.58 ± 0.005
NE-MEV1	6.31	6.30	6.30	6.30 ± 0.005
NE-MEV2	6.79	6.67	6.65	6.70 ± 0.060
NE-MEV3	5.80	5.93	5.99	5.91 ± 0.080
NE-PIT1	6.32	6.33	6.33	6.33 ± 0.005
NE-PIT2	6.54	6.54	6.55	6.54 ± 0.005
NE-PIT3	6.57	6.57	6.59	6.58 ± 0.009
NE-PRA1	6.41	6.41	6.41	6.41 ± 0.000
NE-PRA2	6.71	6.70	6.70	6.70 ± 0.005
NE-PRA3	6.56	6.55	6.54	6.55 ± 0.008

Table B.4 illustrates that the average pH of **NE1**, **NE2** and **NE3** varied between the values of 5.91 – 6.70 and therefore none of the **NEs** should irritate the skin once applied topically, since the pH values fell in the range between 3 and 9 as stated by Barry (2002). Additionally, it can be observed that the **NE2** and **NE3** displayed higher pH values than the **NE1**, which could be attributed to the higher Span® 60 content present in the **NE2** and **NE3**. It has been proposed that that the formulation containing the highest percentage of Span® 60 produced a more basic pH than formulations consisting of less Span® 60 (Danimayotsu *et al*., 2017). The **NE1** could therefore be characterised as more acidic opposed to **NE2** and **NE3**.

B.6.3 Zeta-potential

The zeta-potential of a dispersion indicates electrostatic charges present between droplets (Malvern Instruments Limited, 2015), and typically caused by Brownian motion and Van der Waals forces (Cao & Wang, 2011). Stability of **NEs** can be determined by measuring the zeta-potential, which will establish if the **NE** will tolerate environmental conditions when used (Asmawatia *et al*., 2014). The zeta-potential measures surface charged (inter) activities, together with long-lasting stability between elements present in the formulation by electrophoretic motion calculations of dispersed oil droplets (Kumar, 2014). Therefore, a higher zeta-potential value indicates a more stable dispersion, meaning that the **NE** is electrostatically stable; reducing the risk of flocculation (Asmawatia *et al*., 2014). The ideal zeta-potential diverges from ± 20 mV and depends on the dispersant, including the chemistry of the surface area of the ingredients of the formulation (Kumar, 2014). It is suggested that dispersions with a zeta-potential of more than 30 mV or less than - 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). A high zeta-

potential suggests powerful repulsion forces between the elements within the formulation, which indicates higher stability opposed to a zeta-potential value closer to zero, which increase the risk of flocculation and aggregation (Ribeiro *et al*., 2015). Zeta-potential measurement can be influenced by pH variations, the type (salt) and concentration of the API and the concentration of other constituents (surfactants, gelling agents, etc.) in the formulation (Asmawatia, *et al*., 2014).

A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used to measure the zeta-potential of **NE1**, **NE2** and **NE3**. A volume of the **NEs** was diluted with Milli-Q® water (1:100) and mixed by rotating the flask efficiently. The solutions were then extracted with a syringe and injected in their own individual clear disposable zeta-cell. The sample measurements were performed and completed in triplicate.

Table B.5: Zeta-potential (mV) values of **NE1**, **NE2** and **NE3** that contains respective statins

Formulations	Zeta-potential (mV)			
	1	2	3	Average
NE-ATO1	-35.2000	-33.9000	-35.0000	-34.70 ± 0.57
NE-ATO2	-31.1000	-31.1000	-33.7000	-31.97 ± 1.23
NE-ATO3	-33.2000	-33.7000	-34.9000	-33.93 ± 0.71
NE-MEV1	-37.3000	-40.9000	-39.5000	-39.23 ± 1.48
NE-MEV2	0.0284	0.0452	0.0913	0.06 ± 0.03
NE-MEV3	-42.7000	-44.8000	-43.6000	-43.70 ± 0.86
NE-PIT1	-33.8000	-35.2000	-35.6000	-34.87 ± 0.77
NE-PIT2	-28.5000	-27.6000	-27.7000	-27.93 ± 0.40
NE-PIT3	-28.5000	-30.1000	-30.9000	-29.83 ± 0.10
NE-PRA1	-41.2000	-39.8000	-40.3000	-40.43 ± 0.58
NE-PRA2	0.1190	0.1540	0.0342	0.10 ± 0.05
NE-PRA3	-34.2000	-33.3000	-34.8000	-34.10 ± 0.62

From Table B.5, it is observed that all dispersions (**NE1**, **NE2** and **NE3**) displayed negative zeta-potential values within the required range (below - 30 mV), except for **NE-MEV2**, **NE-PIT2, NE-PRA2** and **NE-PIT3**. **NE1** in general (except for **NE-MEV3**), presented with higher measured zeta-potential values regarding atorvastatin, pitavastatin and pravastatin and could be considered as more stable dispersions (Eid *et al*., 2014; Silva *et al*., 2012) opposed to **NE2** and **NE3**, which indicates that there will be adequate repulsion between the droplets of **NE1** with a lesser probability of flocculation and sedimentation (Silva *et al*., 2012). When observing Figure B.3, it can be specified that the curves appear symmetrical, which indicates a narrow data distribution.

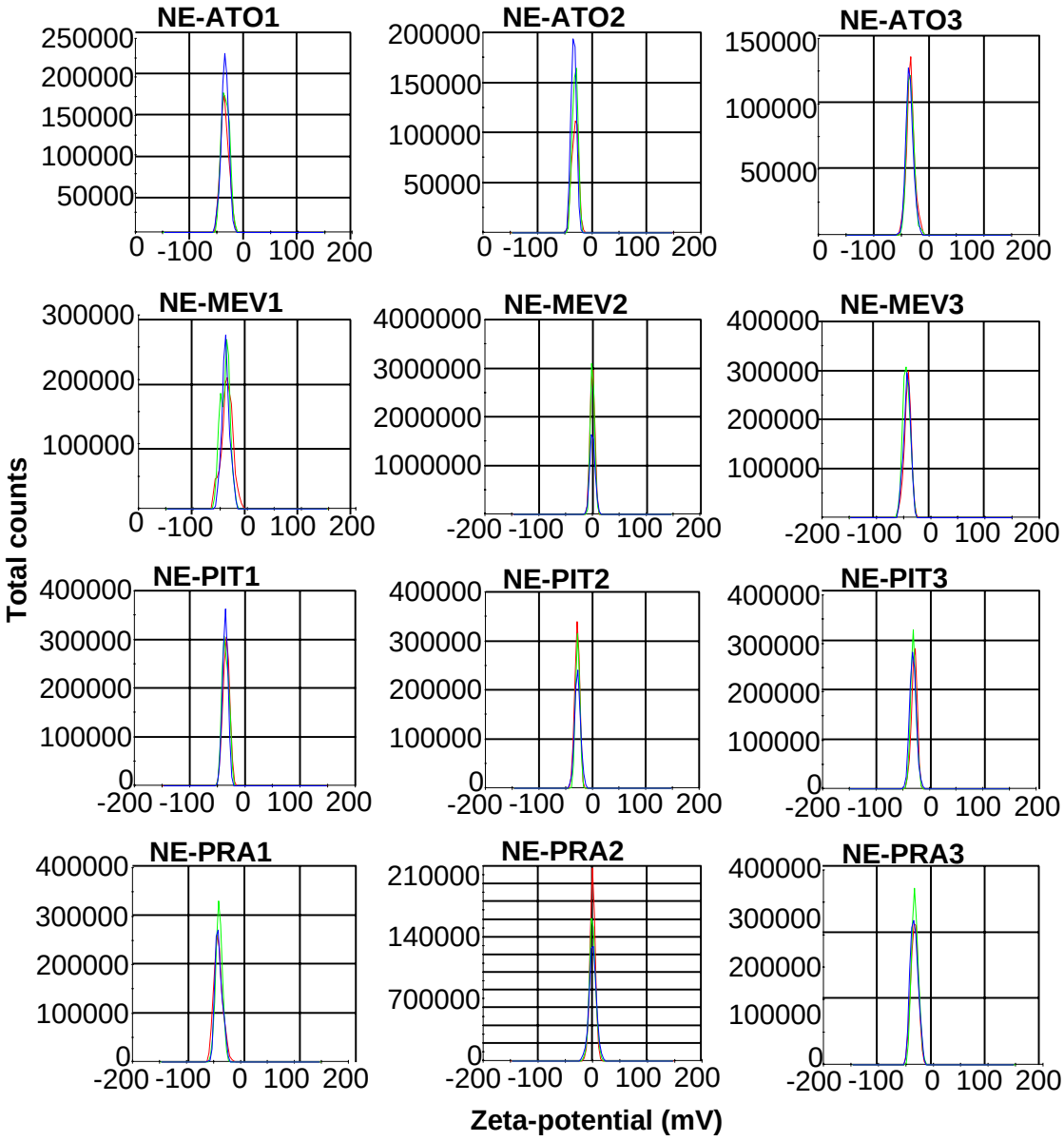


Figure B.3: Average zeta-potential (mV) of **NE1**, **NE2** and **NE3**

Table B.5 and Figure B.3 indicated the difference between the zeta-potential for **NE1**, **NE2** and **NE3**. The order of zeta-potential for the **NE1s** was **NE-PRA1** > **NE-MEV1** > **NE-PIT1** > **NE-ATO1** and for the **NE2s** was **NE-ATO2** > **NE-PIT2** > **NE-PRA2** > **NE-MEV2** and for **NE3s** was **NE-MEV3** > **NE-PRA3** > **NE-ATO3** > **NE-PIT3**. The zeta-potential of **NE1s** (**NE-ATO1**, **NE-PIT1** and **NE-PRA1**) measured higher than **NE2** and **NE3**. This indicates that **NE1s** were more stable **NEs**.

B.6.4 Droplet size and distribution

Droplet size and distribution is an essential physiochemical property regarding a **NE** (Kumar, 2014). The size of the droplet and droplet distribution (polydispersity index (PdI)) can be determined by measuring the droplet diameter through evaluation of the light scattering (Malvern Instruments Limited, 2015). Stability can be evaluated by calculating the droplet size (Solans *et al*., 2005). Additionally, smaller droplet sizes enhance an emulsion's integral stability, as they

decrease the occurrence of sedimentation, creaming, coagulation and flocculation (Kumar, 2014; Solans *et al*., 2005). The required parameters for droplet sizes of **NEs** are < 500 nm (Nalini *et al*., 2017). PdI can be established by using a scale ranging from 0 – 1, where a low PdI, which is closer to zero) is indicative of homogenous or monodispersed dispersions; however, a PdI closer to 1 is indicative of a more polydispersed dispersion (Gaumet *et al*., 2008).

A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used in this study to establish the droplet size and distribution. This instrument functions by using photon correlation spectroscopy (PCS), i.e. a Zetasizer, which calculates light fluctuations that follow between droplets in the **NE** as a result of Brownian motion (Gaur *et al*., 2014; Malvern Instruments Limited, 2015). PCS measures and expresses the droplets distribution as the PdI. Dilutions of the **NEs** were made and analysed; similar to the method used to determine zeta-potential (see Section B.6.3) in triplicate.

In Table B.6 and Figure B.4, the average droplet size (nm) and average PdI were measured regarding **NE1**, **NE2** and **NE3**, respectively. With the investigation of the average droplet size of **NE1s**, it was observed that **NE-PIT1** had the largest average droplet size, followed by **NE-PRA1**, **NE-ATO1** and **NE-MEV1**. **NE2s** displayed average droplet sizes in the order of **NE-PRA2** > **NE-ATO2** > **NE-MEV2** > **NE-PIT2**, while **NE3s** displayed average droplet sizes in the order of **NE-ATO3** > **NE-MEV3** > **NE-PRA3** > **NE-PIT3**. When **NE1s** were compared to **NE2s**, the **NE2s** obtained smaller average droplet sizes than the **NE1s**. When the **NE1s** were compared to the **NE3**, the **NE3s** (**NE-ATO3**, **NE-MEV3** and **NE-PRA3**) displayed larger average droplet sizes than **NE1s**, with the exception of **NE-PIT3**. Hence, smaller droplet size values of nano-emulsions allows uniform deposition and penetration of active ingredients through the skin surface (Che Marzukiet *et al*., 2019).

Table B.6: Average droplet size and PdI of the **NE1**, **NE2** and **NE3** with respective statins

Formula	Average droplet size (nm)	Average PdI
NE-ATO1	145.50 ± 1.28	0.417 ± 0.004
NE-ATO2	112.87 ± 0.68	0.241 ± 0.008
NE-ATO3	182.87 ± 1.05	0.248 ± 0.100
NE-MEV1	144.20 ± 0.64	0.395 ± 0.013
NE-MEV2	112.80 ± 1.49	0.340 ± 0.010
NE-MEV3	174.30 ± 1.08	0.199 ± 0.009
NE-PIT1	149.63 ± 1.64	0.386 ± 0.014
NE-PIT2	94.27 ± 0.45	0.265 ± 0.006
NE-PIT3	139.60 ± 0.99	0.220 ± 0.010
NE-PRA1	148.17 ± 0.67	0.237 ± 0.035
NE-PRA2	124.53 ± 0.49	0.192 ± 0.007
NE-PRA3	154.93 ± 0.62	0.191 ± 0.102

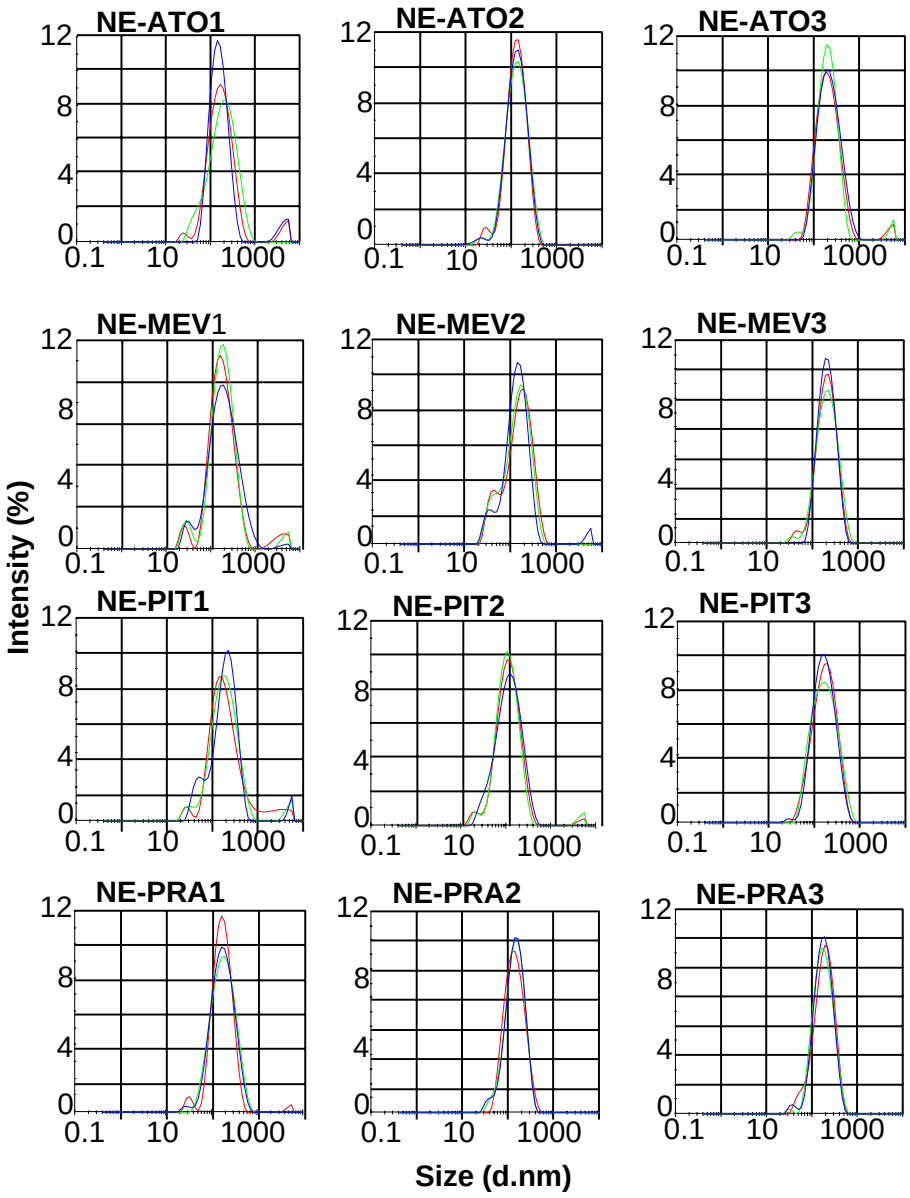


Figure B.4: Average droplet size (nm) of **NE1**, **NE2** and **NE3**

PdI values of the **NE1s** were compared and presented as **NE-ATO1** > **NE-MEV1** > **NE-PIT1** > **NE-PRA1**. The **NE2s** exhibited PdI values of **NE-MEV2** > **NE-PIT2** > **NE-ATO2** > **NE-PRA2**, while **NE3s** displayed PdI values in the order of **NE-ATO3** > **NE-PIT3** > **NE-MEV3** > **NE-PRA3**. PdI values indicated that **NE1s** > **NE2s**, and **NE1s** > **NE3s**. As a result, **NE1s** displayed larger PdI values (less homogenous) compared to **NE2** and **NE3**. However, **NE1s** still obtained PdI values < 0.5, which is regarded as acceptable criteria (Elmataeeshy *et al*., 2018). The PdI, as measured through PCS, expresses the droplet distribution, which is a crucial characteristic of an ideal formulation (Gaumet *et al*., 2008). It should also be mentioned that the droplet size can also have an effect on the distribution of the droplets throughout the dispersion (Gaumet *et al*., 2008).

B.6.5 Viscosity

The viscosity can ultimately influence API release regarding a formulation due to the flow resistance on the skin (Chime *et al*., 2014). Hence, the viscosity of a **NE** is strongly affected by factors such as solvation and aggregation of nano-droplets (Shakeel *et al*., 2007). Due to the large water component present in an o/w **NE**, low viscosity values are produced (Chime *et al*., 2014). Conclusively, **NEs** are characterised as low viscosity dispersions (Shakeel *et al*., 2007, Thakur *et al*., 2012).

A Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA) linked to a thermostatic water bath (25 °C) was used to determine the viscosity of **NE1**, **NE2** and **NE3**. Each formulation was placed in the water bath for 45 min prior to the test. A cylindrical spindle (SC4-18) was found to be the most adequate to measure the viscosity of **NE1**, **NE2** and **NE3**. Approximately 20 ml The **NEs** was transferred in a sample chamber, which fitted on a small sample adapter of the viscometer. The spindle was linked to the viscometer to hang down into the hollow of the sample adapter. Viscosity was measured at a rotation speed of 200 rpm. Centipoise (cP) at room temperature (± 25 °C) is utilised to express the viscosity readings. Rheocalc T1.2.19 was preprogramed to read and assemble multipoint data at 10 s intervals for 3 min. These obtained values were used to establish the average viscosity. The average viscosity measurements for **NE1**, **NE2** and **NE3** are illustrated in Table B.7.

The viscosity of water is measured as 1 cP, and from Table B.7 it is observed that all the formulated **NEs** were more viscous than water (V&P Scientific Inc., 2010), since the viscosity was higher than 1 cP. Tween® 80 is known to increase the viscosity of a formulation (Kennedy & Kennedy, 2007). Additionally, Span® 60 can also change the physical characteristics of formulations by reducing interfacial surface tension and therefore increasing viscosity (Zhao *et al*., 2013). Since the **NE2s** contained lower amounts of Span® 60, these formulas produced less viscous formulations than the **NE1** and **NE3**, which contained more Tween® 80. The torque between the dispersions ranged between 21.83 – 31.08%.

Table B.7: Viscosity (cP) and torque (%) readings of **NE1**, **NE2** and **NE3**

	NE1 (3:3)				**NE2 (3:2)**				**NE3 (2:3)**			
Statin	ATO	MEV	PIT	PRA	ATO	MEV	PIT	PRA	ATO	MEV	PIT	PRA
Viscosity (cP)	3.98	3.45	4.20	3.77	4.18	3.27	3.77	3.39	4.59	4.65	4.04	3.81
	3.93	3.49	4.01	3.72	4.20	3.26	3.81	3.42	4.63	4.68	4.07	3.81
	3.91	3.51	4.04	3.75	4.20	3.30	3.82	3.41	4.57	4.63	4.02	3.78
	3.93	3.48	4.04	3.78	4.22	3.27	3.81	3.43	4.61	4.66	4.07	3.78
Average	3.94 ± (0.03)	3.48 ± (0.02)	4.07 ± (0.07)	3.76 ± (0.02)	4.20 ± (0.01)	3.28 ± (0.02)	3.80 ± (0.02)	3.41 ± (0.01)	4.60 ± (0.02)	4.67 ± (0.02)	4.05 ± (0.02)	3.80 ± (0.02)
Torque (%)	26.5	23.0	28.0	25.1	27.9	21.8	25.1	22.6	30.6	31.0	26.9	25.4
	26.2	23.3	26.7	24.8	28.0	21.7	25.4	22.8	30.9	31.2	27.1	25.4
	26.1	23.4	26.9	25.0	28.0	22.0	25.5	22.7	30.5	30.9	26.8	25.2
	26.2	23.2	26.9	25.2	28.1	21.8	25.4	22.9	30.7	31.2	27.1	25.2
Average	26.25 ± (0.15)	23.23 ± (0.15)	27.13 ± (0.51)	25.03 ± (0.15)	28.00 ± (0.07)	21.83 ± (0.11)	25.35 ± (0.15)	22.75 ± (0.11)	30.68 ± (0.15)	31.08 ± (0.13	26.98 ± (0.13)	25.30 ± (0.10)

B.6.6 Morphology

Transmission electron microscopy (TEM) has been used for over 50 years (Bogner *et al*., 2007) and was used during this study to investigate the morphological characteristics of the optimised **NE** from the three formulas (**NE1** vs. **NE2** vs. **NE3**). Hence, due to the high expenses of performing high-resolution transmission electron microscope (HRTEM), the assay was only conducted on the optimised **NE** (see Section B.8.1.6).

B.6.7 Entrapment efficiency

Entrapment efficiency (%EE) can be defined as the ratio of the entrapped drug to the ratio total drug. It is essential to obtain a %EE higher than 50%, to decrease the possibility of bioactive outflow and to prevent flaws in the formulation (Divakaran, 2012). It has been proposed that **NEs** that contain lipophilic encapsulated APIs have the ability to produce a %EE of close to 100% (Loureiro *et al*., 2015). HPLC samples were prepared by placing 20 ml of each formulated **NE** (**NE1** or **NE2** or **NE3**), e.g. **NE-ATO**, **NE-MEV**, **NE-PIT** and **NE-PRA** into Eppendorf® tubes, where after they were centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) at a g-force of 25 000 g for 45 min at 25 °C. Thereafter, it was possible to differentiate between the unentrapped API (supernatant fluid) and fluid that contained the entrapped API. Exactly 200 µl of the supernatant were diluted with 5 ml THF. A volume of each dilution was transferred to vials and injected in an HPLC to be analysed. To calculate the %EE, Equation B.1 (Kurakula *et al*., 2012) was used, where Cf is the quantity of unentrapped API and Ct is the totalised quantity of the API.

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

A standard linear curve obtained from a standard solution (see Section A.3.1.1) for each of the respective statins was utilised to analyse the data in conjunction with Equation B.1 to calculate the %EE. The results of the determined %EE are listed in Table B.8.

From Table B.8 the %EE of **NE1**, **NE2** and **NE3** was compared and it was observed that **NE1**, **NE2** and **NE3** yielded a high %EE. It can be concluded that **NE1** obtained the highest %EE and **NE2** obtained the lowest %EE. Therefore, larger amounts of API in **NE1** can be transported to the intended area (Kurakula *et al*., 2012). Statins were efficaciously entrapped in the **NE** formulas, as the %EE was close to 100% (Loureiro *et al*., 2015).

Table B.8: The calculated entrapment efficacy (%EE) for **NE1**, **NE2** and **NE3**

Formula	%EE
NE-ATO1	98.77
NE-ATO2	95.04
NE-ATO3	96.11
NE-MEV1	98.99
NE-MEV2	98.04
NE-MEV3	98.15
NE-PIT1	93.32
NE-PIT2	91.82
NE-PIT3	92.14
NE-PRA1	91.52
NE-PRA2	90.45
NE-PRA3	90.49

B.7 Outcome of NEs

Different methods were performed to characterise and analyse the three **NEs** (**NE1**, **NE2** and **NE3**) containing the different statins. The results were then compared to establish the optimised formula, which would be investigated further. The **NEs** were visually inspected and no presence of sedimentation or flocculation occurred. The decrease in the translucent nature of the dispersions could be attributed to the incorporation of 2% (m/m) API. The pH results indicated that all the **NEs** were in range between 3 and 9 (Barry, 2002) and should therefore not irritate the skin when applied topically.

All the **NEs** had zeta-potential values within the required range (below - 30 mV), except, **NE-MEV2**, **NE-PIT2**, **NE-PRA2** and **NE-PIT3** as mentioned in Section B.6.3. The **NEs** succeeded in the required parameters for droplet sizes of < 500 nm (Nalini *et al*., 2017), which indicate lower risks of sedimentation, creaming, flocculation and coagulation (Kumar, 2014; Solans *et al*., 2005). It can be concluded that **NEs** ranged with a PdI from 0 – 1, which is indicative of homogenous or monodispersed dispersions as discussed in Section B.6.4. Although the **NE3s** presented as more viscous dispersions, the **NE1s** viscosity results were in a similar range. The **NE1s** obtained the highest %EE of each statin formula compared to **NE2s** and **NE3s**. The statins in **NE1s** were successfully entrapped in the formulas, as the %EE was close to 100% (Loureiro *et al*., 2015). Additionally, when the %EE of the **NE3s** were compared to that of the **NE2s**, it was concluded that the **NE3s** had a higher entrapment for atorvastatin, mevastatin, pitavastatin and pravastatin than for the **NE2s**.

From the aforementioned it is clear that **NE1** had more optimal characteristics overall. Therefore, **NE1** was chosen as the optimised formula that will be used for further investigation in this study.

The optimised **NE** formula should have the capability to deliver the API efficiently (Gutiérrez *et al*., 2008). However, it is essential to establish whether the API can affect the characteristic of the optimised formula. Therefore, an optimised placebo (**NE-PLAS1**, which is **NE1** without the API) was compared to the **NE1** in terms of visual inspection, pH, surface potential measurement (zeta-potential), droplet size and distribution, viscosity and morphological analysis (TEM) (Kumar 2014; Nejadmansouria *et al*., 2016).

B.8 The optimised NE1 vs. NE-PLAS1

The optimised o/w **NE1** formula, which contains a specific statin, e.g. atorvastatin, mevastatin, pitavastatin and pravastatin in each batch was identified, analysed and determined and compared to **NE-PLAS1** (API absent) in the following sections.

Table B.9: Formula of the optimised o/w **NE1** and **NE-PLAS1**

		NE1	**NE-PLAS1**
Phase	**Constituent**	**Quantity**	**Quantity**
Water	Milli-Q® water	40.000 ml	40.000 ml
	Tween® 80	2.804 ml	2.804 ml
Oil	Statin	1 g	-
	Span® 60	3 g	3 g
	Grapeseed oil	4.350 ml	4.350 ml

Identical formulation method and formula was utilised to prepare the **NE1** and the placebo (**NE-PLAS1**), except the API was not included in **NE-PLAS1** as seen in Table B.9.

B.8.1 Methods utilised during characterisation of the optimised o/w NEs

To establish if the APIs affected the dispersions, **NE-PLAS1** was characterised and compared to those of **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) by means of droplet size and distribution, visual inspection, pH, zeta-potential, viscosity and morphological analysis (TEM) (Nejadmansouria *et al*., 2016).

B.8.1.1 Visual inspection

Figure B.5 illustrates the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) and the placebo (**NE-PLAS1**). No sedimentation or flocculation occurred regarding the **NE-PLAS1** and the **NE1s**. The dispersions presented with a white and milky appearance, with no visible oil droplets. Therefore, it can be concluded that the oil droplets were effectively dispersed in the water phase, with or without the selected statins included in the formula.

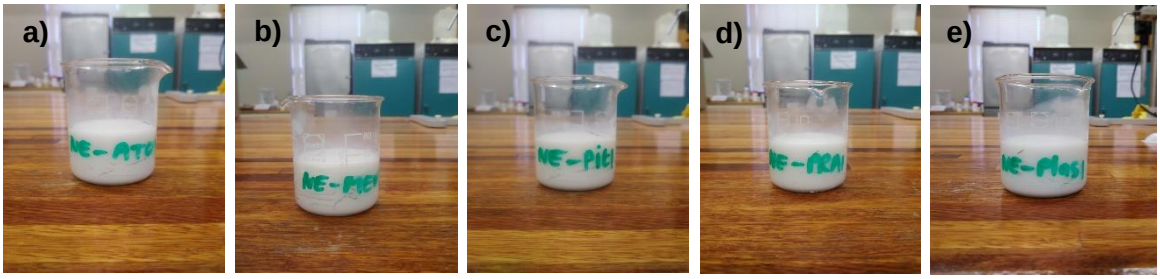


Figure B.5: Visual illustration of the **NE1s** (a) **NE-ATO1**, b) **NE-MEV1**, c) **NE-PIT1**, d) **NE-PRA1** and e) **NE-PLAS1**

B.8.1.2 pH

An identical method was utilised with the same equipment as mentioned in Section B.6.2 to determine the pH values of the **NE1s** and **NE-PLAS1**. According to Barry (2002), human skin can sustain pH values of 3 – 9, which will serve as a guideline in this study Barry (2002).

Table B.10 illustrates the average pH measurements for **NE-PLAS1** compared to **NE1s**. The **NE-PLAS1** and the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) indicated average pH values in range of 6 – 8. The order was displayed as **NE-PRA1** > **NE-PIT1** > **NE-MEV1** > **NE-PLAS1** > **NE-ATO1**.

In conclusion, the addition of the API did slightly influence the pH of the **NE1** formula, compared to **NE-PLAS1**. The pH values of the affected formulations still ranged between the accepted pH values (3 – 9) (Barry, 2002). Therefore, these dispersions should not affect the integrity of the skin or act as a skin irritant.

Table B.10: Average pH of the **NE1s** and **NE-PLAS1**

Formula	pH			
	1	2	3	Average
NE-ATO1	6.12	6.13	6.14	6.13 ± 0.008
NE-MEV1	6.31	6.30	6.30	6.30 ± 0.005
NE-PIT1	6.32	6.33	6.33	6.33 ± 0.005
NE-PRA1	6.41	6.41	6.41	6.41 ± 0.000
NE-PLAS1	6.25	6.27	6.26	6.26 ± 0.008

B.8.1.3 Zeta-potential (mV)

It is suggested that dispersions with a zeta-potential of more than 30 mV or less than - 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). Results were obtained via the same method and equipment as utilised in Section B.6.3.

Table B.11: Average zeta-potential (mV) values of the **NE1s** and **NE-PLAS1**

Formula	Zeta-potential (mV)			
	1	2	3	Average
NE-ATO1	-35.2	-33.9	-35.0	-34.70 ± 0.57
NE-MEV1	-37.3	-40.9	-39.5	-39.23 ± 1.48
NE-PIT1	-33.8	-35.2	-35.6	-34.87 ± 0.77
NE-PRA1	-41.2	-39.8	-40.3	-40.43 ± 0.58
NE-PLAS1	-54.0	-51.5	-52.3	-52.60 ± 1.04

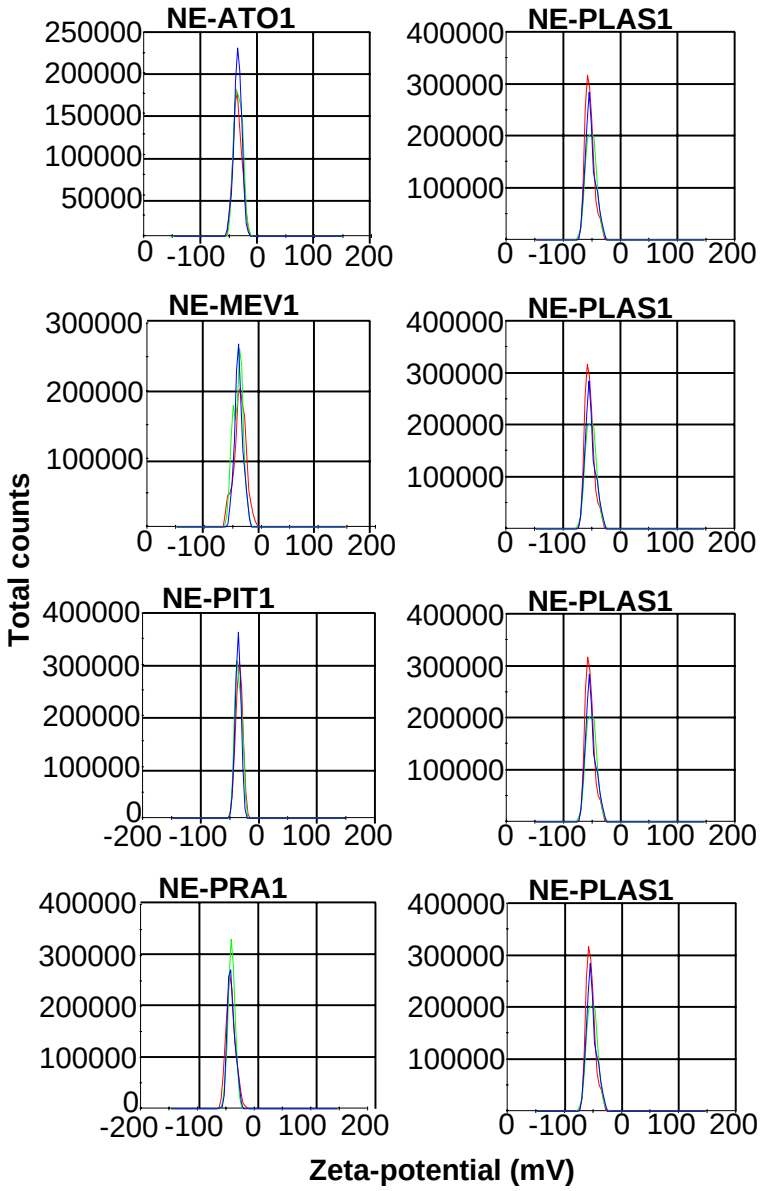


Figure B.6: The average zeta-potential (mV) of **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) and **NE-PLAS1**

Table B.11 illustrates the zeta-potential for the **NE1s** in comparison to the **NE-PLAS1**. It can be concluded that **NE-PLAS1** measured a value less than - 30 mV. It is suggested that dispersions with a zeta-potential of more than 30 mV or less than - 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). The **NE-PLAS1** measured a higher value (Figure B.6) compared to **NE1s**, however both the **NE-PLAS1** and **NE1s** were within the required range suggesting that stability is acceptable.

B.8.1.4 Droplet size and distribution

An identical method, together with the same equipment as used in Section B.6.4 were used to determine the droplet size and distribution. Stability is evaluated by calculating the droplet size (Solans *et al*., 2005). The required parameters for droplet sizes of **NEs** are < 500 nm (Nalini *et al*., 2017). The PdI scale range from 0 – 1, where a low PdI, which is closer to zero, indicates a homogenous or monodispersed dispersions (Gaumet *et al*., 2008).

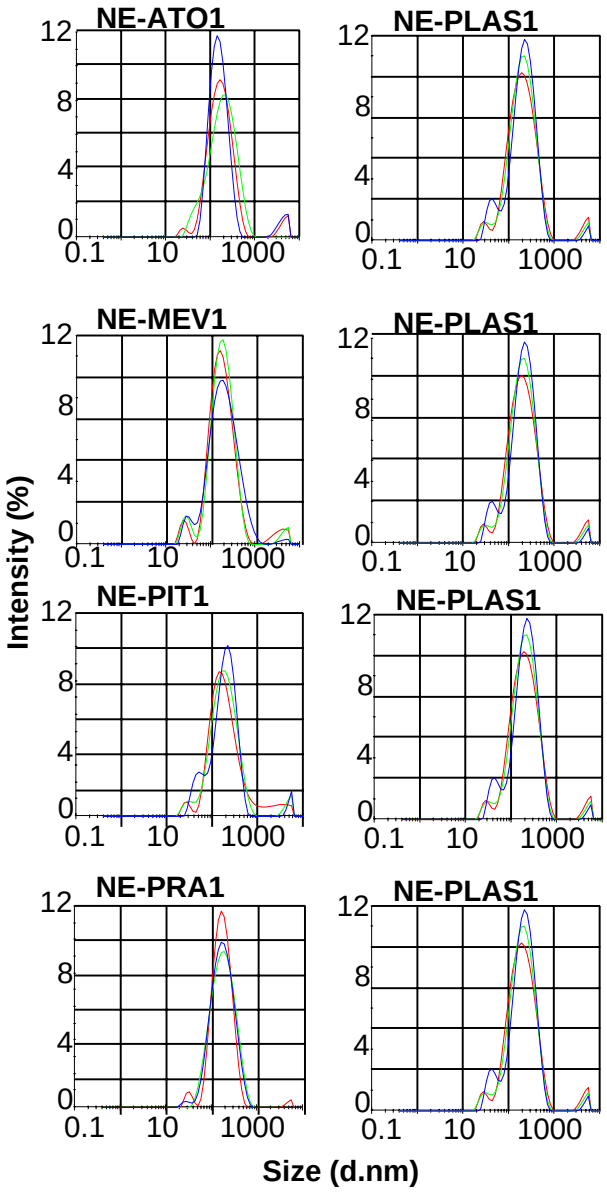


Figure B.7: Average droplet size (nm) of the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) compared to **NE-PLAS1**

Table B.12: Average droplet size and PdI of the **NE1s** and **NE-PLAS1**

Formula	Average	
	Droplet size (nm)	**Polydispersity index (PdI)**
NE-ATO1	145.50 ± 1.28	0.417 ± 0.004
NE1-MEV1	144.20 ± 0.64	0.395 ± 0.013
NE-PIT1	149.63 ± 1.64	0.386 ± 0.014
NE-PRA1	154.93 ± 0.62	0.191 ± 0.102
NE-PLAS1	158.10 ± 0.85	0.398 ± 0.021

Table B.12 and Figure B.7 indicated the average droplet size (nm) and average PdI that was measured for the **NE1s** and the **NE-PLAS1**. **NE-PLAS1** had an average droplet size of 158.1 ± 0.85 nm and an average PdI of 0.398 ± 0.021. It can be concluded that **NE-PLAS1** obtained a larger droplet size and a higher PdI value than the **NE1s**. The higher PdI value of the **NE-PLAS1** is indicative of a more polydispersed dispersion (Gaumet et al., 2008) as opposed to the homogenous **NE1s** (**NE-MEV1**, **NE-PIT1** and **NE-PRA1**). **NE1s** and **NE-PLAS1** ranged within the required parameters for droplet sizes (< 500 nm) (Nalini *et al*., 2017), as well as PdI values (< 0.5) that ranged from 0 – 1, which is generally still indicative of homogenous or monodispersed dispersions. Therefore, these characteristics indicate low risks of sedimentation, creaming, flocculation and coagulation (Kumar, 2014; Solans *et al*., 2005).

B.8.1.5 Viscosity of NE-PLAS1

The method and equipment was identical to that of Section B.6.5 to compare the **NE1s** and **NE-PLAS1**.

Table B.13: The viscosity readings of the **NE1s** and **NE-PLAS1**

Formula	Viscosity (cP)	Speed (rpm)	Torque (%)	Temp (°C)
NE-ATO1	3.94 ± 0.03	200 ± 0.000	26.25 ± 0.15	22.47 ± 0.07
NE-MEV1	3.48 ± 0.02	200 ± 0.000	23.23 ± 0.15	22.05 ± 0.50
NE-PIT1	4.07 ± 0.07	200 ± 0.000	27.13 ± 0.51	21.71 ± 0.13
NE-PRA1	3.76 ± 0.02	200 ± 0.000	25.03 ± 0.15	22.97 ± 0.04
NE-PLAS1	3.32 ± 0.02	200 ± 0.000	22.10 ± 0.12	22.12 ± 0.13

Table B.13 indicates that **NE-PLAS1** measured a viscosity (cP) value lower than that of the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**). This occurrence suggests that when the API was added to the formula, the viscosity increased.

B.8.1.6 Morphology of NE1 and NE-PLAS1

TEM is an operative imaging method adapted to capture images at a significantly greater resolution (0.2 nm) opposed to that of light microscopes (Egerton, 2005). It should also be mentioned that TEM is generally used in the morphological and structural research of **NEs** (Kalra, 2013). TEM was performed to establish the structural form and droplet size of the **NE1s** compared to the **NE-PLAS1** by capturing images of these prepared **NEs**. A FEI Tecnai G2 20S-Twin 200 kV HRTEM (Czech Republic, EU) with an Oxford INCA X-Sight EDS System was utilised during this study.

The **NEs** were prepared distinctly for each statin and were diluted (1:100) with Milli-Q® water. A small volume of the solution was extracted and transferred to a microscopic copper grid carbon-coated 300 mesh by using a micropipette and left to dry for 10 – 15 min (excess removed with filter paper). Finally, staining was done with osmium tetroxide (Chime *et al*., 2014), which provided the images with high-contrast quality (Nomaki *et al*., 2015). Additionally, the osmium tetroxide preserved unsaturated fatty acids in the **NEs**, making viewing of oil droplets effortless (Nomaki *et al*., 2015). After drying (20 – 30 min), the coloured copper grids were studied at sufficient voltage (± 200 kV) with the use of TEM to capture images with a Gatan bottom mount camera and digital micrograph software.

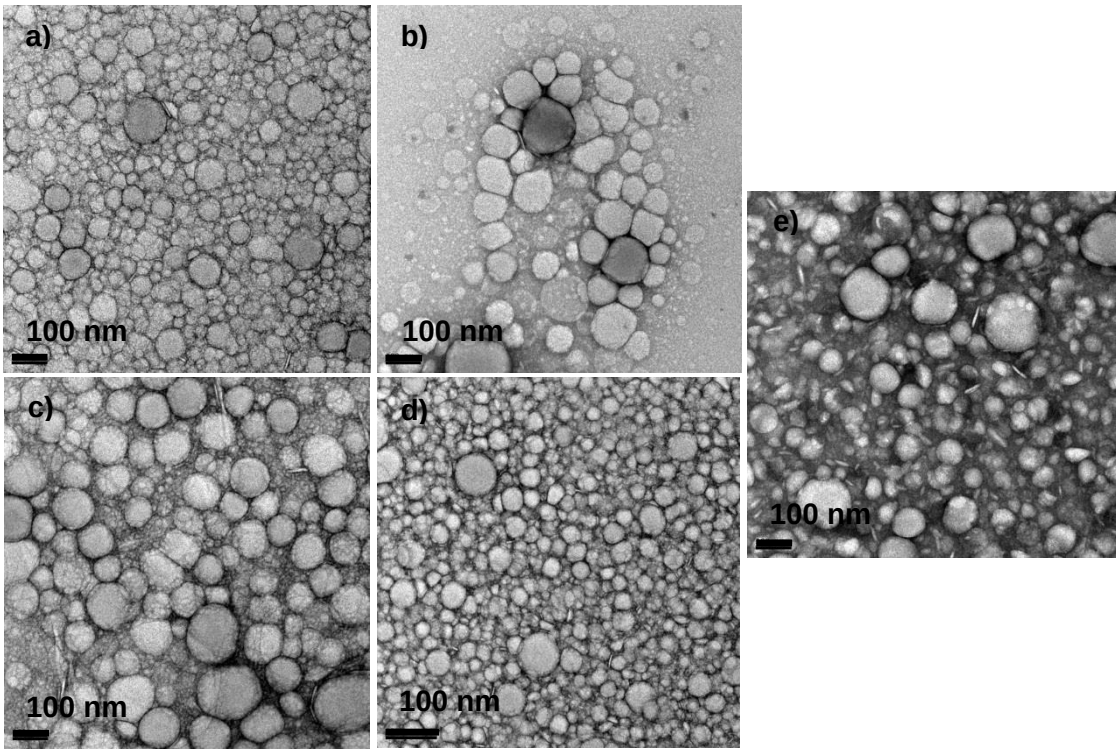


Figure B.8: TEM micrographs of: a) **NE-ATO1**, b) **NE-MEV1**, c) **NE-PIT1**, d) **NE-PRA1** and e) **NE-PLAS1**

TEM micrographs are illustrated in Figure B.8 regarding the optimised **NE1s** and **NE-PLAS1** to indicate the morphology. The droplet sizes of **NE-PLAS1** ranged from 51.85 – 162.33 nm. The droplet sizes of the **NE1s** ranged from as follow: **NE-ATO1** (30.49 – 128.29 nm), **NE-MEV1** (58.58 – 126.59 nm), **NE-PIT1** (17.56 – 182.58 nm) and **NE-PRA1** (32.29 – 171.98 nm), which were compared to **NE-PLAS1**. All **NEs** (**NE1** and **NE-PLAS1**) exhibited spherical shaped dispersed droplets and were narrowly distributed. It is evident that **NE-PLAS1** illustrated larger droplet sizes compared to **NE-ATO1** and **NE-MEV1**. However, **NE-PIT1** and **NE-PRA1** displayed larger droplet sizes opposed to **NE-PLAS1**.

According to literature, the oil droplets within the water phase of **NEs** should be below 500 nm (Nalini *et al*., 2017). The **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1)**, as well as the **NE-PLAS1** indicated droplet sizes within the range; therefore, **NE1s** were sufficiently formulated.

B.9 Conclusion

Different methods were used to characterise and investigate the **NE1s**, **NE2s** and **NE3s**, which contained respective statins to establish an optimised **NE** formula. Based on visual inspection, pH, zeta-potential, droplet size and distribution, viscosity, TEM and %EE results, it was concluded that the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) were the most favourable formula and therefore selected as the optimised **NE**.

A control group, **NE-PLAS1**, was formulated and characterised to establish if the APIs affected the formula of the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**). The following characteristics were determined to compare the optimised **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**) to the **NE-PLAS1**, namely visual inspection, pH, droplet size and distribution, zeta-potential, viscosity and morphological analysis (TEM) (Kumar 2014; Nejadmansouria *et al*., 2016).

The **NE1s** and **NE-PLAS1** had a white and milky colour, with no visible oil droplets in the dispersions when visually inspected, in conclusion, both formulas (**NE1** and **NE-PLAS1**), with or without statins, were dispersed sufficiently.

The pH of **NE1s** were not affected by the selected statins when compared to the **NE-PLAS1**; thus, the pH levels still remained in range according to literature, which is values 3 – 9 (Barry, 2002) and should not act as an irritant towards the skin barrier, when the dispersion is topically applied.

The zeta-potential of the **NE1s** and **NE-PLAS1** were within the ideal range, since it is suggested that dispersions with a zeta-potential of more than 30 mV or less than - 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). The **NE1s** and **NE-PLAS1** also ranged in the required parameter (< 0.5) regarding the PdI measurement from 0 – 1, which indicates homogenous or monodispersed dispersions (Elmataeeshy *et al*., 2018). Both, **NE1s** and the **NE-PLAS1** displayed nano-sized droplets in the required range (< 500 nm) (Nalini *et al*., 2017). This parameter indicated that **NE1s** entails a low risk for sedimentation, creaming, flocculation and coagulation (Kumar, 2014; Solans *et al*., 2005). As a result, the **NE1s** were adequately sonicated and formulated.

Regarding viscosity, the **NE-PLAS1** displayed a lower viscosity opposed to the **NE1s**; which suggested that the selected APIs raised the viscosity of the optimised formula.

The characterisation methods established that the **NE1s** (**NE-ATO1**, **NE-MEV1, NE-PIT1** and **NE-PRA1**) and the **NE-PLAS1** succeeded in the test requirements for an optimal **NE** formula. There were no significant effects by inclusion or exclusion of the selected statins in the optimised formula; however, the **NEs** are considered as low viscosity dispersions (Shakeel *et al*., 2007, Thakur *et al*., 2012). This could suggest challenges regarding the topical application of the **NE1s** to the skin and patient convenience for that matter. To improve these predicaments, a **NG** was formulated (Appendix C) based on the optimised **NE1**.

References

Abolmaali, S.S., Tamaddon, A.M., Farvadi, F.S., Daneshamuz, S. & Moghimi, H. 2011. Pharmaceutical nanoemulsions and their potential topical and transdermal applications. *Iranian journal of pharmaceutical sciences*, 7:139-150.

Alexander, A., Dwivedi, S., Ajazuddin, Giri, T.K., Saraf, S., Saraf, S. & Tripathi, D.K. 2012. Approaches for breaking the barrier of drug permeation through transdermal drug delivery. *Journal of controlled release*, 164:26-40.

Aparicio, R., Roda, L., Albi, M.A., Gutiérrez, F.J.J.o.a. & chemistry, f. 1999. Effect of various compounds on virgin olive oil stability measured by Rancimat. *Journal of agricultural and food chemistry*, 47(10):4150-4155.

Asmawatia, B., Mustaphaa, W.A.W., Yusopa, S.M., Maskata, M.Y. & Shamsuddinc, A.F. 2014. Characteristics of cinnamaldehyde nanoemulsion prepared using APV-high pressure homogenizer and ultra turrax. *AIP conference proceedings*, 1614:244-250.

Baibhav, J., Gurpreet, S., Rana, A.C., Seema, S. & Vikas, S. 2011. Emulgel: a comprehensive review on the recent advances in topical drug delivery. *International research journal of pharmacy*, 2:66-70.

Bail, S., Stuebiger, G., Krist, S., Unterweger, H. & Buchbauer, G.J.F.c. 2008. Characterisation of various grape seed oils by volatile compounds, triacylglycerol composition, total phenols and antioxidant capacity. *Food chemistry*, 108(3):1122-1132.

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

Basera, K., Bhatt, G., Kothiyal, P. & Gupta, P. 2015. Nanoemulgel: a novel formulation approach for topical delivery of hydrophobic drugs. *World journal of pharmacy and pharmaceutical sciences*, 4:1871-1886.

Bogner, A., Jouneau, P.H., Thollet, G., Basset, D., Gauthier, C. 2007. A history of scanning electron microscopy developments: Towards „„wet-stem"" imaging. Micron, 38:390-401.

Braamskamp, M.J., Wijburg, F.A. & Wiegman, A. 2012. Drug therapy of hypercholesterolaemia in children and adolescent. *Drugs*, 72(6):759-772.

Cao, G. & Wang, Y. 2011. Van der Waals attraction potential. (*In* Cao, G. & Wang, Y., *ed*. Nanostructures and nanomaterials: synthesis, properties, and applications. 2nd ed. Singapore: World Scientific. p. 43-45).

Che Marzuki, N.H., Wahab, R.A. & Abdul Hamid, M., 2019. An overview of nanoemulsion: concepts of development and cosmeceutical applications. *Biotechnology & Biotechnological Equipment*, 33(1):779-797.

Chime, S.A., Kenechukwu, F.C. & Attama, A.A. 2014. Nanoemulsions: advances in formulation, characterization and application in drug delivery. (*In* Sezer, A.D., *ed*. Application of nanotechnology in drug delivery. p. 77-126). http://cdn.intechopen.com/pdfs-wm/47116.pdf Date of access: 20 Jan. 2019.

Čižinauskas, V., Elie, N., Brunelle, A. & Briedis, V. 2017. Skin Penetration Enhancement by Natural Oils for Dihydroquercetin Delivery. *Molecules*, 22(9):1536.

Debnath, S., Satayanarayana & Kumar, G.V. 2011. Nanoemulsion-a method to improve the solubility of lipophilic drugs. *Pharmanest: an international journal of advances in pharmaceutical sciences*, 2:72-83.

Divakaran, D. 2012. Preparation and characterization of nanoemulsions encapsulating polyphenol extracts. Karnal: Deemed University. (Thesis-M.Tech).

Dos Santos Freitas, L., Jacques, R.A., Richter, M.F., da Silva, A.L. & Caramão, E.B.J.J.o.C.A. 2008. Pressurized liquid extraction of vitamin E from Brazilian grape seed oil. *Journal of Chromatography A*, 1200(1):80-83.

Egerton, R.F. 2005. Physical principles of electron microscopy: An introduction to TEM, SEM, and AEM
file:///C:/Users/22819509/Downloads/Physical_Principles_of_Electron_Microscopy__An_Introduction_to_TEM__SEM__and_AEM2.pdf Date of access: 22 Nov. 2019.

Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti-inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of nanomedicine and nanotechnology*, 5:1-10.

Elmataeeshy, M.E., Sokar, M.S., Bahey-El-Din, M.B. & Shaker, D.S. 2018. Enhanced transdermal permeability of terbinafine through novel nanoemulgel formulation. *Future journal of pharmaceutical sciences*, 4(1):18-28.

Fiori, L., Solana, M., Guella, G., Strim, C. & Tosi, P. 2010. Grape seed oil: supercritical extraction, chemical analysis and fractionation. (*In*. 12th European Meeting on Supercritical Fluids organised by: Institut National Polytechnique de Lorraine. p. 1-10).

Gabera, M., Medhata, W., Hanya, M., Sahera, N., Fang, J., Elzoghby, A. 2017. Protein-lipid nanohybrids as emerging platforms for drug and gene delivery: Challenges and outcomes. *Journal of controlled release*, 254:75-91.

Gaumet, M., Vargas, A., Gurny, R. & Delie, F. 2008. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, 69:1-9.

Gaur, S., Garg, A., Yadav, D., Beg, M. & Gaur, K. 2014. Nanoemulsion gel as novel oil based colloidal nanocarrier for topical delivery of bifonazole. *Indian research journal of pharmacy and science*, 1:36-54.

Gupta, A., Eral, H.B., Hatton, T.A. & Doyle, P.S. 2016. Nanoemulsions: formation, properties and applications. *Soft Matter*, 12(11):2826-2841.

Gutiérrez, J.M., González, C., Maestro, A., Solè, I., Pey, C.M. & Nolla, J. 2008. Nano-emulsions: new applications and optimization of their preparation. *Current options in colloid and interface science*, 13:245-251.

Hach Company. 2010. What is pH and how is it measured? : a technical handbook for industry. http://www.hach.com/asset-get.download.jsa?id=7639984488.pdf Date of access: 21 May 2019.

Honary, S. & Zahir, F. 2013. Effect of zeta potential on the properties of nano-drug delivery systems-a review (Part 2). *Tropical journal of pharmaceutical research*, 12(2):265-273.

Hussein, S., Abdrabba, S.J.I.J.o.A.S. & Mathematics. 2015. Physico-chemical characteristics, fatty acid, composition of grape seed oil and phenolic compounds of whole seeds, seeds and leaves of red grape in Libya. *International journal of applied science and mathematics*, 2(5):2394-2894.

Hyma, P., Jahan, N., Raheemunissa, Sreelekha, G. & Babu, K. 2014. Emulgel: a review. *International journal of pharmaceutical archive*, 3:1-11.

Kaur, G., Bedi, P.M.S., Narang, J.K. 2017. Topical nanoemulgel: a novel pathway for investigating alopecia. Journal of nanomedicine and nanotechnology, 8:1-472. Kalra, A. 2013. Preparation and evaluation of oil-in-water self-nanoemulsifying systems with potential for pulmonary delivery.

https://etd.ohiolink.edu/!etd.send_file?accession=mco1364764383&disposition=inline.pdf Date of access: 25 Sept. 2019.

Kaya, C., Kola, O., Ozer, M.S., Altan. 2008. Some characteristics and fatty acids composition of wild apricot (Prunus pseudoarmeniaca L.) kernel oil. *Asian journal of chemistry*, 20(4):2597-2602.

Kela, S.K. & Kaur, C.D. 2013. Pharmaceutical nanoemulsions an ardent carrier for drug delivery. *Indo american journal of pharmaceutical research*, 3:9202-9212.

Kennedy, R.A. & Kennedy, M.L. 2007. Effect of selected non-ionic surfactants on the flow behaviour of aqueous veegum suspensions. American association of pharmaceutical scientists. *PharmSciTech*, 8:E1-E6.

Kim, M.C., Ahn, Y., Jang, S.Y., Cho, K.H., Hwang, S.H., Lee, M.G., Ko, J.S., Park, K.H., Sim, D.S., Yoon, N.S., Yoon, H.J., Kim, K.H., Hong, T.J., Park, H.W., Kim, J.H., Jeong, M.H., Cho, J.G., Park, J.C. & Kang, J.C. 2011. Comparison of clinical outcomes of hydrophilic and lipophilic statins in patients with acute myocardial infarction. *The Korean journal of internal medicine*, 26:294-303.

Koocheki, A., Kadkhodaee, R. 2011. Effect of alyssum homolocarpum seed gum, Tween 80 and NaCl on droplets characteristics, flow properties and physical stability of ultrasonically prepared corn oil-in-water emulsions. *Food hydrocolloids*, 25:1149-1157.

Kumar, S. 2014. Role of nano-emulsion in pharmaceutical sciences. A review. *Asian journal of research in pharmaceutical sciences and biotechnology*, 2(1):1-15.

Kumarn, G.P., Rajeshwarrao, P. & Padmavathi, T. 2011. India college of pharmacy, nonionic surfactant vesicular systems for effective drug delivery-an overview. *Acta pharmaceutica sinica B*, 1(4):208-219.

Kurakula, M., Srinivas, C., Kasturi, N. & Diwan, P.V. 2012. Formulation and evaluation of prednisolone proliposomal gel for effective topical pharmacotherapy. *International journal of pharmaceutical sciences and drug research*, 4:35-43.

Loureiro, A., Nogueira, E., Azoia, N.G., Sárria, M.P., Abreu, A.S., Shimanovich, U., Rollett, A., Härmark, J., Hebert, H., Guebitz, G., Bernardes, G.J.L., Preto, A., Gomes, A.C. & Cavaco- Paulo, A. 2015. Size controlled protein nanoemulsions for active targeting of folate receptor positive cells. *Colloids and Surfaces B: Biointerfaces*, 135:90-98.

Lu, W., Chiang, B., Haung, D. & Li, P. 2014. Skin permeation of D-limonene-based nanoemulsions as a transdermal carrier prepared by ultrasonic emulsification. *Ultrasonics sonochemistry*, 21:826-832.

Malvern Instruments Limited. 2015. A basic guide to particle characterization. http://www.cif.iastate.edu/sites/default/files/uploads/Other_Inst/Particle%20Size/Particle%20Characterization%20Guide.pdf Date of access: 4 Jul 2019.

Mancini, G.B.J., Tashakkor, A.Y., Baker, S., Bergeron, J., Fitchett, D., Frohlich, J., Genest, J., Gupta, M., Hegele, R.A., Ng, D.S., Pearson, G.J., Pope, J. 2013. Diagnosis, prevention, and management of statin adverse effects and intolerance: Canadian working group consensus update. *Canadian journal of cardiology*, 29:1553-1568.

Marais, A. 2016. Hypercholesterolaemia: from inauspicious to appropriate to detrimental: case study. *South African pharmaceutical journal*, 83(1):30-32.

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

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

McFarland, A.J., Anoopkumar-Dukie, S., Arora, D.S., Grant, G.D., McDermott, C.M., Perkins, A.V. & Davey, A.K. 2014. Molecular mechanisms underlying the effects of statins in the central nervous system. *International journal of molecular sciences*, 15(11):20607-20637.

Mueller, J., Oliveira, J.S.L., Baker, R., Trappa, M., Schroeter, A., Brezesinski, G. & Neubert, R.H.H. 2016. The effect of urea and taurine as hydrophilic penetration enhancers on stratum corneum lipid models. *Biochimica et biophysica acta*, 1858:2006-2018.

Münch, S., Wohlrab, J. & Neubert, R.H.H. 2017. Dermal and transdermal delivery of pharmaceutically relevant macromolecules. *European journal of pharmaceutics and biopharmaceutics*, 119:235-242.

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

Nalini, T., Kumari, V.S. & Basha S.K. 2017. Novel nanosystems for herbal drug delivery. *World journal of pharmacy and pharmaceutical sciences*, 6(5):1447-1463.

Nejadmansouria, M., Hosseinia, S.M.H., Niakosaria, M., Yousefib, G.H. & Golmakani, M.T. 2016. Physicochemical properties and storage stability of ultrasound-mediated WPI-stabilized fish oil nanoemulsions. *Food hydrocolloids*, 61:801-811.

Nomaki, H., Toyofuku, T., Tsuchiya, M., Matsuzaki, T., Uematsu, K. & Tame, A. 2015. Three-dimensional observation of foraminiferal cytoplasmic morphology and internal structures using uranium-osmium staining and micro-X-ray computed tomography. *Marine micropaleontology*, 121:32-40.

Hach Company. 2010. What is pH and how is it measured? : a technical handbook for industry. http://www.hach.com/asset-get.download.jsa?id=7639984488.pdf. Date of access: 24 Aug 2019.

Ng, K.W. & Lau, W.M. 2015. Skin deep: the basics of human skin structure and drug penetration. (In Dragicevic-Curic, N. & Maibach, H.I., eds. Percutaneous penetration enhancers: chemical methods in penetration enhancement: drug manipulation strategies and vehicle effects. Heidelberg: Springer. p. 3-12).

Nomaki, H., Toyofuku, T., Tsuchiya, M., Matsuzaki, T., Uematsu, K. & Tame, A. 2015. Three-dimensional observation of foraminiferal cytoplasmic morphology and internal structures using uranium-osmium staining and micro-X-ray computed tomography. *Marine micropaleontology*, 121:32-40.

Ochoa, A.A., Hernandez-Becerra, J.A., Cavazos-Garduno, A., Vernon-Carte, E.J., Garc, H.S. 2016. Preparation and characterization of curcumin nanoemulsions obtained by thin-film hydration emulsification and ultrasonication methods. http://www.scielo.org.mx/pdf/rmiq/v15n1/1665-2738-rmiq-15-01-00079.pdf. Date of access: 22 Oct. 2019.

O'Sullivan, S. 2007. Statins: A review of benefits and risks. *Trinity student medical journal*, 8:52-56.

Özgün, S. 2013. Nanoemulsions in Cosmetics. *Anadolu Univ*, 1(6):3-11.

Rao, S., Prasad, T., Mohanta, G. & Manna, P. 2011. An overview of statins as hypolipidemic drugs. *International journal of pharmaceutical sciences and research*, 3(3):178-183.

Reddy, A.K., Debnath, S. & Babu, M.N. 2013. Nanoemulsions a novel approach for lipophilic drugs - a review. *Asian journal of pharmaceutical research*, 3:84-92.

Reza, H. 2011. Nanoemulsion as a novel transdermal drug delivery system. *International journal of pharmaceutical sciences and research*, 2(8):1938-1946.

Ribeiro, R.C.D., Gomes-Barreto, S.M.A., Ostrosky, E.A., Da Rocha-Filho, P.A., Veríssimo, L.M., Ferrari, M. 2015. Production and characterization of cosmetic nanoemulsions containing opuntia ficus-indica (l.) mill extract as moisturizing agent. *Molecules,* 20:2492-2509.

Schachter, M. 2005. Chemical, pharmacokinetic and pharmacodynamic properties of statins: an update. *Fundamental & clinical pharmacology*, 19(1):117-125.

Setya, S., Talegaonkar, S. & Razdab, B.K. 2014. Nanoemulsions: formulation methods and stability aspects. *World journal of pharmacy and pharmaceutical sciences*, 3:2214-2228.

Silva, H.D., Cerqueira, M.A., Vicente, A.A. 2012. Nanoemulsions for food applications: development and characterization. *Food and bioprocess technology*, 5:854-867.

Shakeel, F., Baboota, S., Ahuja, A., Ali, J., Aqil, M. & Shafiq, S. 2007. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. American association of pharmaceutical scientists. *PharmSciTech*, 8:E1-E9.

Sharma, N., Bansal, M., Visht, S., Sharma, P. & Kulkarni, G. 2010. Nanoemulsion: A new concept of delivery system. *Chronicles of Young Scientists*, 1(2):2.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N. & Garcia-Celma, M.J. 2005. Nano-emulsions. *Current opinion in colloid and interface science*, 10:102-110.

Sotiropoulou, E.I., Varelas, V., Liouni, M. & Nerantzis, E.J.E.M.N.-M.P. 2012. Grape seed oil: from a winery waste to a value added cosmetic product-A review. Edible medicine non-medicinal plants 2:867-878.

Smith, S.C., Blair, S.N., Bonow, R.O., Brass, L.M., Cerqueira, M.D., Dracup, C., Fuster, V., Gotto, A., Grundy, S.M., Miller, N.H., Jacobs, A., Jones, D., Krauss, R.M., Mosca, L., Ockene, I., Pasternak, R.C., Pearson, T., Pfeffer, M.A., Starke, R.D., Taubert, K.A. 2001. AHA/ACC Scientific Statement: AHA/ACC Guidelines for preventing heart attack and death in patients with atherosclerotic cardiovascular disease. *Journal of the American college of cardiology*, 38(5):1581-1583.

Tadros, T., Izquierdo, P., Esquena, J. & Solans, C. 2004. Formation and stability of nano-emulsions. *Advances in colloid and interface scienc*e, 108(19):303-318.

Thakur, N., Garg, G., Sharma, P.K. & Kumar, N. 2012. Nanoemulsions: a review on various pharmaceutical application. *Global journal of pharmacology*, 6:222-225.

V&P Scientific, Inc. 2010. Viscosity tables. http://www.vp-scientific.com/Viscosity_Tables.htm Date of access: 5 May. 2018.

Walter, J.R. & Xu, S. 2015. Therapeutic transdermal drug innovation from 2000 to 2014: current status and outlook. *Drug discovery today*, 20(11):1293-1299.

Weiss, S.C. 2011. Conventional topical delivery systems. *Dermatologic therapy*, 24:471-474.

Wilbur, R.L. 2017. The Difference Between Topical and Transdermal Medications. https://genscopharma.com/wp-content/uploads/2017/05/Topical-v-Transdermal-White-Paper-05-26-17-EMAIL.pdf Date of access: 4 Jun. 2019.

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E., *ed*. Aultons pharmaceutics: the design and manufacture of medicines. 3rd ed. London: Churchill Livingstone. p. 675-697).

Zhang, D. 2009a. Polyoxyethylene sorbitan fatty acid esters. (*In* Rowe, R.C., Sheskey, P.J. & Quinn, M.E., *eds*. Handbook of pharmaceutical excipients. 6th ed. USA: Pharmaceutical Press. p. 549-553).

Zhang, D. 2009b. Sorbitan esters. (*In* Rowe, R.C., Sheskey, P.J. & Quinn, M.E., *eds*. Handbook of pharmaceutical excipients. 6th ed. USA: Pharmaceutical Press. p. 675-678).

Zhao, Q., Kuang, W., Long, Z., Fang, Liu, D., Yang, B., Zhao, M. 2013. Effect of sorbitan monostearate on the physical characteristics and whipping properties of whipped cream. *Food chemistry*, 141:1834-1840.

Hielscher Ultrasound technology. 1999. Ultrasonic Production of Stable Nanoemulsions. https://www.hielscher.com/ultrasonic-production-of-stable-nanoemulsions.htm Date of access: 15 Jan'. 2019.

Zhao, Q., Kuang, W., Longa, Z., Fang, M., Liu, D., Yang, B., Zhao, M. 2013. Effect of sorbitan monostearate on the physical characteristics and whipping properties of whipped cream. *Food chemistry*, 141:1834-1840.

APPENDIX C

THE CHARACTERISATION OF RESPECTIVE STATIN AND GRAPESEED OIL INFUSED O/W NANO-EMULGELS

C.1 Introduction

This chapter will focus on the characterisation and formulation of a nano-emulgel (**NG1**) that was based on the optimised nano-emulsion (**NE1**). **NE1** was successfully formulated, characterised and selected as described in Appendix B. Literature suggests that the bioavailability values of certain APIs are ± 3.5 times higher, when the transdermal route is followed opposed to the oral route, due to the exclusion of hepatic first-pass metabolism (Chellapa *et al.*, 2015).

When using the transdermal drug delivery route, the stratum corneum (thickness of 10 – 20 µm) serves as a barrier due to its highly complicated protein-lipid matrix (Chellapa *et al.*, 2015). This barrier can be breached by utilising **NEs** (low spreadability) or by combining a gelling agent into the formula to produce a **NG** (Chellapa *et al.*, 2015). **NGs** can display both characteristics of **NEs** and gels (Kaur *et al*., 2017).

NEs are isotropic systems, which consist of good stability and physiochemical characteristics (Chellapa *et al.*, 2015). The small droplet sizes regarding **NEs** produces a system that is kinetically stable and abstains from instabilities, e.g. flocculation and coalescence (Roberts *et al*., 2017). **NEs** are frequently researched as an alternative drug delivery system to enhance the penetration of poorly soluble APIs. **NEs** are very advantageous; the delivery system provides prolonged retention time at the administration area, which transports through the skin with less potential side-effects (Sutradhar, 2013). **NEs** are considered less convenient due to low viscosity (Ali *et al*., 2014) and poor spreadability (Chellapa *et al.*, 2015). Hence, these limitations have restrained the application of **NEs** clinically (Khurana *et al*., 2013). Therefore, the approach of incorporating a gelling agent within the **NEs** can help in overcoming this problem (Mou *et al*., 2008; Singh *et al*. 2017), it can improve formulation stability (by increasing viscosity and decreasing interfacial tension) and skin adhesion (by increasing the solubilisation capacity), which may assist in better skin penetration (Eid *et al*., 2014) with minimal effort (Pund *et al*., 2015). Carbopol® Ultrez 20 was the gelling-agent that is used in this study.

Carbopol® Ultrez 20 can be described as a white powder that wets and dissolves easily (Lubrizol, 2018). Additionally, this gelling-agent causes swelling, which increase the viscosity of the **NE** and therefore enhances stability, as well as permeability of the formulations when applied to the skin (Chudasama, 2011). Carbopol® Ultrez 20 can remain stable over a wide pH range, which decreases potential obstacles. This gelling-agent also assists in dissolving substances in the formulation (Lubrizol, 2018) and presents with a rich glowing appearance that makes it suitable

to use in shampoos, lotions, creams and gels (Lubrizol, 2018). Carbopol® Ultrez 20 has been utilised to higher the viscosity of **NEs**, as well as enhance permeability of formulations when applied topically (Chudasama, 2011).

NGs are classified as semi-solid dosage systems with a consistency somewhere between a solid and a liquid (Kaur *et al*., 2013). Semi-solid formulations offer direct contact when applied to the skin, which has a double function, e.g. assisting drug delivery and acting as a carrier system (Gupta & Garg, 2002). A **NG** can be described as the product of a nano-emulsion-based-hydrogel, by incorporating a **NE** within a hydrogel, which ultimately increases skin permeability (Chellapa *et al.*, 2015). **NGs** are known to increase patient compliance, as they are effortlessly applied and removed from the skin, opposed to other topical and transdermal formulations, e.g. ointments, creams, etc. (Pund *et al*., 2015; Williams, 2013). This gel structure regulates the release of APIs, which increase the effectiveness of limited half-life APIs (Chellapa *et al.*, 2015; Maree, 2018). **NGs** applied topically includes adhesion characteristics and induced solubilisation capability, which causes higher concentration gradients of APIs; therefore, a higher concentration can penetrate the skin (Chellapa *et al.*, 2015).

Surfactants such as Span® 60 (lipophilic) and Tween® 80 (hydrophilic) were included in the **NE** formula to obtain stable dispersions, as mentioned in Appendix B. These surfactants were also utilised to formulate **NGs** in this study. Surfactants are used to reduce the tension between the water and oil phases of the o/w **NE** (Setya *et al*., 2014), which lead to enhanced stability properties (Baibhav *et al*., 2011).

The characteristics of the chosen optimised **NE** will be compared to the characteristics of the **NG**. The codes for **NE1s** will remain as described in Appendix B.5 and the following codes will be used to define the **NGs** that will be studied.

Table C.1: The codes that describes the nano-emulsions and nano-emulgels

Statin	Optimised nano-emulsion (NE1)	Nano-emulgel code (NG1)
Atorvastatin	**NE-ATO1**	**NG-ATO1**
Mevastatin	**NE-MEV1**	**NG-MEV1**
Pitavastatin	**NE-PIT1**	**NG-PIT1**
Pravastatin	**NE-PRA1**	**NG-PRA1**
Placebo	**NE-PLAS1**	**NG-PLAS1**

C.2 Formulation goal

NEs entail many advantages for transdermal drug delivery; however, the low viscosity (Ali *et al*., 2014) and poor spreadability (Chellapa *et al.*, 2015) can lead to poor patient compliance (Pund

et al., 2015) and reduced API delivery when applied topically (Ali *et al*., 2014). Hence, the limitation has restrained the application of **NEs** clinically (Khurana *et al*., 2013). Therefore, the approach of incorporating a gelling agent within the **NEs** can help in overcoming this problem (Mou *et al.,* 2008). The goal was to formulate a semi-solid, more specifically a **NG**, to increase viscosity and potentially improve skin permeation.

C.2.1 The method and formula of the nano-emulgel

NG1 was formulated by using the **NE1** obtained from Appendix B, which is combined with a gelling agent (Carbopol® Ultrez 20). Table C.3 indicates the formula used to formulate **NG1**. An illustration of the process used during the manufacturing of the **NG1** is presented in Figure C.1.

Table C.2: Ingredients used during the formulation of **NG1** in conjunction with batch numbers, suppliers and function

Ingredients	Batch numbers	Suppliers	Function
Atorvastatin	20170518	DB Fine chemicals	API
Mevastatin	USMV-1703006	DB Fine chemicals	API
Pitavastatin	AFM-170502	DB Fine chemicals	API
Pravastatin	0651001	DB Fine chemicals	API
Span® 60	5361721034	Sigma & Aldrich	Lipophilic surfactant/emulsifier
Tween® 80	BCBV7863	Sigma & Aldrich	Hydrophilic surfactant/emulsifier
Grapeseed oil	0712E040876	CJP Chemicals	Oil phase (natural oil) and penetration enhancer
Milli-Q® water	Direct Pure UP	In-Lab	Water phase/solvent
Carbopol® Ultrez 20	0102052576	Sigma-Aldrich	Gelling agent/ thickening agent

Table C.3: Formula of the o/w **NG1**

Phase	Constituent	Quantity
		NE1
Water	Milli-Q® water	40.000 ml
	Tween® 80	2.804 ml
	Carbopol® Ultrez 20	0.21 g
Oil	Statin	1.00 g
	Span® 60	3.000 ml
	Grapeseed oil	4.350 ml

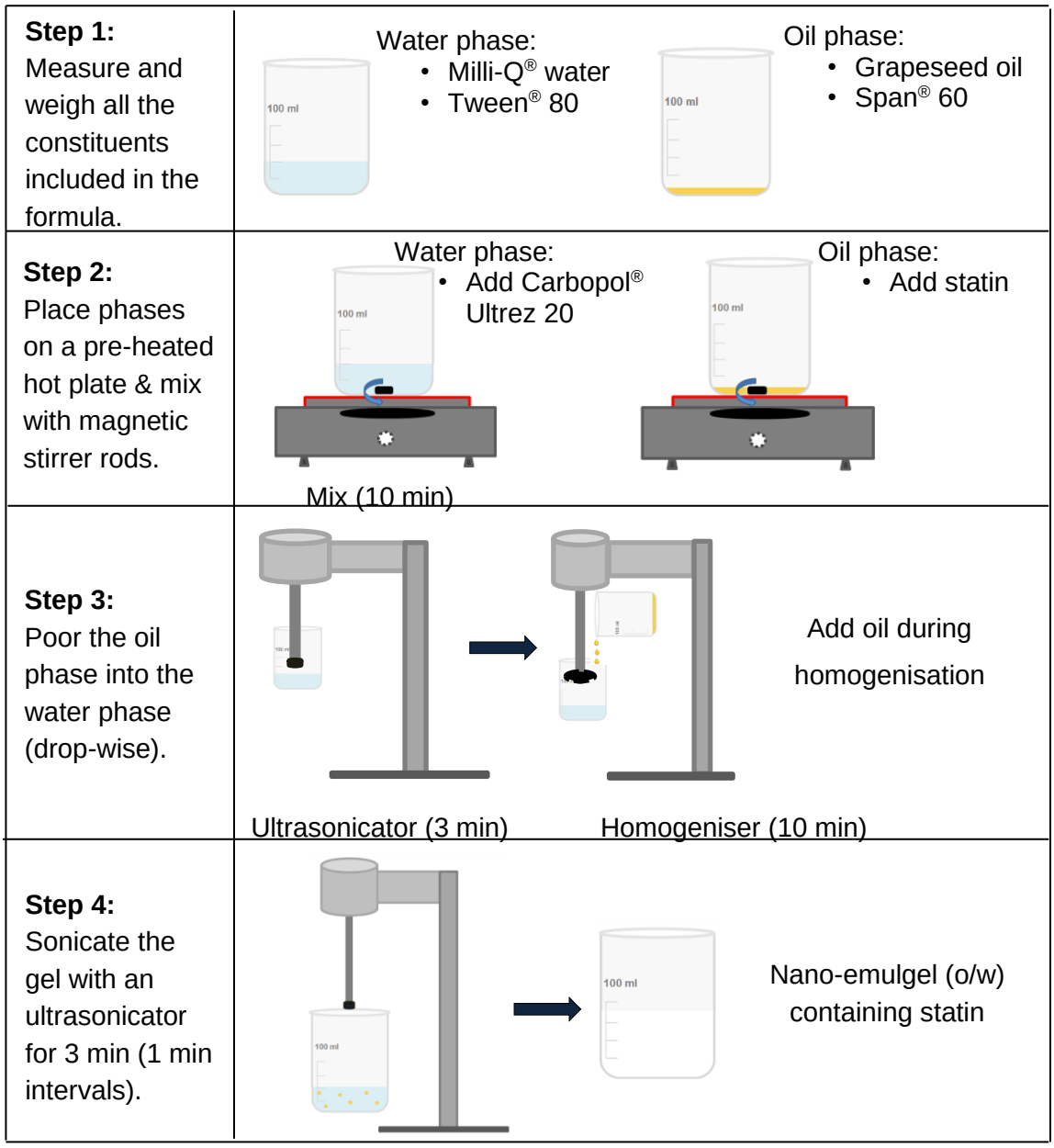


Figure C.1: Schematic representation of the **NG** formulation method

C.3 Methods utilised in characterisation of nano-emulgels

The following characteristics were analysed concerning the **NG1s**, which were compared to the **NE1s** (Kumar 2014; Nejadmansouria *et al*., 2016):

- Visual inspection
- pH
- Zeta-potential measurement (surface potential)
- Droplet size and distribution

- Viscosity
- Light microscopy

C.3.1 Visual inspection

In Figure C.2, the **NG1s** were compared to the **NE1s** via visual inspection. The **NG1s**, which contained Carbopol® Ultrez 20 (thickening agent); exhibited a smooth, shiny, white, gel-like appearance. The viscosity of **NG1s** appeared to be higher opposed to the **NE1s**, which exhibited with a whitish-milky appearance. The visual inspection concluded that **NG1s** (separately containing identical statins) showed no signs of sedimentation, flocculation and visible oil droplets, therefore, the oil droplets were effectively dispersed within in the water phase.

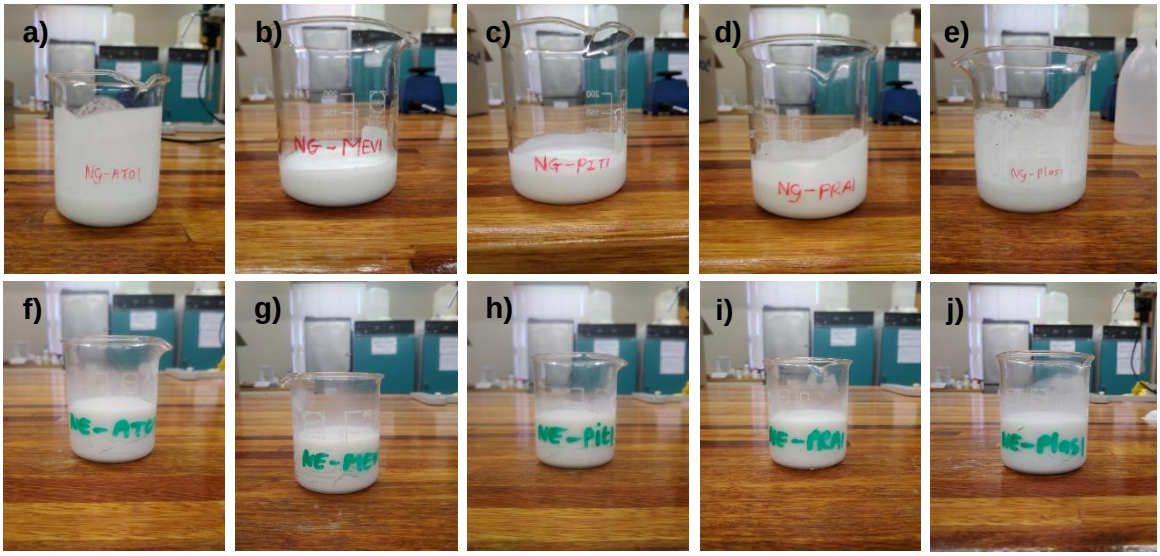


Figure C.2: Visual illustration of the **NG1s** (a) **NG-ATO1**, b) **NG-MEV1**, c) **NG-PIT1**, d) **NG-PRA1**, e) **NG-PLAS1** and the **NE1s** (f) **NE-ATO1**, g) **NG-MEV1**, h) **NE-PIT1**, i) **NG-PRA1** and j) **NE-PLAS1**)

C.3.2 pH

The skin normally has a pH value of ± 5 (Ng & Lau, 2015; Williams, 2013) and according to Barry (2002), human skin can sustain pH values of 3 – 9, which will serve as a parameter in this study. The pH is measured to determine whether the applied formulation will act as an irritant towards human skin. The pH of the **NG1s** was measured with a Mettler Toledo® pH meter (Mettler Toledo, CU) by placing the Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) into the **NG** to determine the pH value.

Table C.4: Measured pH values for **NG1s** and **NE1s**

Formula	pH			
	1	2	3	Average
NE-ATO1	6.12	6.13	6.14	6.13 ± 0.008
NG-ATO1	5.55	5.45	5.45	5.48 ± 0.047
NE-MEV1	6.31	6.30	6.30	6.30 ± 0.005
NG-MEV1	6.15	6.17	6.18	6.17 ± 0.012
NE-PIT1	6.32	6.33	6.33	6.33 ± 0.005
NG-PIT1	5.07	5.04	5.06	5.06 ± 0.012
NE-PRA1	6.41	6.41	6.41	6.41 ± 0.000
NG-PRA1	5.10	5.10	5.11	5.10 ± 0.005
NE-PLAS1	6.25	6.27	6.26	6.26 ± 0.008
NG-PLAS1	5.45	5.43	5.45	5.44 ± 0.009

Table C.4 illustrates the average pH values obtained for **NG1s** and **NE1s**. **NG1s** varied between the values 5.06 – 6.17, which was lower than the pH values of **NE1** (6.13 – 6.41). This occurrence can be ascribed to Carbopol® Ultrez 20, which is an acidic polymer that is known to decrease the pH levels after being included in the formula (Lubrizol, 2009; Marais, 2018). To reach maximum viscosity, the **NG1s** pH level is ideally between 6 and 7; therefore, the **NG1s'** pH levels for each statin had to be neutralised (Lubrizol, 2009) by using NaOH. **NE-PLAS1** and **NG-PLAS1** served as control groups to eliminate and isolate variables. All the formulas in Table C.4 were within the required parameters (pH 3 – 9) (Barry, 2002). Thus, it can be concluded that these formulas should not act as an irritant towards the skin, when applied topically on human skin.

C.3.3 Zeta-potential (mV)

The Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) were used to measure the zeta-potential of the **NE1s** and **NG1s**. Zeta-potential will establish if the preparation will withstand environmental conditions when utilised (Asmawatia *et al*., 2014). Zeta-potential is determined by measuring inter (surface charged) activities and the long-lasting stability between the elements in the formulation; therefore, calculating electrophoretic motion of the dispersed oil particles (Kumar, 2014).

The ideal zeta-potential diverges from ± 20 mV and depends on the dispersant, including the chemistry of the surface area of the ingredients of the formulation (Kumar, 2014). It is suggested that dispersions with a zeta-potential of more than ± 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). A high zeta-potential suggests powerful repulsion forces between the elements within the formulation, which indicates higher stability opposed to a zeta-potential value closer to zero, which increase the risk of flocculation and aggregation (Ribeiro *et al*., 2015;

Maree, 2018). Zeta-potential measurement can be influenced by pH variations, the type (salt) and concentration of the API and the concentration of other constituents (surfactants, gelling agents, etc.) in the formulation (Asmawatia, *et al*., 2014).

During the zeta-potential measurement a volume of the **NG1** was diluted with Milli-Q® water (1:100) and mixed by rotating the flask efficiently. The solutions were then extracted with a syringe and injected in their own individual clear disposable zeta-cell. The sample measurements were performed and completed in triplicate, as mentioned in Appendix B.6.3.

Table C.5: Average zeta-potential (mV) values of **NG1s** and **NE1s**

Formula	Zeta-potential (mV)			
	1	**2**	**3**	**Average**
NE-ATO1	-35.2	-33.9	-35.0	-34.70 ± 0.57
NG-ATO1	-57.1	-57.0	-58.1	-57.40 ± 0.50
NE-MEV1	-37.3	-40.9	-39.5	-39.23 ± 1.48
NG-MEV1	-49.1	-49.8	-49.9	-49.60 ± 0.36
NE-PIT1	-33.8	-35.2	-35.6	-34.87 ± 0.77
NG-PIT1	-56.4	-54.6	-59.1	-56.70 ± 1.85
NE-PRA1	-41.2	-39.8	-40.3	-40.43 ± 0.58
NG-PRA1	-44.6	-42.5	-43.1	-43.40 ± 0.88
NE-PLAS1	-54.0	-51.5	-52.3	-52.60 ± 1.04
NG-PLAS1	-87.5	-88.7	-89.4	-88.53 ± 0.78

The zeta-potential of the **NG1s** and the **NE1s** were compared. It is evident that the **NG1s** (**NG-ATO1**, **NG-MEV1**, **NG-PIT1** and **NG-PRA1**) presented with higher zeta-potential values than the respective **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1**), indicating that the **NG1s** were more stable than their respective **NE1s**. Since the zeta-potential values were higher than - 30 mV (Eid *et al*., 2014; Marais, 2018; Silva *et al*., 2012), it can be concluded that the formulas (**NG1s** and **NE1s**) were well within the required range for acceptable stability. The **NG1s** had the highest zeta-potential as illustrated in Table C.5. **NE-PLAS1** and **NG-PLAS1** were also included as a baseline to compare groups and assess the effect of the absence of selected statins in formulations. The **NG-PLAS1** and **NE-PLAS1** measured higher values compared to their **NG1s** and **NE1s**; however, all of the formulations are within the required range suggesting that stability is acceptable. Zeta-potential values were less negative after the addition of the selected statins, since the statins induced a stronger anionic surface charge, resulting in decreased negative surface charges (Sari *et al*., 2015).

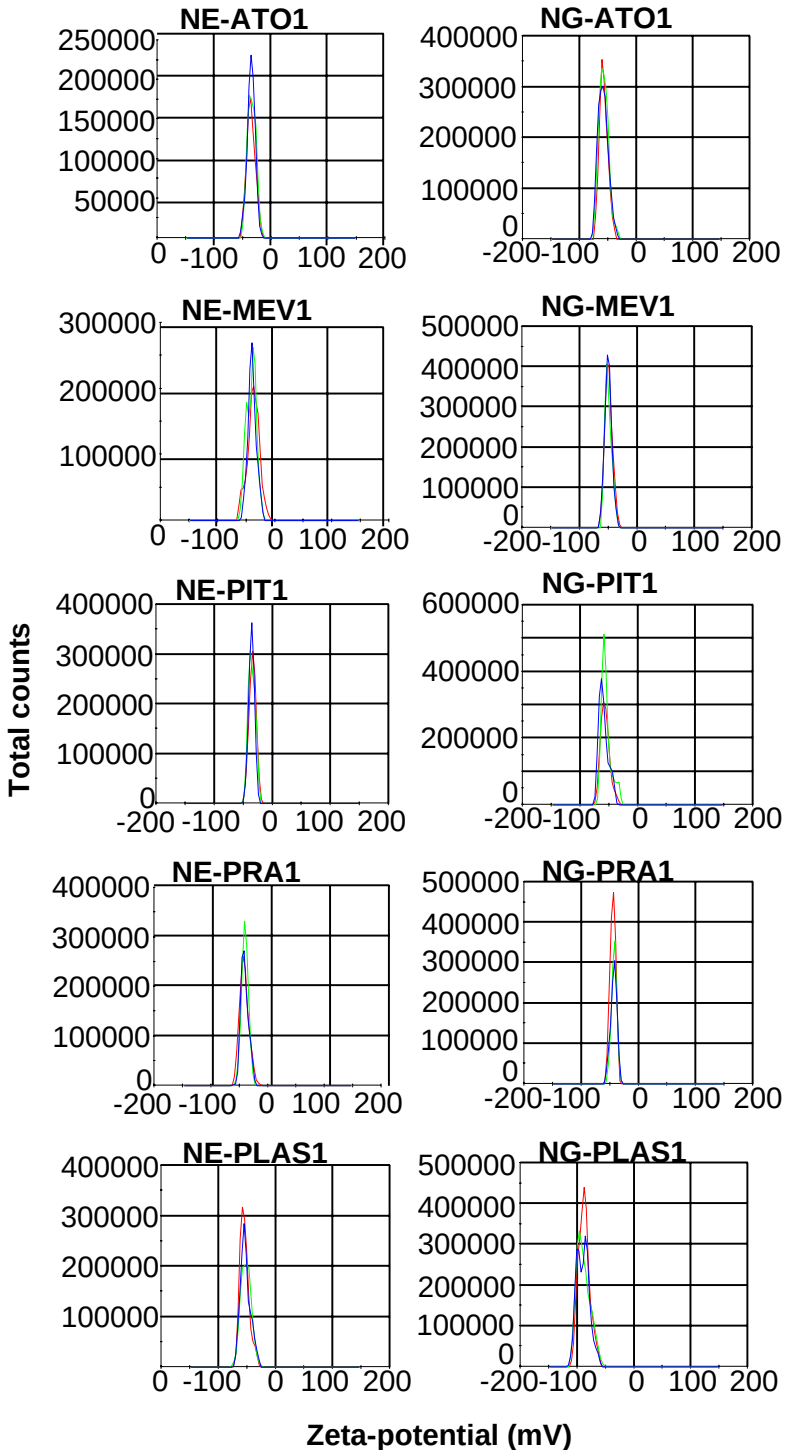


Figure C.3: The average zeta-potential (mV) of the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1**, **NE-PRA1** and **NE-PLAS1**) and the **NG1s** (**NG-ATO1**, **NG-MEV1**, **NG-PIT1**, **NG-PRA1** and **NG-PLAS1**)

C.3.4 Droplet size and distribution

A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used in this study to establish the droplet size and distribution of **NG1s**, which was compared to **NE1s**. This

instrument functions by using photon correlation spectroscopy (PCS), i.e. a Zetasizer, which calculates light fluctuations that follow between droplets in the **NE** as a result of Brownian motion (Gaur *et al*., 2014; Malvern Instruments Limited, 2015). PCS measures and expresses the droplets distribution as the PdI.

Stability can be established by calculating the droplet size (Solans *et al*., 2005); additionally, smaller droplet sizes enhance the integral stability of a formulation, as they decrease the occurrence of sedimentation, creaming, coagulation and flocculation (Kumar, 2014; Solans *et al*., 2005). Since, **NGs** are known as the addition of a **NE** system into hydrogel matrix, the required parameters for droplet sizes of **NEs** are < 500 nm (Nalini *et al*., 2017). PdI can be determined by utilising a scale ranging from 0 – 1, where a low PdI (closer to 0) is indicative of homogenous or monodispersed dispersions; however, a PdI closer to 1 is indicative of a more polydispersed dispersion (Gaumet *et al*., 2008).

Dilutions of the **NG1s** and **NE1s** were analysed, respectively by adding drops to a measured quantity (100 ml) of Milli-Q® water, which were subsequently mixed thoroughly by rotating the flask (similar to the method used to determine zeta-potential). Sample measurement was performed in triplicate.

Table C.6: Average droplet size and PdI of **NG1s** and **NE1s**

Formula	Average	
	Droplet size (nm)	**Polydispersity index (PdI)**
NE-ATO1	145.500 ± 1.280	0.417 ± 0.004
NG-ATO1	146.130 ± 2.830	0.228 ± 0.000
NE-MEV1	144.200 ± 0.640	0.395 ± 0.013
NG-MEV1	183.230 ± 3.350	0.340 ± 0.002
NE-PIT1	149.630 ± 1.640	0.386 ± 0.014
NG-PIT1	200.900 ± 3.330	0.384 ± 0.001
NE-PRA1	148.170 ± 0.67	0.237 ± 0.035
NG-PRA1	233.030 ± 8.780	0.399 ± 0.000
NE-PLAS1	158.100 ± 0.850	0.398 ± 0.021
NG-PLAS1	162.670 ± 1.180	0.291 ± 0.001

The average droplet size (nm) and average PdI for the **NG1s** and **NE1s** are illustrated in Table C.6. **NE-PLAS1** and **NG-PLAS1** served as control groups to eliminate and isolate the variables. After comparing the average droplet sizes of the **NE1s** and the **NG1s**, it is evident that the average droplet sizes of the **NG1s** were larger than those of the **NE1s**. Therefore, by the addition of Carbopol® Ultrez 20 to the **NE1s** to produce the **NG1s**, the droplet sizes of the **NG1s**

increased. This occurrence can be ascribed to the increased formation of cross-links in **NG1s** (Eid *et al*., 2014).

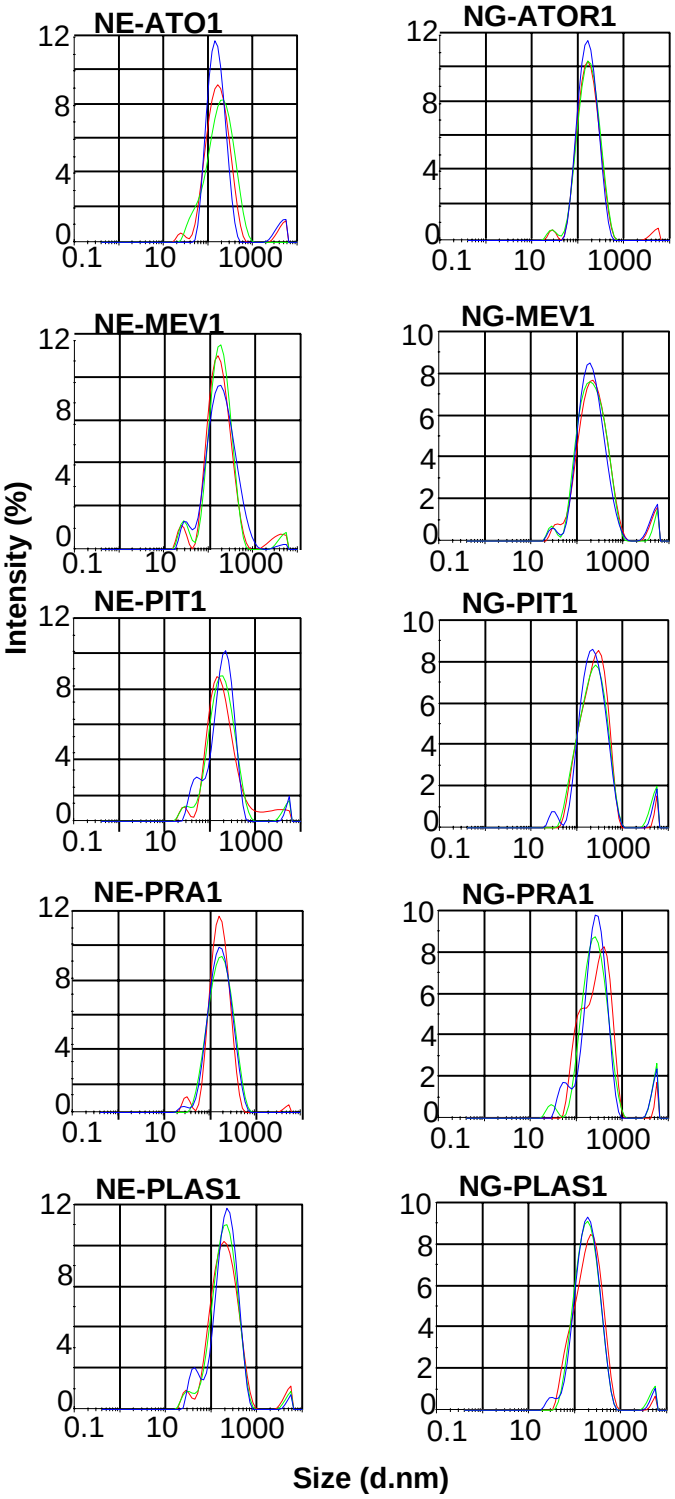


Figure C.4: Average droplet size (nm) of the **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1**, **NE-PRA1** and **NE-PLAS1**) and the **NG1s** (**NG-ATO1**, **NG-MEV1**, **NG-PIT1**, **NG-PRA1** and **NG-PLAS1**)

It can be observed, when the average PdI of the **NG1s** were compared to the average PdI of the **NE1s** that the **NE1s** had insignificantly higher PdI values; therefore, indicating that the **NE1s** were more polydispersed dispersions (Gaumet *et al.,* 2008) opposed to their **NG1s** that were more homogenous formulations, except for **NE-PRA1**, which presented with a slightly lower PdI value than **NG-PRA1**. This occurrence correlates with literature, stating that formulations containing Carbopol® Ultrez 20 (**NG1s**) do not have significant PdI value variations (Eid *et al*., 2014).

C.3.5 Viscosity

Viscosity is a fundamental concept to understand, which can be defined asthe resistance of a fluid to flow (Chime *et al*., 2014). The addition of substances (oil, APIs, surfactant) to water can ultimately influence the resistance of a fluid to flow. Due to the large water component present in an o/w **NE**, they are characterised as low viscosity dispersions (Shakeel *et al*., 2007, Thakur *et al*., 2012) and during visual examination, **NEs** presents watery (Thakur *et al*., 2012). Increasing the water content ultimately lowers the viscosity of a **NE** and a **NG**, while reducing the amount of surfactant increases interfacial tension between water and oil resulting in increased viscosity (Chime *et al*., 2014). When referring to **NEs**, the viscosity can be utilised as a characteristic of these systems (Shakeel *et al*., 2007). The addition of a gelling-agent to the formula of the **NE1s** produced a **NG1** (Chellapa, 2015). The gelling-agent in the formulation has the tendency to swell, which enhances the water phase's viscosity (Chellapa, 2015); producing a formulation with a higher viscosity (Mahalingam *et al*., 2008; Mitsui, 1997).

Carbopol® Ultrez 20 is an acidic polymer that is known to decrease the pH levels after being included in the formula (Lubrizol, 2009). To reach maximum viscosity, the **NG1s** pH level is ideally between 6 and 7, therefore, the pH levels for each **NG1** containing the respective statin had to be neutralised (Lubrizol, 2009; Shin *et al*., 2000) by using NaOH. When incorporating a gelling agent, the **NG** should be highly viscous, uniform and consistent (Dhawan *et al*., 2014; Marais 2018). Viscosity was calculated after the pH was adjusted and measured due to the addition of NaOH, which increased the viscosity (Lubrizol, 2009).

A Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA) linked to a thermostatic water bath (25 °C) was used to determine the viscosity of **NE1s** and **NG1s**. Each formulation was placed in the water bath (25 °C) for 45 min prior to the test. The appropriate spindle regarding respective **NG1s** had to be selected and fitted to the viscometer. T-bar spindles in conjunction with the Helipath stand accessory facilitates the measurement of viscosity of the **NG1s** that are slow flowing or non-flowing, e.g. creams, pastes, and gels (Brookfield, 2014, Marais, 2018). A T-bar spindle (T-C spindles were used, excluding pravastatin, where a T-D spindle was utilised) was found to be the most suitable to measure the viscosity of the **NG1s**,

where a cylindrical spindle (SC4-18) was utilised for measuring the viscosity of **NE1s**. A volume of the **NG1s** was transferred in the sample chamber, which fitted on a small sample adapter of the viscometer. The T-bar spindle was linked to the viscometer to hang down into the hollow of the sample adapter. To measure the viscosity, the T-bar spindle was set to rotate at a speed of 10 rpm. Viscosity readings were then measured in cP at 20 sec intervals for a period of 2 min. Rheocalc T 1.2.19 software was used to collect multipoint data to analyse and determine average viscosity values. This method was followed for each formula respectively. The average viscosity measurements for **NG1s** and **NE1s** can be compared as indicated in Table C.7.

Table C.7: Average viscosity readings of **NG1s** and **NE1s**

Formula	Viscosity (cP)	Speed (rpm)	Torque (%)	Temp (°C)
NE-ATO1	3.94 ± 0.03	200.00 ± 0.00	26.25 ± 0.15	22.47 ± 0.07
NG-ATO1	2 322.25 ± 9.93	30.00 ± 0.00	61.90 ± 0.25	24.51 ± 0.03
NE-MEV1	3.48 ± 0.02	200.00 ± 0.00	23.23 ± 0.15	22.05 ± 0.50
NG-MEV1	1 270.00 ± 90.07	30.00 ± 0.00	67.73 ± 4.81	23.70 ± 0.00
NE-PIT1	4.07 ± 0.07	200.00 ± 0.00	27.13 ± 0.51	21.71 ± 0.13
NG-PIT1	1 638.00 ± 10.32	30.00 ± 0.00	87.35 ± 0.55	21.70 ± 0.00
NE-PRA1	3.76 ± 0.02	200.00 ± 0.00	25.03 ± 0.15	22.97 ± 0.04
NG-PRA1	10 987.50 ± 618.72	30.00 ± 0.00	58.60 ± 3.30	21.42 ± 0.04
NE-PLAS1	3.32 ± 0.02	200.00 ± 0.00	22.10 ± 0.12	22.12 ± 0.13
NG-PLAS1	1 891.00 ± 84.11	30.00 ± 0.00	23.05 ± 0.45	24.80 ± 0.00

Table C.7 illustrates the viscosity readings (cP) obtained for the different **NG1s** and **NE1s** containing the respective statins. Both control groups, **NE-PLAS1** and **NG-PLAS1** eliminated and isolated the variables concerning viscosity with the absence of selected statins. All of the formulas are within the required range suggesting that stability is acceptable. The **NG1s** obtained significantly larger viscosity values opposed to the **NE1s**, which agrees with literature that combining an optimal **NE1** with a gelling agent enhances the water phase's viscosity, as it decreases interfacial and surface tension even more than **NEs** (Chellapa, 2015). Since the **NG1s** pH levels were modified (neutralised) (Lubrizol, 2009) with NaOH between 6 and 7, which the viscosity was drastically enhanced. The speed (rpm) to which the spindles were rotating in the **NG1s** were also slower compared to the speed of the **NE1s**. Mixing speed and time are essential considerations regarding **NG1s**. It is suggested that 800 – 1 200 rpm for ± 20 min is optimal. However, very high shear mixers can shorten the mixing time, which is not recommended, as this type of intense mixing can result in permanent loss of viscosity (Lubrizol, 2009; Marais, 2018). Various types of spindles were utilised due to the viscosity differences. Torque (%) readings of **NG1s** were also substantially higher, which indicates that the extent to which resistance was offered from the **NGs** against the spindle were more than those of the **NE1s**. The aim to enhance

the viscosity of the respective **NE1s** by addition of a gelling agent into the water phase was achieved when observing Table C.7.

C.3.6 Light microscopy of NG1s and NE1s

Light microscopy is considered an absolute method to determine shape, droplet distribution and size of particles during stability and characterisation studies (Particle sciences, 2010). The light microscope operates by using light and an organisation of lenses to enlarge samples to an optical size (Carllson, 2007). Light microscopy can establish whether aggregation in a formulation occurs, e.g. semi-solid dosage forms (Silva *et al*., 2012).

Light microscopy was utilised to observe the morphology of the **NG1s** (**NG-ATO1**, **NG-MEV1**, **NG-PIT1**, **NG-PRA1** and **NG-PLAS1**) in this study. Since, morphological analysis such as TEM (higher magnification capability) could not be performed on **NG1s** due to the high viscosity opposed to **NE1s**, which can be considered harmful to the TEM equipment. The respective **NE1s** (**NE-ATO1**, **NE-MEV1**, **NE-PIT1**, **NE-PRA1** and **NE-PLAS1**) were not investigated by means of light microscopy, as the morphology of these dispersions was investigated by utilising TEM, which is fully discussed in Appendix B.8.1.6.

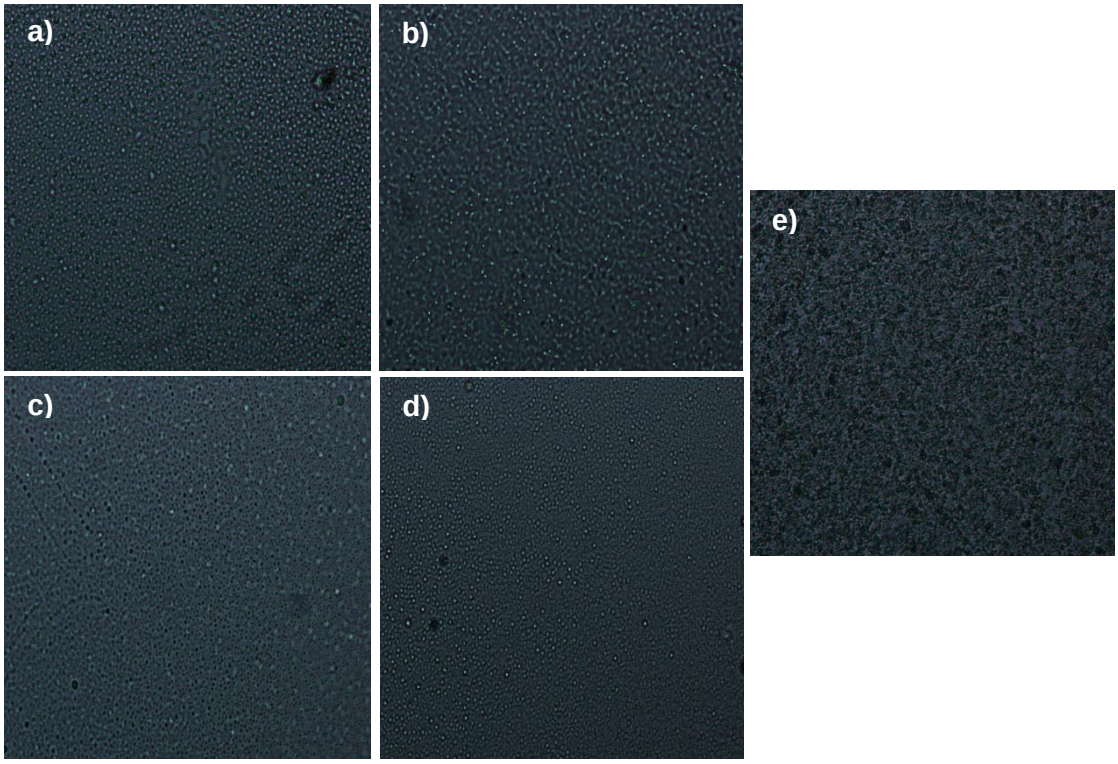


Figure C.5: Light microscopy micrographs of the **NG1s**: a) **NG-ATO1**, b) **NG-MEV1**, c) **NG-PIT1**, d) **NG-PRA1** and e) **NG-PLAS1**

The light microscopy investigation was performed by transferring a small volume of the respective **NG1s** on microscope slides, which was covered with a 16 mm cover slip. The slides were then studied on the microscope regarding each **NG1**, by executing numerous magnifications. It was evident that a 50 x magnification was suitable to study the morphology of the **NG1s**. This characterisation test was performed by using a Nikon Eclipse 50i microscope, fitted with a Nikon DSfi1 camera (Nikon, Japan Linkam THMS600), equipped with a T95 LinkPad temperature controller (Surrey, ENG). Figure C.5 illustrates the micrographs that were captured by using NIS-elements D (4.00.01, 64-bit) software.

The micrographs in Figure C.5 indicate the visible and distributed droplets of the **NG1s**. Due to the magnification alterations between TEM and light microscopy, these droplets appear out of perspective, opposed to the TEM micrographs in Appendix B.8.1.6. **NG-PLAS1** was also included in the study as a positive control group to indicate that the **NG1s** functioned as proposed.

C.4 Conclusion

Characterisation methods, e.g. visual inspection, pH, zeta-potential, droplet size and distribution, viscosity and light microscopy were implemented and performed to analyse and characterise **NG1s** (Kumar 2014; Nejadmansouria *et al*., 2016). The **NG1s** were compared to their optimised **NE1s**, which was characterised and discussed in Appendix B. It is evident that the addition of a gelling agent in optimised **NE1s**, which produced the **NG1s**, resulted in a gel-like formulation with a significantly higher viscosity (Mahalingam *et al*., 2008; Mitsui, 1997) that will according to literature enhance skin permeability (Chellapa *et al*., 2015; Eid *et al*., 2014). **NG1s** entail the ideal characteristic to improve stability by preventing particle separation (Mitsui, 1997). This occurrence can be supported by the high obtained viscosity measurements and visual inspection for **NG1s**.

Both control groups, **NE-PLAS1** and **NG-PLAS1** eliminated and isolated the variables and ensured that the **NG1s** and **NE1s** functioned as suggested, compared to their experimental groups that contained respective statins. These positive control groups (**NG-PLAS1** and **NE-PLAS1**) also established that the APIs (statins) did not affect the formula concerning the characterisation of the study.

It is evident that the pH of **NG1s** tested well below **NE1s**, as they contained Carbopol® Ultrez 20, which has the propensity to decrease pH of a formulation, since it is an acidic polymer (Lubrizol, 2009). The pH levels of **NG1s** were neutralised (6 – 7) (Lubrizol, 2009) by using NaOH in order to reach maximum viscosity. Thus it can be expected that the **NG1s** will not irritate the skin, since human skin can tolerate pH values within 3 – 9 (Barry, 2002).

The zeta-potential of **NG1s** and **NE1s** were well within the required range, since it is suggested that dispersions with a zeta-potential of more than ± 30 mV can remain stable over time (Eid *et al*., 2014; Silva *et al*., 2012). It can be concluded that **NG1s** obtained zeta-potential values well above those of the **NE1s**, which is an indicator of higher stability (the higher the zeta-potential, the lower the risk of flocculation) (Asmawatia *et al*., 2014; Chellapa *et al*., 2015; Eid *et al*., 2014).

Both, **NG1s** and **NE1s** displayed nano-sized droplets in the required range (< 500 nm) (Nalini *et al*., 2017). Generally, **NG1s** are more stable opposed to the optimised **NE1s**. Additionally, the oil droplets, which encapsulate lipophilic APIs, were more efficiently distributed in the gel-structure and efficacies of APIs with a limited half-life are extended, since **NG1s** regulates the release of the APIs, topically (Chellapa *et al*., 2015).

From these findings, it is evident that the **NG1s** were more stable opposed to **NE1s**. It can be proposed that **NGs** may enhance patient compliance due to the type of formulation, which is not sticky compared to other topical delivery systems, e.g. ointments and creams. Appendix D will investigate human skin permeability concerning **NG1s** and **NE1s** by means of *in vitro* skin diffusion studies.

References

Abolmaali, S.S., Tamaddon, A.M., Farvadi, F.S., Daneshamuz, S. & Moghimi, H. 2011. Pharmaceutical nanoemulsions and their potential topical and transdermal applications. *Iranian journal of pharmaceutical sciences*, 7:139-150.

Ali, M.S., Alam, M.S., Alam, N. & Siddiqui, M.R. 2014. Preparation, characterization and stability of dutasteride loaded nanoemulsion for treatment of benign prostatic hypertrophy. *Iranian journal of pharmaceutical research*, 13:1125-1140.

Asmawatia, B., Mustaphaa, W.A.W., Yusopa , S.M., Maskata, M.Y. & Shamsuddinc, A.F. 2014. Characteristics of cinnamaldehyde nanoemulsion prepared using APV-high pressure homogenizer and ultra turrax. AIP conference proceedings, 1614:244-250.

Baibhav, J., Gurpreet, S., Rana, A.C., Seema, S. & Vikas, S. 2011. Emulgel: a comprehensive review on the recent advances in topical drug delivery. *International research journal of pharmacy*, 2:66-70.

Barry, B. 2002. Transdermal drug delivery. (In Aulton, M.E., ed. Pharmaceutics: the science of dosage form design. 2nd *ed*. London: Churchill Livingstone. p. 499-533.

Carllson, K. 2007. Light microscopy: Physics of biomedical microscopy. https://www.kth.se/social/files/542d1251f276544bf2492088/Compendium Light.Microscopy.pdf Date of access: 8 Oct 2019.

Chellapa, P., Mohamed, A.T., Keleb, E.I., Elmahgoubi, A., Eid, A.M., Issa, Y.S. & Elmarzugi, N.A. 2015. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR journal of pharmacy*, 5(10):43-47.

Chime, S.A., Kenechukwu, F.C. & Attama, A.A. 2014. Nanoemulsions: advances in formulation, characterization and application in drug delivery. (*In* Sezer, A.D., *ed.* Application of nanotechnology in drug delivery. p. 77–126). http://cdn.intechopen.com/pdfs–wm/47116.pdf Date of access: 4 Oct. 2019.

Chudasama, A., Patel, V., Nivsarkar, M., Vasu, K. & Shishoo, C. 2011. Investigation of microemulsion system for transdermal delivery of itraconazole. *Journal of advanced pharmaceutical technology & research*, 2(1):30-38.

Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti-inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of nanomedicine and nanotechnology*, 5:1-10.

Dhawan, B., Aggarwal, G. & Harikumar, S.L. 2014. Enhanced transdermal permeability of piroxicam through novel nanoemulgel formulation. *International journal of pharmaceutical investigation*, 4(2):65-76.

Elmataeeshy, M.E., Sokar, M.S., Bahey-El-Din, M.B. & Shaker, D.S. 2018. Enhanced transdermal permeability of terbinafine through novel nanoemulgel formulation. *Future journal of pharmaceutical sciences*, 4(1):18-28.

Gaumet, M., Vargas, A., Gurny, R. & Delie, F. 2008. Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*, 69:1-9.

Gaur, S., Garg, A., Yadav, D., Beg, M. & Gaur, K. 2014. Nanoemulsion gel as novel oil based colloidal nanocarrier for topical delivery of bifonazole. *Indian research journal of pharmacy and science*, 1:36-54.

Gupta, P. & Garg, S. 2002. Recent advances in semisolid dosage forms for dermatological application. *Pharmaceutical technology*, 144-162.

Kaur, G., Bedi, P.M.S., Narang, J.K. 2017. Topical Nanoemulgel: A Novel Pathway for Investigating Alopecia. *Journal of nanomedicine and nanotechnology*, 8:1-472.

Kaur, P., Kaur, L. & Khan, M.U. 2013. Topical formulations and hydro-gel: an overview. International journal of advances in pharmacy. *Biology and chemistry*, 2:201-2060.

Kumar, S. 2014. Role of nano-emulsion in pharmaceutical sciences*. A review Asian journal of research in pharmaceutical sciences and biotechnology,* 2(1):1-15.

Khurana, S., Jain, N. & Bedi, P. 2013. Nanoemulsion based gel for transdermal delivery of meloxicam: physico-chemical, mechanistic investigation. *Life sciences*, 92(6-7):383-392.

Lubrizol. 2018. Carbapol® 20 polymer. http://www.lubrizol.com Date of access: 4 Aug 2019.

Lubrizol. 2009. Viscosity of Carbopol® Ultrez 20 polymers in aqueous systems. http://www.lubrizol.com>lubrizol>Techicaldatasheet Date of access: 4 June 2019.

Mahalingam, R., Li, X. & Jasti, B.R. 2008. Semisolid dosages: ointments, creams and gels. (*In* Gad, S.C., *ed*. Pharmaceutical manufacturing handbook: production and processes. New Jersey: John Wiley & Sons. p. 267-309).

Malvern Instruments Limited. 2015. A basic guide to particle characterization. http://www.cif.iastate.edu/sites/default/files/uploads/other_inst/particle%20size/particle%20characterization%20guide.pdf Date of access: 4 Oct. 2019.

Marais, S. 2018. The use of apricot oil emulsions for the transdermal delivery of selected statins. Potchefstroom: NWU. (Dissertation-MSc).

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

Mitsui, T. 1997. Polymers. (*In* Mitsui, T., *ed*. New cosmetic science. Netherlands: Elsevier. p. 138-140).

Mou, D., Chen, H., Du, D., Mao, C., Wan, J., Xu, H. & Yang, X. 2008. Hydrogel-thickened nanoemulsion system for topical delivery of lipophilic drugs. *International journal of pharmaceutics*, 353(1-2):270-276.

Nalini, T., Kumari, V.S. & Basha S.K. 2017. Novel nanosystems for herbal drug delivery. *World journal of pharmacy and pharmaceutical sciences*, 6(5):1447-1463.

Nejadmansouria, M., Hosseinia, S.M.H., Niakosaria, M., Yousefib, G.H. & Golmakani, M.T. 2016. Physicochemical properties and storage stability of ultrasound-mediated WPI-stabilized fish oil nanoemulsions. *Food hydrocolloids*, 61:801-811.

Ng, K.W. & Lau, W.M. 2015. Skin deep: the basics of human skin structure and drug penetration. (In Dragicevic-Curic, N. & Maibach, H.I., eds. Percutaneous penetration enhancers: chemical methods in penetration enhancement: drug manipulation strategies and vehicle effects. Heidelberg: Springer. p. 3-12).

Particle sciences. 2010. Particle size determination in semisolid formulations. https://particlescieces.com>docs.pdf Date of access: 8 Oct. 2019.

Pund, S., Pawar, S., Gangurde, S. & Divate, D. 2015. Transcutaneous delivery of leflunomide nanoemulgel: mechanistic investigation into physicomecanical characteristics, *in vitro* anti-psoriatic and anti-melonoma activity. *International journal of pharmaceutics*, 487:148-156.

Roberts, M.S., Mohammeda, Y., Pastore, M.N., Namjoshi, S., Yousef, S., Alinaghi, A., Haridass, I.N. & Abd, E., Leite-Silva, V.R., Benson, H.A.E. & Grice, J.E. 2017. Topical and cutaneous delivery using nanosystems. *Journal of controlled release*, 247:86-105.

Sari, T.P., Mann, B., Kumar, R., Singh, R.R.B., Sharma, R., Bhardwaj, M. & Athira, S., 2015. Preparation and characterization of nanoemulsion encapsulating curcumin. *Food Hydrocolloids*, 43:540-546.

Setya, S., Talegaonkar, S. & Razdab, B.K. 2014. Nanoemulsions: formulation methods and stability aspects. *World journal of pharmacy and pharmaceutical sciences*: 3:2214-2228.

Shakeel, F., Baboota, S., Ahuja, A., Ali, J., Aqil, M. & Shafiq, S. 2007. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. *American association of pharmaceutical scientists: PharmSciTech*, 8:E1–E9.

Shin, S., Kim, Y. & Oh, I. 2000. Mucoadhesive and physicochemical characterization of carbopol-poloxamer gels containing triamcinolone acetonide. *Drug development and industrial pharmacy*, 26(3):307-312.

Silva, H.D., Cerqueira, M.A., Vicente, A.A. 2012. Nanoemulsions for food applications: development and characterization. *Food and bioprocess technology*, 5:854-867.

Singh, R.P., Parpani, S., Narke, R. & Chavan, R. 2017. Emulgel: a recent approach for topical drug delivery system. *Asian journal of pharmaceutical research and development*, 2(2):112- 123.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N. & Garcia-Celma, M.J. 2005. Nano-emulsions. *Current opinion in colloid and interface science*, 10:102-110.

Sutradhar, K.B. & Amin, L. 2013. Nanoemulsion: increasing possibilities drug delivery. *European journal of nanomedicine*, 5(2):97-110.

Thakur, N., Garg, G., Sharma, P.K. & Kumar, N. 2012. Nanoemulsions: a review on various pharmaceutical application. *Global journal of pharmacology*, 6:222-225.

Williams, A.C. 2013. Topical and transdermal drug delivery. (*In* Aulton, M.E., *ed*. Aulton„s pharmaceutics: the design and manufacture of medicines. 3rd ed. London: Churchill Livingstone. p. 675-697).

APPENDIX D

DIFFUSION STUDIES OF O/W GRAPESEED OIL NANO-EMULSIONS AND NANO-EMULGELS CONTAINING SELECTED STATINS

D.1 Introduction

The skin is the largest and heaviest organ in the body, which covers the entire external surface (Arda *et al*., 2014; Goodwin, 2011; Johnsen, 2010) with an expected body surface area of about 2 m² (Lai-Cheong & McGrath, 2009). The skin consists of three layers: epidermis, dermis, and the hypodermis (Lai-Cheong & McGrath, 2009). The epidermis, comprising the stratum corneum, forms the outermost layer of the skin (Johnsen, 2010), which influences transdermal drug delivery due to the stratum corneum acting as a barrier for drug delivery (Arda *et al*., 2014; Wickett & Visscher, 2006; Wilbur, 2017). Therefore, API absorption through the skin relies highly on the appropriate delivery system (Weiss, 2011), e.g. **NEs** and **NGs**.

NE1s are considered as appropriate pharmaceutical formulations intended for dermal and transdermal delivery (Chime *et al*., 2014). These formulations promote skin penetrations and transport of poor soluble APIs via sustained exposure at the target area (Sutradhar, 2013). **NE1s** and **NG1s** were formulated with the same amount of API in each formulation and statistically compared to one another. Only **NG1s** includes a gelling agent, which is known to further improve skin permeability and patient compliance due to enhanced stability and increased viscosity (Mahalingam *et al*., 2008; Mitsui, 1997) for it to be applied on the skin (Chellapa, 2015). Transdermal drug delivery refers to the diffusion of APIs through the skin, e.g. stratum corneum, epidermis and dermis to permeate into the systemic circulation at a regulated continuous rate to attain systemic therapeutic effects without the accumulation of the API within skin layers (Ali *et al*., 2015).

Membrane release studies, skin diffusion studies and tape stripping were thus performed to determine if the formulas: 1) were suitable to release the APIs from both formulas (**NE1s** and **NG1s**), 2) delivered the APIs transdermally and 3) permeated into the skin to deliver the APIs topically.

D.2 Methods

D.2.1 HPLC analysis of statin samples

An HPLC analytical method was developed and successfully validated as described in Appendix A. In this study, this method was used to detect the amount of statins obtained in the injected samples that was gathered through the use of Franz cell diffusion- and release studies,

which also included tape stripping. The collected samples were analysed with an HPLC system (Agilent HP 1100 series HPLC equipped with a pump, autosampler, UV detector and ChemStation software). The obtained samples were extracted from the receptor phase during diffusion studies and filtered with 0.45 µm polytetrafluoroethylene (PTFE) filters (Agela Technologies, Newark, DE) before HPLC analysis were initiated, due to the biological material.

The laboratory temperature was controlled (25 °C) and the specific column that has been utilised in this study was: Venusil XBP C_{18} (2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE). The HPLC method used (to detect the statins obtained in the receptor compartment, including the skin layers) followed a flow rate of 1.0 ml/min, a 10 µl injection volume, detection wavelength (UV) of 240 nm and a run time of 10.5 min with PBS (pH 7.4). The retention times (min) of the statins were ± 4.17 (atorvastatin), ± 6.64 (mevastatin), ± 3.39 (pitavastatin) and ± 3.85 (pravastatin). The mobile phase contained ACN/Milli-Q® water with 0.1% orthophosphoric acid, which started at 25% ACN for 1.0 min, then it increased linearly to 90% after 3.0 min, which was subsequently held at 90% for 6.5 min and thereafter, it was re-equilibrated to start conditions.

Throughout analysis, a standard solution of the selected statin was prepared (± 20 mg statin in 100 ml methanol) followed by two dilutions. Dilution 1 was prepared by adding 5 ml standard solution in a 50 ml volumetric flask, which was diluted (up to volume) with methanol. Dilution 2 was prepared by adding 5 ml of Dilution 1 in a 50 ml volumetric flask, where after it was made up to volume with methanol. As a result, the standard solution, including Dilutions 1 and 2 were injected as follow: 2.5 µl, 5.0 µl, 7.5 µl and 10.0 µl to acquire a standard curve for analysis.

D.2.2 Franz cell method

In vitro studies are suitable due to the ethical and economic aspects involved with *in vivo* studies (Karadzovska & Riviere, 2013). The prediction of drug outcome via *in vivo* studies is critical to develop suitable transdermal and topical formulations that requires robust and validated *in vitro* techniques and models (Bartosova & Baljar, 2012). *In vitro* Franz cell diffusion has become one of the most critical methods for researching transdermal drug administration. The most general used *in vitro* method concerning transdermal delivery is the diffusion cell (Karadzovska & Riviere, 2013; Simon *et al*., 2016). Franz cells are proposed to be used as diffusion cells in general (Simon *et al*., 2016). These cells consist of a donor and receptor compartment, which are divided by human skin (indicated for permeation studies) or synthetic membranes (indicated for release studies) (Simon *et al*., 2016). The receptor compartment contains a buffer (i.e. PBS at pH 7.4), which simulates blood underneath the skin (Williams, 2013). The donor compartment contains the formulation, which simulates the applied formulation on the stratum corneum (Williams, 2013). Franz cells provide significant information regarding the relations between the skin, formulation

and API, and assess skin permeability components (Klein, 2013). This specific method supports the development, dose and quality assessment of formulations (Cabral *et al*., 2014). In this study, the vertical Franz diffusion cell method was implemented to establish the release and diffusion of the statins from the **NE1s** and **NG1s**. *In vitro* methods are almost an exact simulation of an *in vivo* environment (Modi & Shah, 2015). When performing an *in vitro* study, it is important to bear a resemblance to *in vivo* conditions as closely as possible (Modi & Shah, 2015). Temperature is a variable that affects diffusion rate and should be carefully observed (Shahzad *et al*., 2015), as fluctuation in temperature could affect the rate of diffusions and the results obtained from the respective *in vitro* study (Shahzad *et al*., 2015). The water bath containing Franz cells was utilised to mimic *in vivo* environment at 37 °C (skin temperature) (Williams, 2013). A second water bath preheated to 32 °C was used to pre-heat the formulas, which is considered normal for external skin temperature (Williams, 2013).

D.2.2.1 Donor phase preparation

Table D.1 presents the formulas (**NE1** and **NG1**) that include the selected statins, which was tested individually during *in vitro* diffusion studies. During this study, eight formulas containing a specific statin, together with two placebos (containing no API; e.g. **NE-PLAS1** and **NG-PLAS1**), which served as the control group, were formulated.

Table D.1: **NE1s** and **NG1s** utilised in the donor phase during the Franz diffusion cell studies

Formula	**NE**	**NG**
Statins	**NE-ATO1**	**NG-ATO1**
	NE-MEV1	**NG-MEV1**
	NE-PIT1	**NG-PIT1**
	NE-PRA1	**NG-PRA1**
Placebo	**NE-PLAS1**	**NG-PLAS1**

D.2.2.2 Receptor phase preparation

The receptor phase during the diffusion studies contained PBS (pH 7.4). The pH selected is similar to that of human blood and is non-toxic for cells and body fluids (OECD, 2011). PBS (pH 7.4) was formulated by adding 3.03 g NaOH in 800 ml Milli-Q® water, which was then placed on a magnetic stirrer. Separately, 13.65 g potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 500 ml Milli-Q® water and placed on a magnetic stirrer. Once both solutions were entirely dissolved, the KH_2PO_4 solution was transferred into the NaOH solution and stirred on a magnetic stirrer. The solution was diluted with an additional 700 ml of Milli-Q® water, with a final volume of 2 000 ml PBS. The pH (7.4) of the PBS was measured with a Mettler Toledo® pH meter

(Mettler Toledo, CU), kitted with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) and calibrated with NaOH. Subsequently, 100 ml of ethanol was dissolved in 900 ml PBS (pH 7.4) to achieve a PBS:ethanol ratio of 9:1. A compound (statins) should freely partition in the receptor phase concerning the relatively lipophilic properties; therefore, ethanol was added (Collier & Bronaugh, 1991). A 10% ethanol to PBS (pH 7.4) is acceptable, as it does not compromise the skin (Thomas *et al*. 2014).

D.2.2.3 Membrane release studies

Membrane release studies were conducted to determine whether the respective statins were released from their formula, e.g. dispersions (**NE1s**) and semi-solid formulations (**NG1s**), to the receptor phase, under controlled *in vitro* conditions. A polyvinylidene fluoride (PVDF) (Pall® Life Sciences, Michigan, USA) membrane was placed between the receptor and donor compartment. The PBS:ethanol (9:1 at pH 7.4) and formulas were placed in separate Grant® water baths (Grant Instruments, UK), which were preheated to temperatures of 37 °C and 32 °C, respectively (1 h before commencing with the release studies). Two Franz cells contained either **NG-PLAS1** or **NE-PLAS1**. The compartments were sealed using Dow Corning® high vacuum grease. A magnetic stirrer was placed inside the receptor compartment to ensure adequate mixing. Afterwards, the synthetic membrane (PVDF with a pore size of 0.45 µm and 25 mm diameter) was placed on the greased side of the receptor compartment and the donor compartment was placed on top of the receptor compartment with the use of vacuum grease to seal the sides of the two connected compartments to prevent leakage. Finally, a horseshoe clamp was positioned over the donor and receptor compartments, to attach the two compartments. The receptor compartment has a filling capacity of ± 2ml and a diffusion surface of 1.075 cm^2. Thus, only 2 ml PBS:ethanol (9:1 at pH 7.4) was transferred to the receptor compartment for each study (one specific statin formula per study). A visual inspection was done to ensure the absence of air bubbles. The donor compartment was filled with 1 ml of **NEs** or **NGs** at a temperature of 32 °C, and enclosed with Parafilm®, as well as a plastic lid to keep the formulation intact. The PBS:ethanol (9:1 at pH 7.4) was returned to the water bath (37 °C). The Franz cells were placed in a Franz cell stand within the preheated water bath (37 °C), which includes a Variomag® magnetic stirring plate (Variomag, USA) to ensure adequate mixing. Every hour for the following 6 h, the receptor phase was fully extracted and refilled with PBS:ethanol (9:1 at pH 7.4) at a stable temperature of 37 °C. The extracted PBS:ethanol (9:1 at pH 7.4) was placed in an HPLC vial to be analysed via HPLC.

D.2.2.4 *In vitro* skin diffusion studies

D.2.2.4.1 Skin collection and ethical aspects

Males tend to have thicker skin layers in comparison to females (Igarashi *et al*., 2007; Olsen *et al*., 1995) and the truncal (torso) skin of men is significantly thicker compared to the skin found on the extremities (Olsen *et al*., 1995). Numerous findings have been reported on the relation between age and the thickness of skin layers (Denda & Takahashi, 1990; Gniadecka & Jemec, 1998; Igarashi *et al*., 2007; Lasagni & Seidenari, 1995); however, contrary to popular belief, a clear relationship has not been established. The stratum corneum in African or Caucasian skin is equally thick (Berardesca & Maibach, 2003); however, in African individuals, it contains more cell layers and has an increased resistance to tape stripping, compared to the stratum corneum of Caucasian skin (Weigand *et al*., 1974). Abdominoplasty is generally performed on females rather than males. Caucasian female skin was used during diffusion studies. This skin was obtained after post-abdominoplasty surgery.

Ethical approval was attained from the North-West University Health Research Ethics Committee (NWU-HREC) to perform transdermal drug delivery studies on human skin, which is regarded as biological waste. The surgeon explained to the participants that participation in this study was in no way mandatory and informed consent forms were signed before collection of the skin. Confidentiality of the participant was protected by eliminating all patient information from the donated skin and replacing it with a reference number. The reference number allocated to the specific participant was documented in a Donor File (log book) to ensure that if the participant wanted to withdraw from the study, the correct skin could be identified and discarded. This information was only accessible by authorised students and personnel. The obtained skin was transported in a locked cooler, which was then transferred to a freezer at a temperature of - 20 °C. Skin obtained from abdominoplasty will allow the results to be compared to those published internationally. Ethics approval for the sub-study (Ethics no: NWU-00111-17-A1-05) was granted by the NWU-HREC under the larger study titled "*The use of human skin (biological waste) to study transdermal and topical delivery*" (Ethics no: NWU-00111-17-A1).

D.2.2.4.2 Skin preparation for *in vitro* diffusion studies

Skin was defrosted and placed on a paper towel with the stratum corneum facing upwards. A Zimmer™ electric dermatome (Zimmer TDS, United Kingdom) was used to remove skin with a thickness of roughly 400 µm. The dermatome was placed on the skin at an angle of 30 – 45°. The dermatomed skin was placed on Whatman® filter paper, enfolded with aluminium foil and returned to the freezer (- 20 °C) until needed for skin diffusion studies. Dermatomed skin was

visually inspected for inadequacies and cut into circles before each respective study. Each circle could be fitted between the receptor and donor compartment of a vertical Franz cell.

D.2.2.4.3 Skin diffusion studies

In vitro skin diffusion studies (vertical Franz diffusion cells) can grant fundamental information on the association between skin diffusion, the delivery system (**NE1s** and **NG1s**) and the API (Ng *et al*., 2010). A similar technique was performed during the *in vitro* skin diffusion studies as for the membrane release studies (Section D.2.2.3). The only difference between the two methods were that membrane release studies used PVDF membranes, whereas *in vitro* skin diffusion studies used dermatomed human skin between the donor and receptor compartments, and that the receptor phase was only extracted once (for each Franz cell) after 12 h, based on the study performed by Burger (2014). The extraction solution was transferred into individual HPLC vials; thereafter, the chromatograms were evaluated and integrated, once HPLC analysis was completed.

D.2.3 Tape stripping

It is critical to quantify the API in the skin regarding topical and transdermal research (Escobar-Chávez *et al*., 2008). Tape stripping procedure provides data, which determines if topical delivery of the API within the stratum corneum-epidermis (SCE) or the epidermis-dermis (ED) occurred (OECD, 2004). The principle of this study was to enable transdermal delivery of selected statins (e.g. atorvastatin, mevastatin, pitavastatin and pravastatin) and after extracting the receptor phases during *in vitro* skin diffusion studies, the horseshoe clamps on each Franz cell were loosened to separate the donor from the receptor compartments. The skin samples were detached and pinned on Parafilm® to a wooden board. Any formulation residue present on the skin samples was gently dabbed off using a piece of paper towel. Tape (3M Scotch® Magic™) was cut into 20 strips, which were used to strip the SCE. The first strip that was dabbed on the skin sample was disposed of (possible contamination); thus, nineteen of the twenty tape strips (containing the SCE) were transferred into a single polytop that contained methanol (5 ml). After stripping the skin sample, the residual skin (ED) was removed from the Parafilm® and cut into pieces and transferred to an individual polytop that contained methanol (5 ml). Every single Franz cell included two polytops, one containing the nineteen tape strips (SCE) and the other containing the residue cut skin (ED). The polytops were stored in the fridge at a temperature of ± 4 °C for approximately 8 h. After 8 h, the content of the polytops were extracted and filtered using 0.45 µm PTFE filters. The filtrated solution was then placed in HPLC vials for analysis. This procedure was performed on each individual Franz cell sample of the respective study. Via HPLC it was possible to establish the concentration of the respective statins in the SCE and the ED.

D.2.4 Data analysis

All samples (membrane release, skin diffusion and tape stripping studies) were analysed by using the HPLC. Before any samples were analysed, a standard solution was injected to obtain a standard curve, which was used to determine the concentration of the individual statins in the particular samples.

During the membrane release studies, the average cumulative amount of the selected statin per area (µg/cm^2) was plotted against time (h) (Shakeel *et al*., 2007). As a result, the average drug flux (µg/cm^2.h) was established by using the slope of the linear regression attained (Ng *et al*., 2010). Hence, the average and median flux, together with the average %released (%) were determined during the membrane release studies to establish whether these statins were released from the formulas (**NE1s** and **NG1s**) and at which estimated rate.

Throughout the skin diffusion studies, results were established for the individual statins. This was used to determine the average %diffused (%), the average concentration diffused (µg/ml), together with the average and median amount per area diffused (µg/cm^2) of all the Franz cells together, which diffused through the skin after 12 h. During tape stripping the average and median concentrations (µg/ml) of the respected statins in the **NE1s** and **NG1s** were determined, which ultimately permeated the SCE and ED.

Hence, the release of the API from the dosage forms via membrane release and the diffusion of the API through the skin via skin diffusion studies could be established and proved beneficial during the testing of nanoparticles (Jug *et al*., 2017). Human skin samples are considered the most beneficial *in vitro* method, for it predicts *in vivo* permeation of topical applied APIs (Simon, 2016). In addition, it is critical to establish whether the target-site was reached. Systemic absorption is not intended for topical delivery, while transdermal delivery necessitates a topically applied formulation to penetrate beyond the skin layers intended for systemic absorption (Kanfer, 2017).

D.2.5 Statistical data analysis

Statistical analysis was performed on the data obtained from membrane release and skin diffusion studies via statistical methods e.g. ANOVA (analysis of variance). ANOVA is a parametric statistical method, which was used to test a specified hypothesis, e.g. the null hypothesis (H_0) (general hypothesis of indifference) and helped to establish if there were any statistically significant differences between the mean values of the experimental groups. During membrane and skin diffusion studies, eight formulas were compared in terms of the respective statins and the particular formula (**NE1** and **NG1**). Tape stripping data was compared in terms of the skin

layer (SCE and ED), in combination with the particular formulas and statins. Aforesaid was performed via a three-way ANOVA by Statistica™ (TIBCO® Statistica™, 2018), followed by a one-way or two-way ANOVA depending on the necessities.

The median and mean was used throughout this study to illustrate the statistical data. The median is a more accurate method to establish the concentration and flux in circumstances where there is a substantial difference between the median and mean values (Dawson & Trapp, 2004). Results of the ANOVA tests were revealed with p-values (Consonni, 2017). P-values (calculated probabilities) are generally utilised to conclude and summarise data and states the probability of the difference in means between groups, to be more similar or more extreme than its experimental value, when the H_0 of this study is regarded as true, using a particular statistical model (Consonni, 2017). P-values below or equal to 0.05 are generally reflected as a statistical significant result (Concato & Hartigan, 2016). Therefore, if the p-value is < 0.05, the H_0 is overruled and the alternative hypothesis (of differences in means) is regarded as true. Acceptable evidence supports the alternative hypothesis and *vice versa*.

Statistical data indicated skewed distributions, which indicated distributions near to normal (Hamada, 2018). When an ANOVA is significant, a follow-up test is required to determine which group mean is different from which other group's means. Therefore, a Tukey's honestly significant difference (HSD) post-hoc test was implemented. Tukey's HSD post-hoc test determines the least amount that means must vary from each other to be truly different. This test compares the means of all possible pairwise combinations of groups and ranks them according to their homogeneity (Hamada, 2018). The means of all the **NE1s** and **NG1s** differed more than the HSD value; therefore, they were considered as honestly significantly different. A non-parametric Kruskal-Wallis test can be used instead of a Tukey's HSD post-hoc test if data deviate from normality and/or homogeneity of variances (the assumptions under which the parametric ANOVA and Tukey-tests are valid).

Procedures attributed to descriptive analysis involve the calculation of the mean concentrations and the median (middle score in distribution), which includes the SD (Sheskin, 2000). Data distribution and outliers can have a substantial effect on the mean, whereas the median is less affected (Driscoll *et al*., 2000). Therefore, describing data only in terms of mean could be problematic. Box-plots were implemented to present a summary of the data, by using the median, extreme values, and the mean to indicate the estimated average SD (Krzywinski & Altman, 2014). The line in the middle of the box represents the 50th percentile or median (Nuzzo, 2016). Whiskers were added to the edges and extend to what was regarded as extreme to illustrate the data range for the outstanding 50% of the data. Data beyond the extremes are classified as outliers (Dawson & Trapp, 2004; Nuzzo, 2016).

D.3 Results and discussions

D.3.1 Membrane release studies

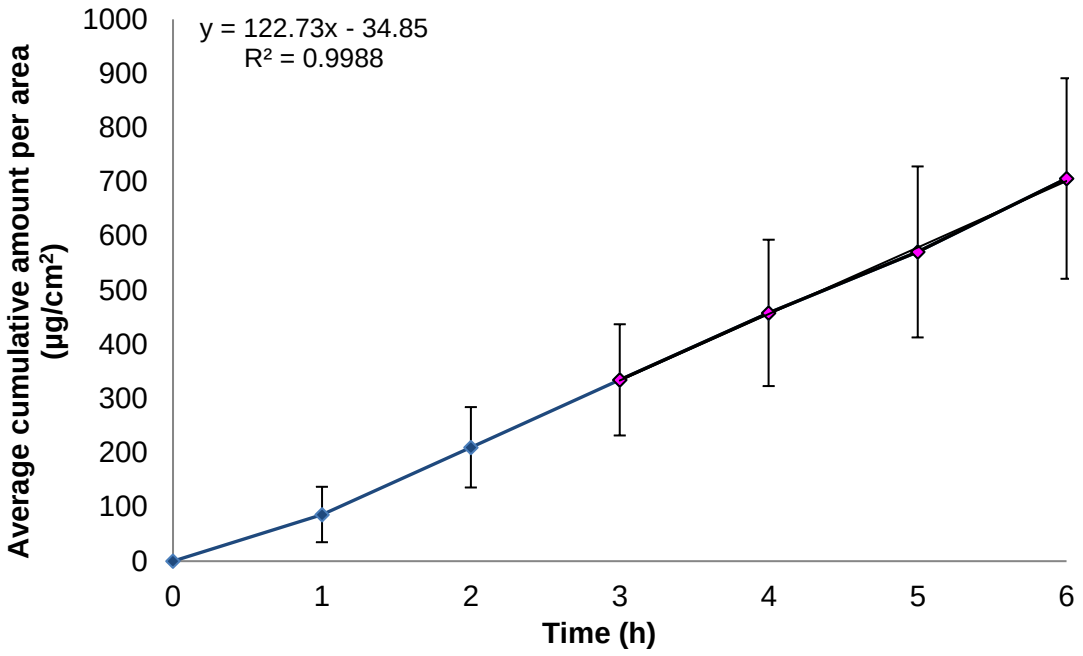


Figure D.1: Average cumulative amount per area (µg/cm^2) of atorvastatin from **NE-ATO1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 11)

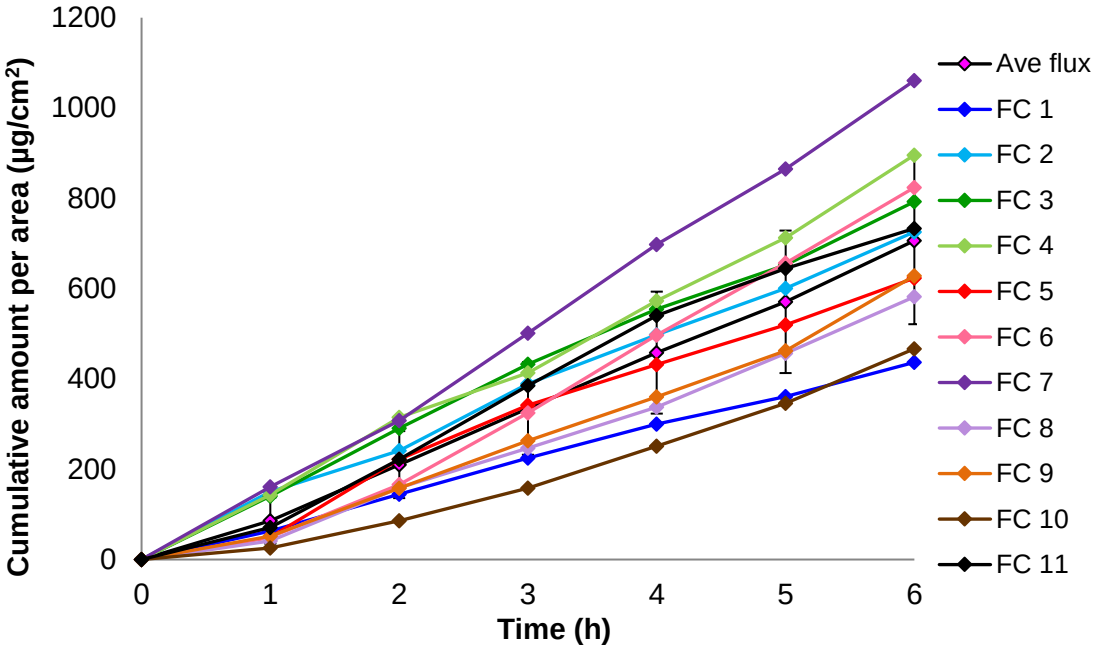


Figure D.2: Cumulative amount per area (µg/cm^2) of atorvastatin from **NE-ATO1** that was released through the membranes of each individual Franz cell over 6 h (n = 11)

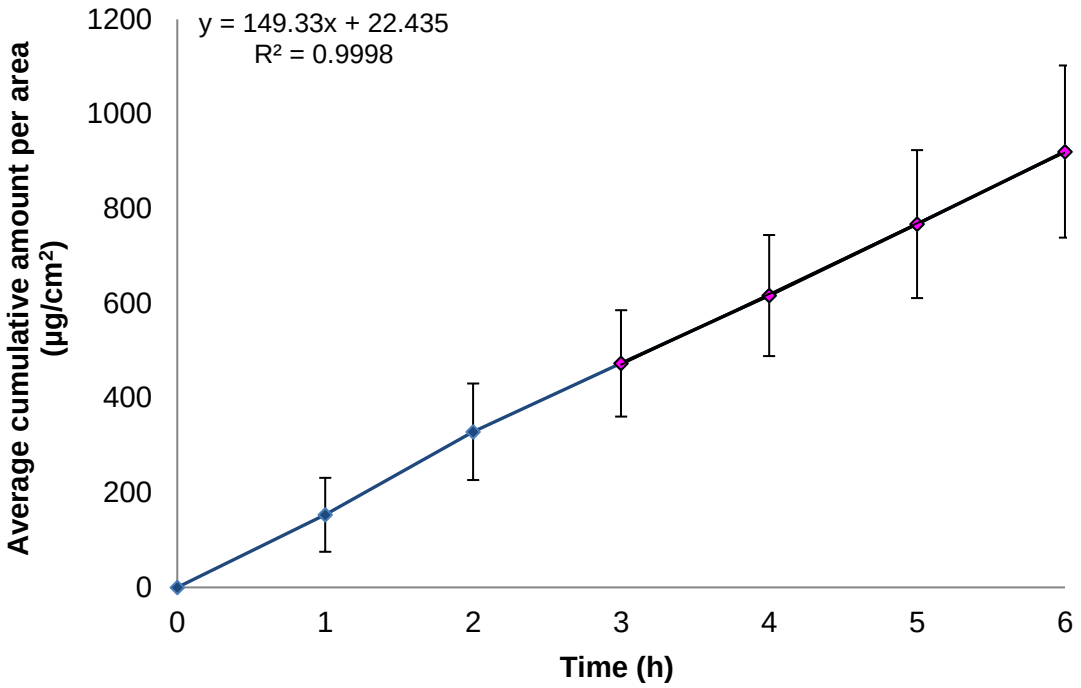


Figure D.3: Average cumulative amount per area (µg/cm^2) of atorvastatin from **NG-ATO1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 9)

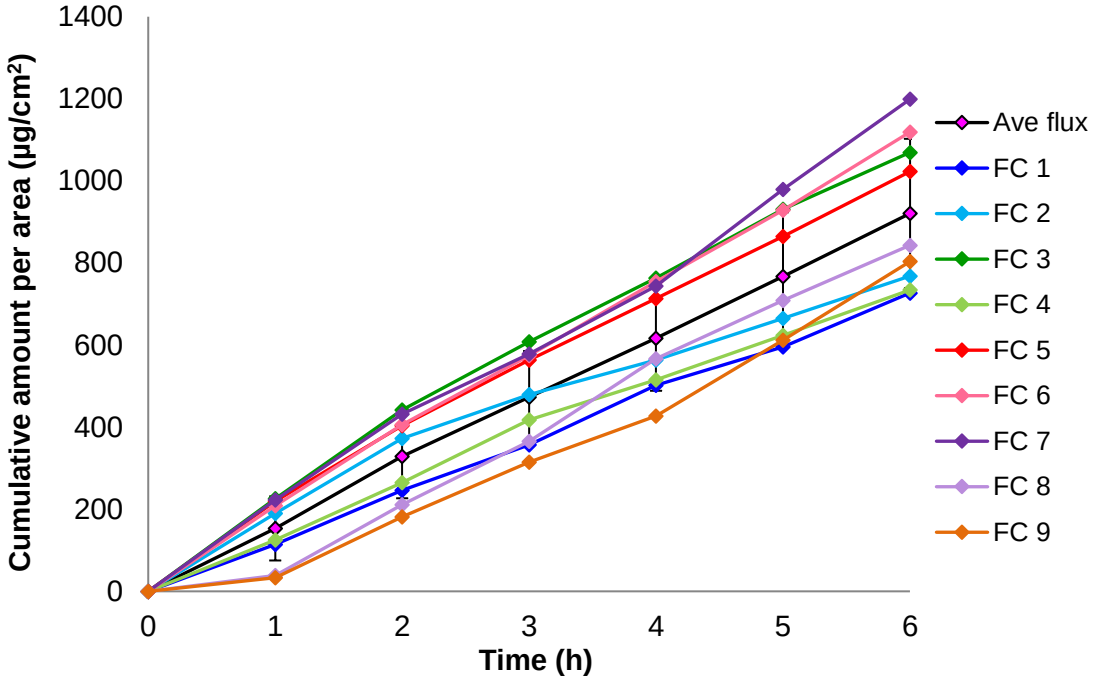


Figure D.4: Cumulative amount per area (µg/cm2) of atorvastatin from **NG-ATO1** that was released through the membranes of each individual Franz cell over 6 h (n = 9)

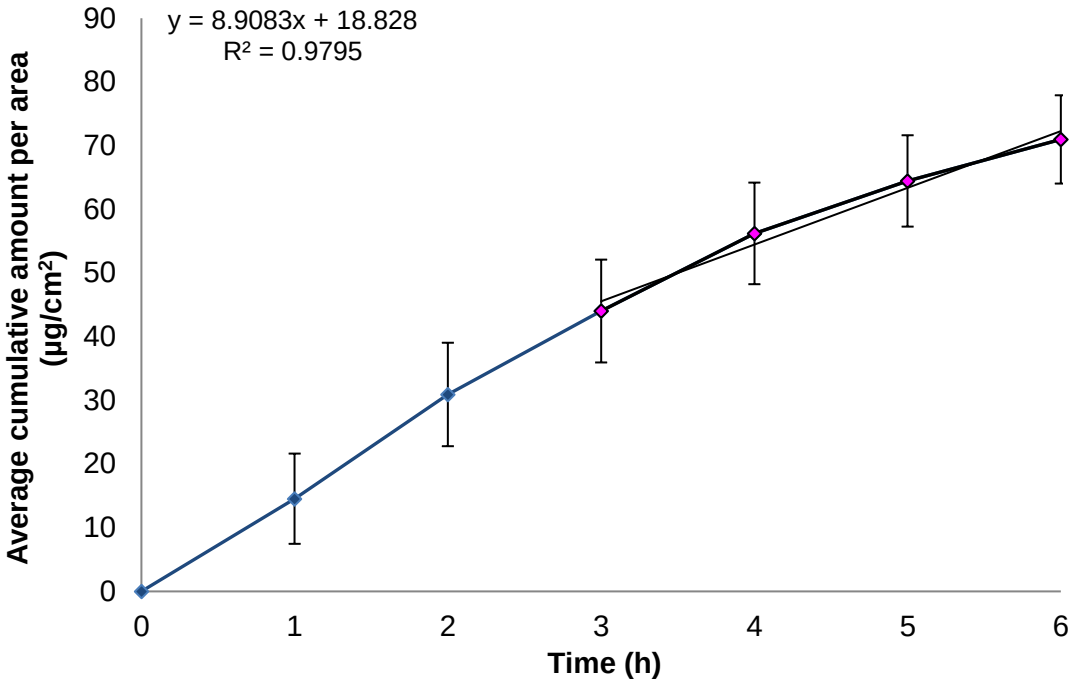


Figure D.5: Average cumulative amount per area (µg/cm^2) of mevastatin from **NE-MEV1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 9)

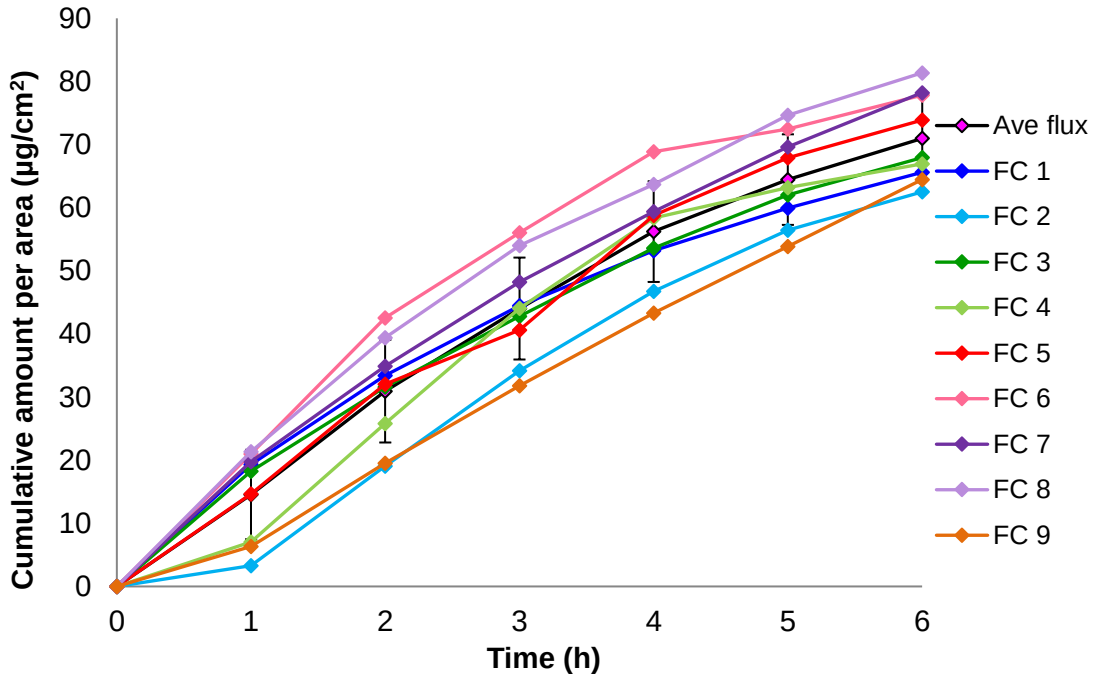


Figure D.6: Cumulative amount per area (µg/cm^2) of mevastatin from **NE-MEV1** that was released through the membranes of each individual Franz cell over 6 h (n = 9)

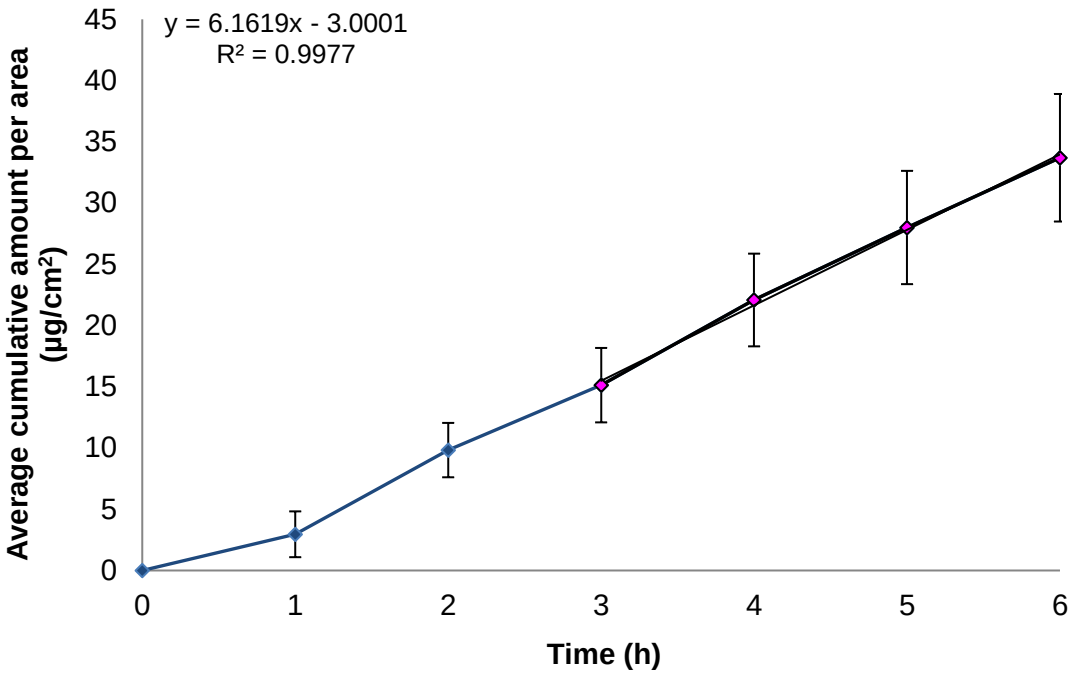


Figure D.7: Average cumulative amount per area (µg/cm^2) of mevastatin from **NG-MEV1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 9)

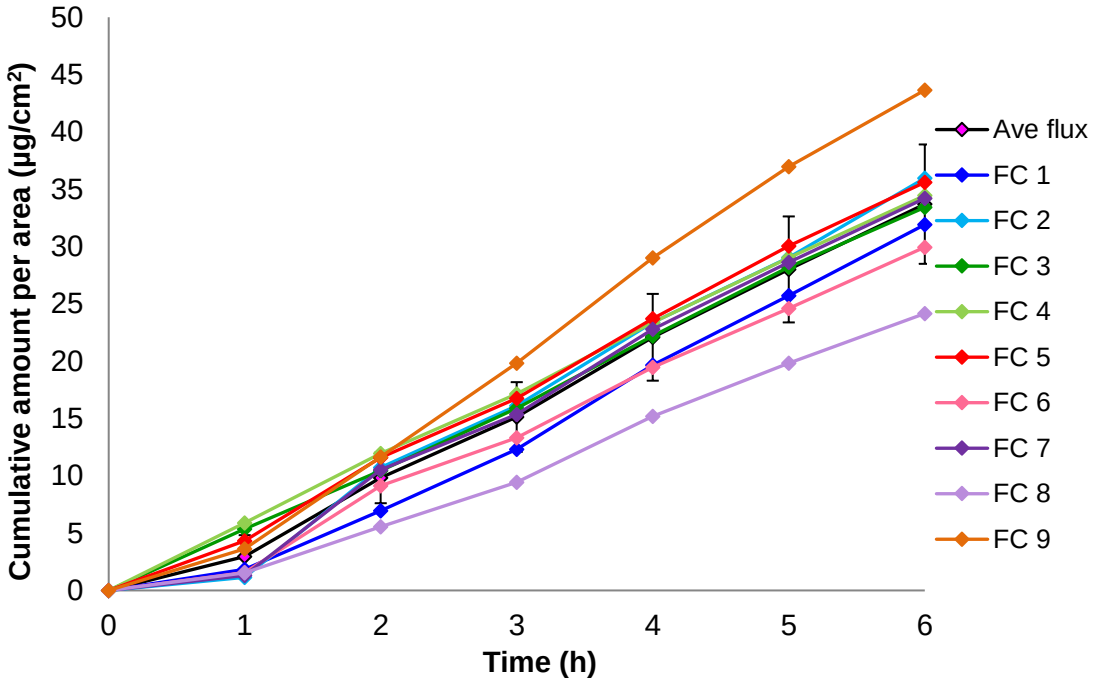


Figure D.8: Cumulative amount per area (µg/cm^2) of mevastatin from **NG-MEV1** that was released through the membranes of each individual Franz cell over 6 h (n = 9)

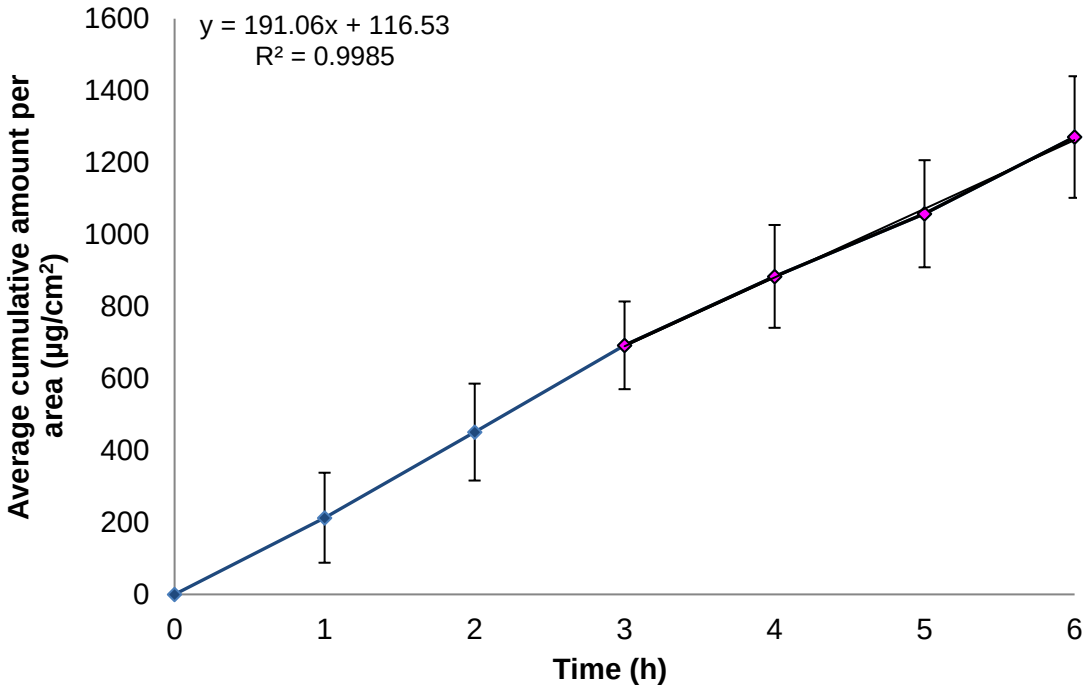


Figure D.9: Average cumulative amount per area (µg/cm^2) of pitavastatin from **NE-PIT1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 9)

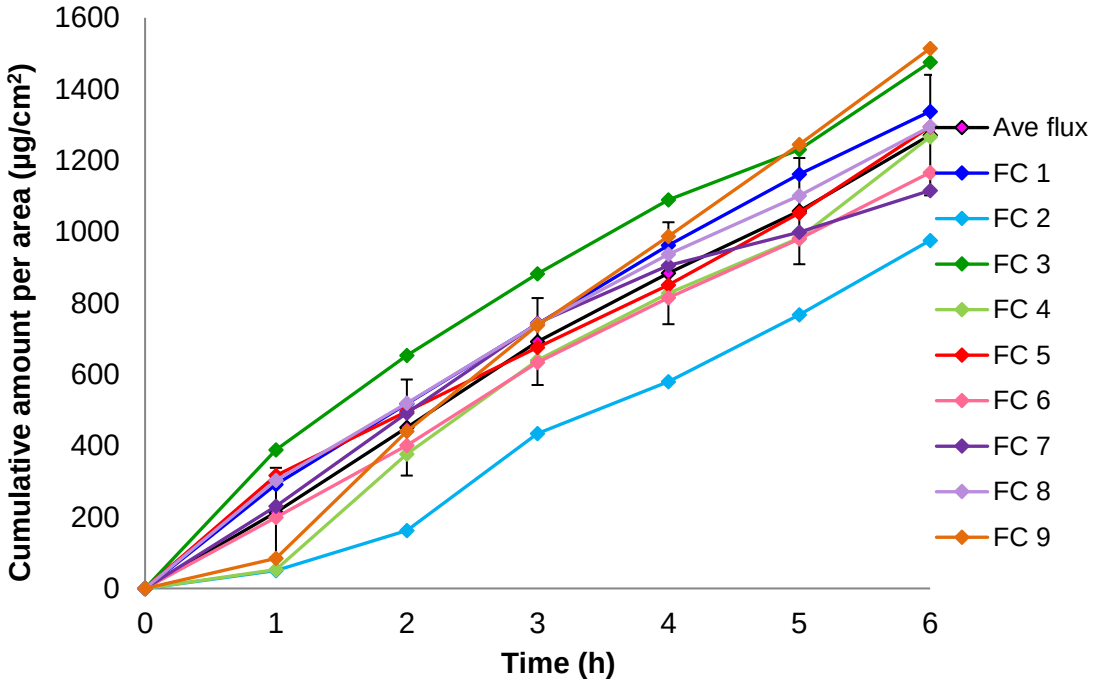


Figure D.10: Cumulative amount per area (µg/cm^2) of pitavastatin from **NE-PIT1** that was released through the membranes of each individual Franz cell over 6 h (n = 9)

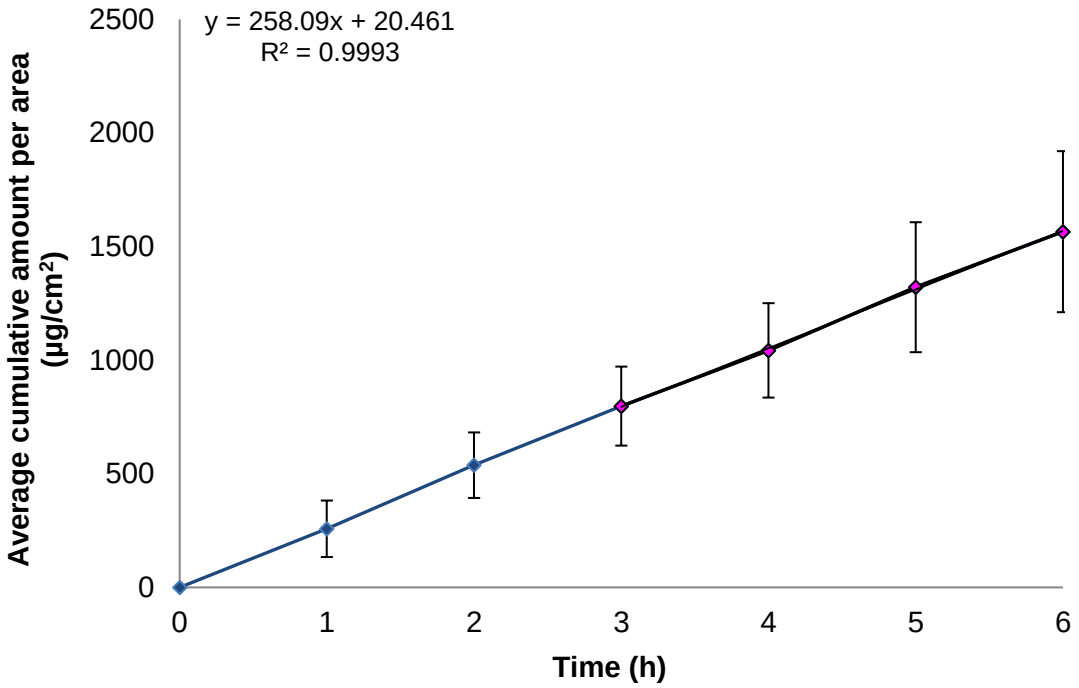


Figure D.11: Average cumulative amount per area (µg/cm^2) of pitavastatin from **NG-PIT1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 9)

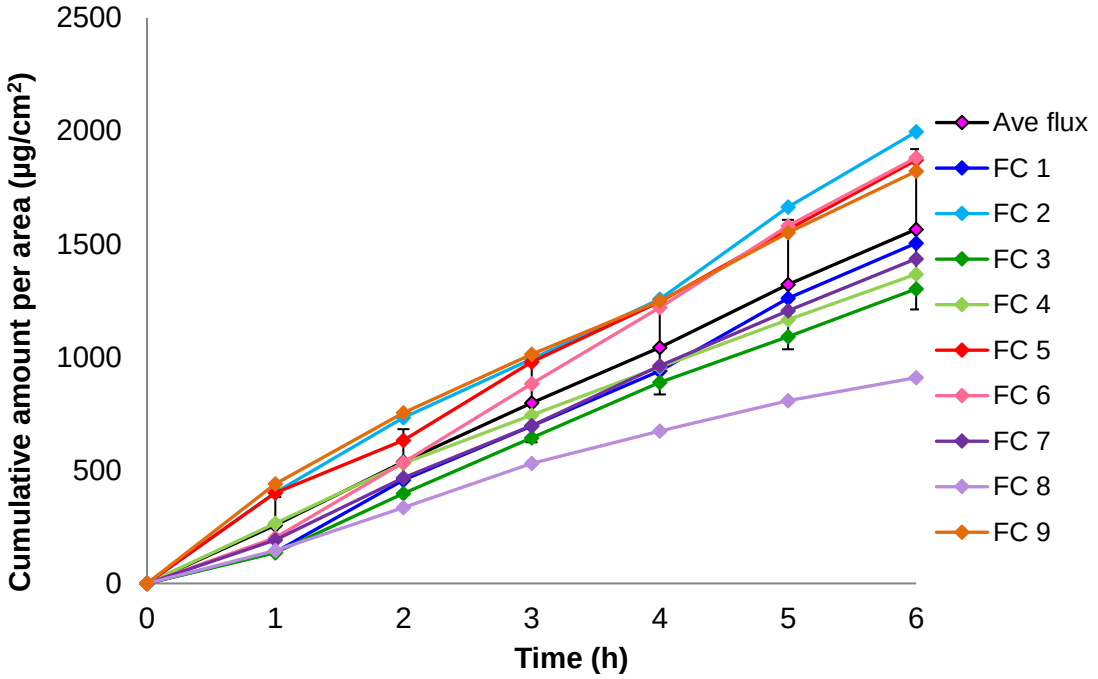


Figure D.12: Cumulative amount per area (µg/cm^2) of pitavastatin from **NG-PIT1** that was released through the membranes of each individual Franz cell over 6 h (n = 9)

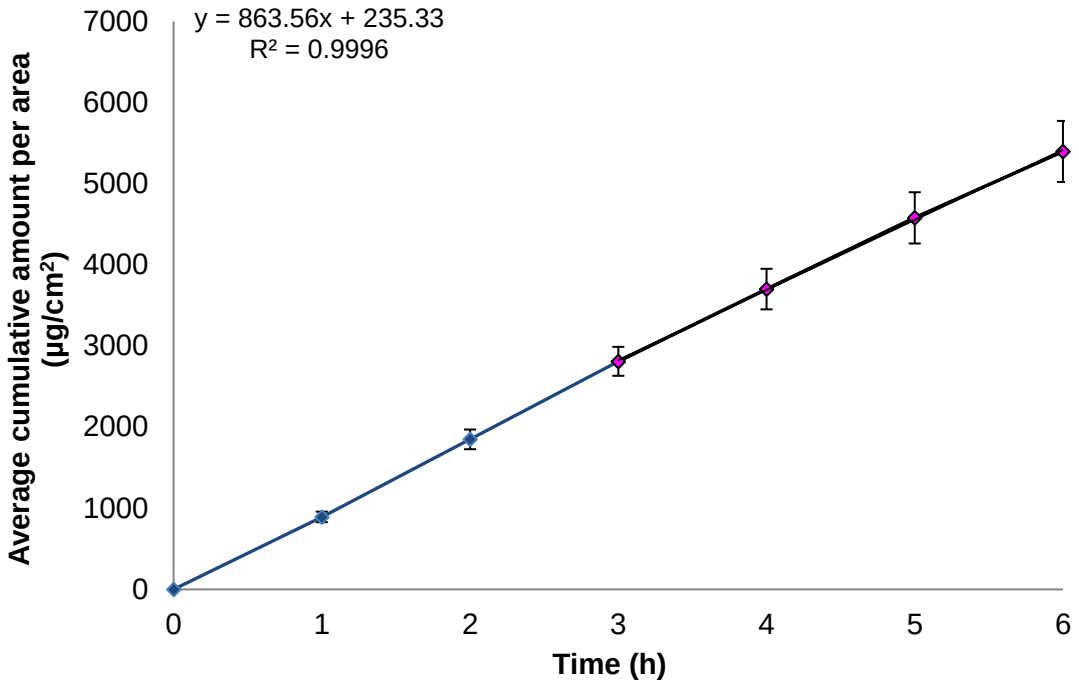


Figure D.13: Average cumulative amount per area (µg/cm^2) of pravastatin from **NE-PRA1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 12)

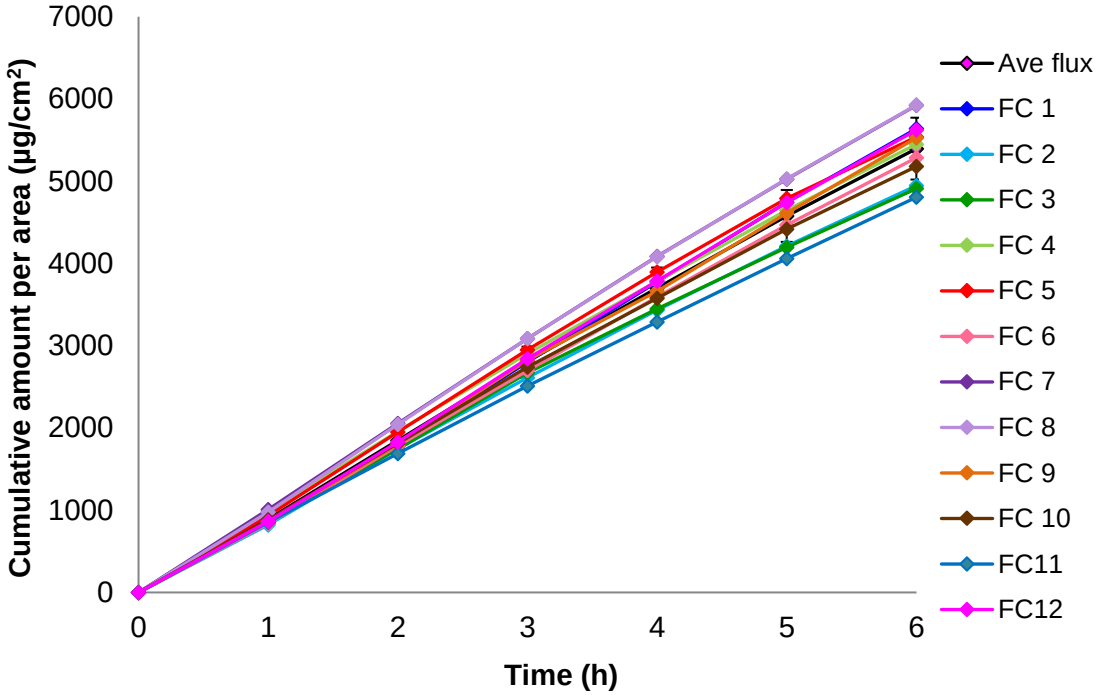


Figure D.14: Cumulative amount per area (µg/cm^2) of pravastatin from **NE-PRA1** that was released through the membranes of each individual Franz cell over 6 h (n = 12)

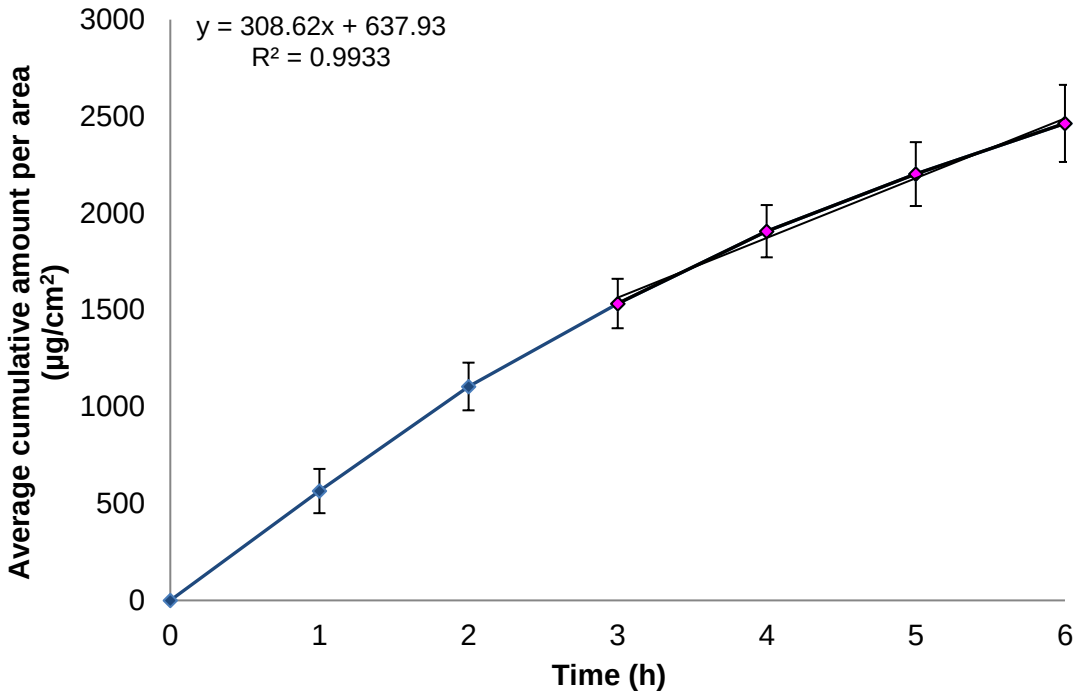


Figure D.15: Average cumulative amount per area (µg/cm^2) of pravastatin from **NG-PRA1** that was released through the membranes to indicate the average flux between 3 – 6 h (n = 10)

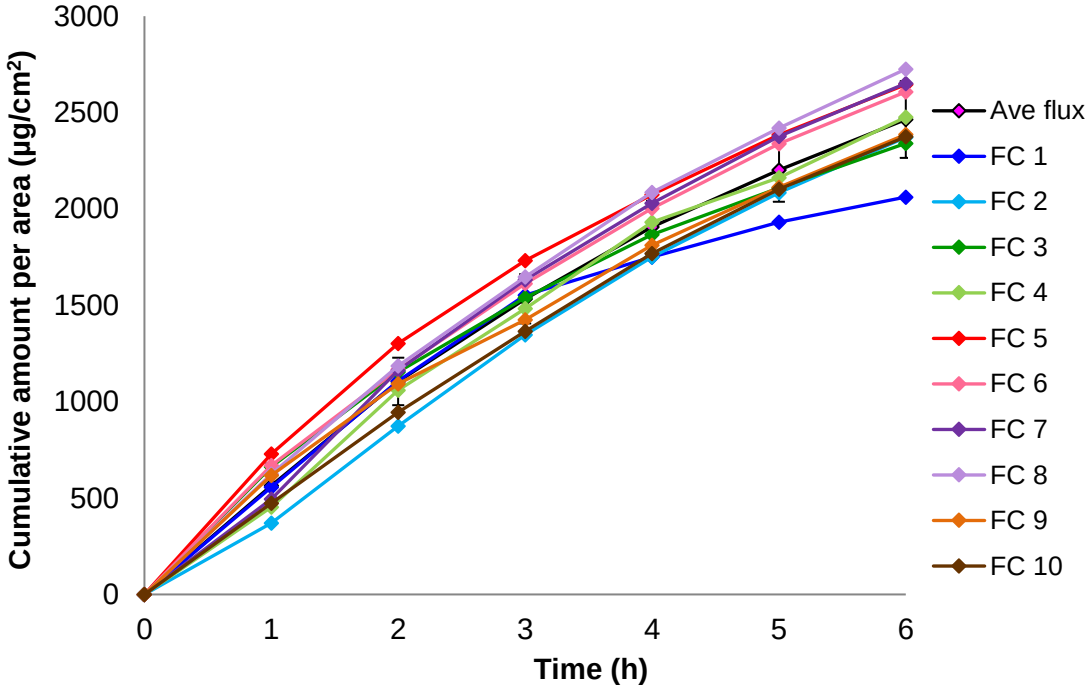


Figure D.16: Cumulative amount per area (µg/cm^2) of pravastatin from **NG-PRA1** that was released through the membranes of each individual Franz cell over 6 h (n = 10)

Membrane release studies were performed with each of the **NE1** and **NG1** formulas containing the selected statins. The results of all eight formulas are tabulated in Table D.2, which illustrates the average percentage released (%); average flux (µg/cm^2.h) and median flux (µg/cm^2.h) obtained from membrane release studies regarding **NE1s** and **NG1s** for each statin over 6 h. Figures D.1 – D.16 represent the cumulative amount per area (µg/cm^2) of each selected statin released from each **NE1** and **NG1** formulas.

Table D.2: The average %released (%), average and median flux (µg/cm^2.h) for each of the statins from the **NE1s** and **NG1s** after 6 h, where *n* is the number of cells used

Formula	*n*	Average percentage released (%)	Average flux (µg/cm^2.h)	Median flux (µg/cm^2.h)
NE-ATO1	11	1.899 ± 0.470	122.73 ± 33.81	116.099
NG-ATO1	9	2.474 ± 0.460	149.33 ± 36.45	158.524
NE-MEV1	9	0.191 ± 0.075	8.91 ± 1.57	11.643
NG-MEV1	9	0.091 ± 0.037	6.16 ± 0.85	6.114
NE-PIT1	9	3.418 ± 1.374	191.06 ± 35.92	197.072
NG-PIT1	9	4.209 ± 1.816	258.09 ± 93.53	259.638
NE-PRA1	12	14.510 ± 5.156	863.56 ± 73.61	911.234
NG-PRA1	10	6.625 ± 2.512	308.62 ± 54.70	385.378

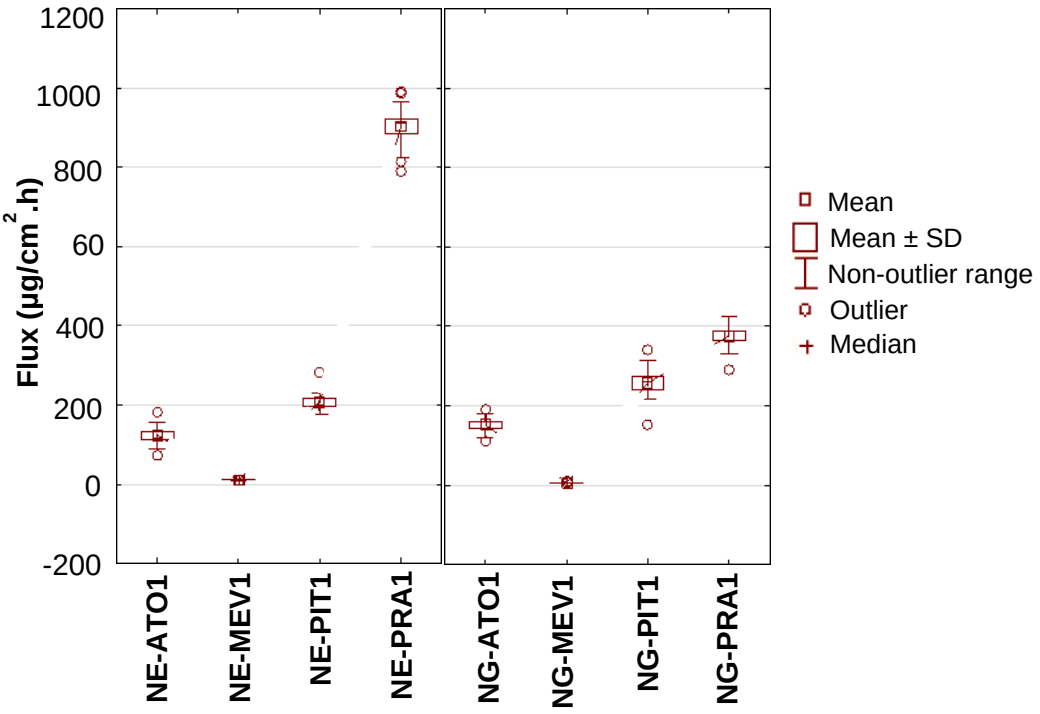


Figure D.17: Box-plots indicating the flux (µg/cm^2.h) of the **NE1s** and the **NG1s** of all four statins during the membrane release studies over 6 h

Median values are considered more accurate than the average values, since outliers/extremes alter the average values, where medians remain unchanged (Dawson & Trapp, 2004). Therefore, only median flux values will be discussed further.

Table D.3: The median flux (µg/cm^2.h) ranking for the **NE1s** and **NG1s** together

1	2	3	4	5	6	7	8
NE-PRA1	**NG-PRA1**	**NG-PIT1**	**NE-PIT1**	**NG-ATO1**	**NE-ATO1**	**NE-MEV1**	**NG-MEV1**

From Table D.3, it is observed that the **NE-PRA1** obtained the highest median flux value and that the **NG-MEV1** obtained the lowest median flux value, when all the formulas were compared. It can also be observed that the **NE1s** containing pravastatin and mevastatin (**NE-PRA1** and **NE-MEV1**) had higher median flux values than their **NG1s**-counterpart. The opposite was true for pitavastatin and atorvastatin, with **NG-PIT1** and **NG-ATO1** presenting with higher median flux values than their **NE1s**-counterpart.

When the **NE1s** and the **NG1s** were compared separately, it was observed that pravastatin had the highest median flux value, followed by pitavastatin, atorvastatin and lastly, mevastatin for both the **NE1s** and the **NG1s**. Solubility is a fundamental physicochemical characteristic of an API due to the close correlation between skin permeability and solubility, since a higher solubility results in better skin penetration (Williams, 2003). When considering physicochemical properties of statins, it was observed that pitavastatin (aqueous solubility of 0.500 mg/ml (Cayman chemical, 2014) and log D of 1.50 (Fong, 2016)) and atorvastatin (aqueous solubility of 0.19 mg/ml (Rodde *et al*., 2014) and log D of 1.00 – 1.25) (Fong, 2016)) both have ideal log D values (log D between 1 and 3 (Naik *et al*., 2000)), but have poor aqueous solubility values, while pravastatin (aqueous solubility of 10.000 mg/ml (Cayman chemical, 2015) and log D of - 0.47 (Fong, 2016)) and mevastatin (aqueous solubility of 0.032 mg/ml (Drugbank, 2005c) and log D of 3.570 (Kim *et al*., 2011)) are the most hydrophilic and lipophilic statins, respectively used in this study. Hence, it can be concluded that the API, more than the type of formula (**NE1s** or **NG1s**) used may have influenced the release of the API from the formula. Nevertheless, all formulas (**NE1s** or **NG1s**) were successful in releasing the statins during the membrane release studies.

In previous studies performed by Maree (2018) and Marais (2018), **NEs** and **NGs** were also formulated containing 2% statins, although the natural oil (apricot oil) used was different. The median flux values obtained for the **NEs** containing apricot oil were: 102.983 µg/cm^2.h for atorvastatin (Maree, 2018), 18.970 µg/cm^2.h for mevastatin (Marais, 2018), 150.388 µg/cm^2.h for pitavastatin and 93.3947 µg/cm^2.h for pravastatin (Maree, 2018), which posed significantly lower median flux values compared to the **NE1s** containing grapeseed oil used during this study (except

for mevastatin). The median flux values for the **NGs** containing apricot oil were: 101.196 µg/cm^2.h for atorvastatin (Maree, 2018), 4.867 µg/cm^2.h for mevastatin (Marais, 2018), 107.344 µg/cm^2.h for pitavastatin and 393.350 µg/cm^2.h for pravastatin (Maree, 2018), which posed significantly lower median flux values compared to the **NG1s** containing grapeseed oil used during this study (excluding pravastatin). Therefore, in general, it can be stated that the natural oil used in this study (grapeseed oil) affected API release more than the natural oil (apricot oil) used during previous studies.

D.3.2 Skin diffusion studies (*in vitro*)

Skin diffusion studies were performed with selected statins on four **NE1s** and four **NG1s**, separately. The results for all eight formulas are illustrated in Table D.4. The amount per area (µg/cm^2) of selected statins that diffused through the skin via individual Franz cells (FC) from the **NE1s** and **NG1s** during the *in vitro* skin diffusion studies after 12 h and their average values are illustrated in Figures D.18 – D.25.

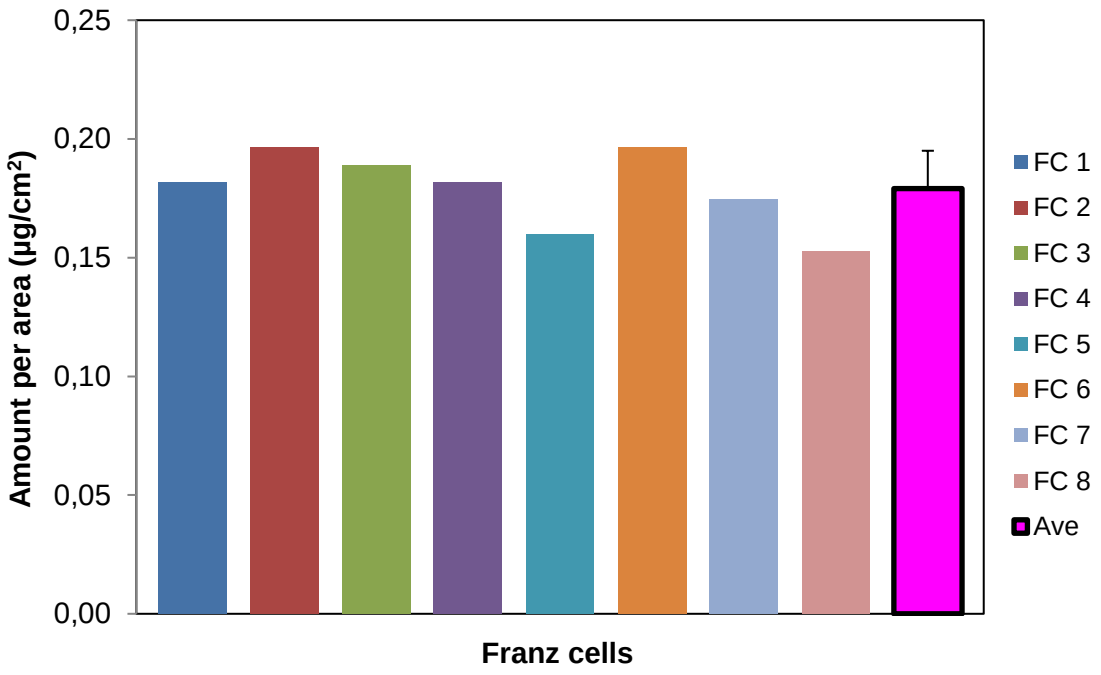


Figure D.18: The amount per area diffused (µg/cm^2) of atorvastatin from **NE-ATO1** after the 12 h diffusion study (n = 8)

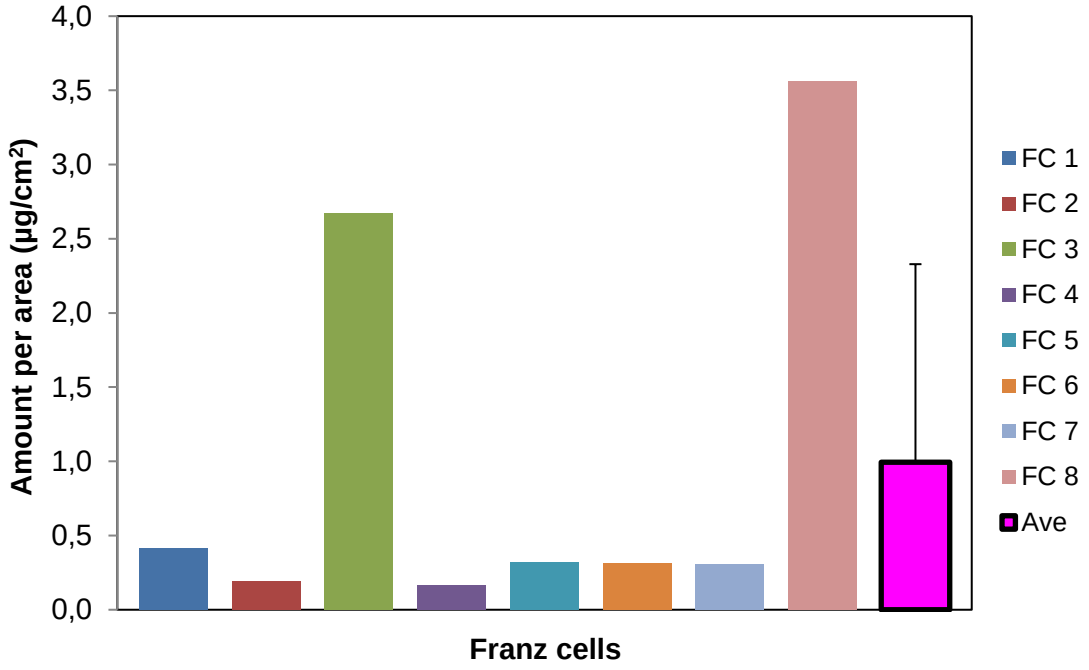


Figure D.19: The amount per area diffused (µg/cm^2) of atorvastatin from **NG-ATO1** after the 12 h diffusion study (n = 8)

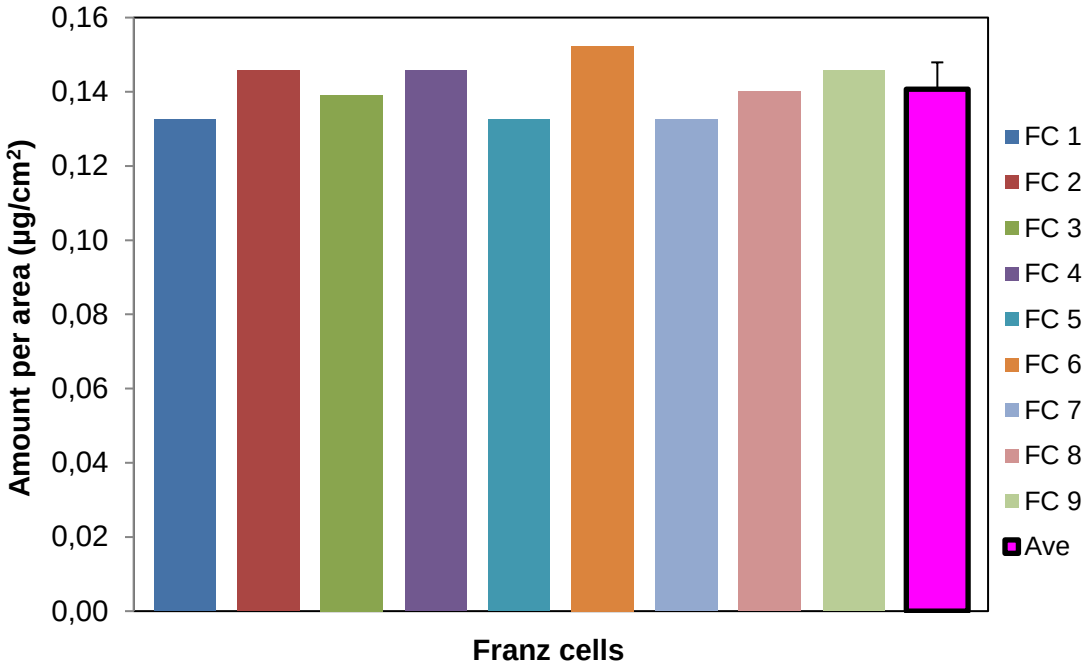


Figure D.20: The amount per area diffused (µg/cm^2) of mevastatin from **NE-MEV1** after the 12 h diffusion study (n = 9)

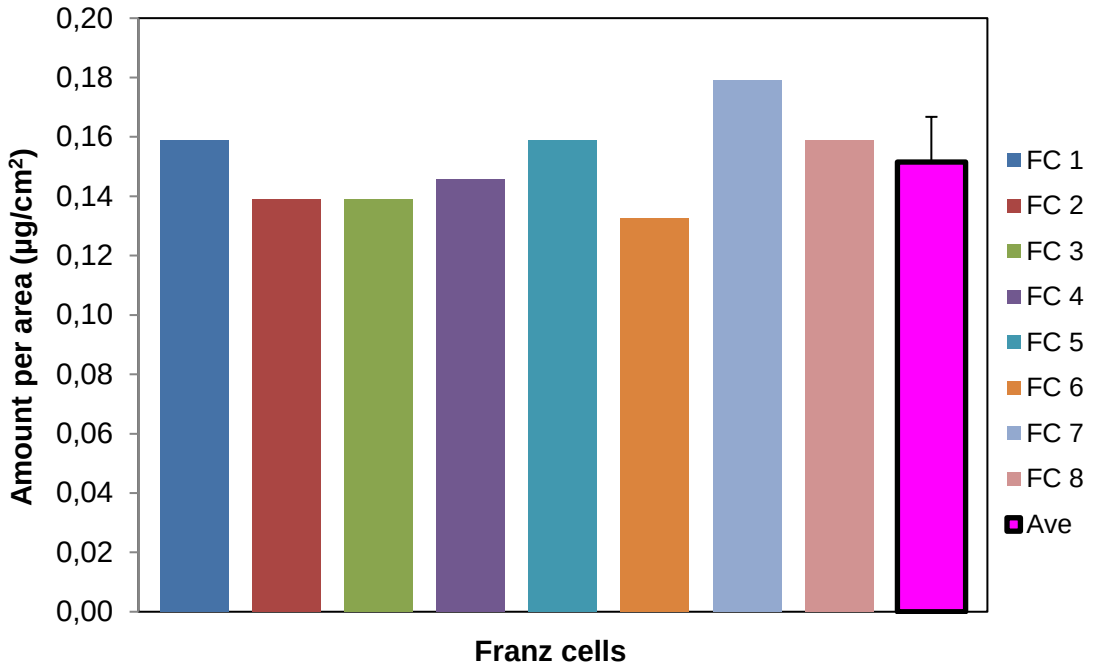


Figure D.21: The amount per area diffused (µg/cm^2) of mevastatin from **NG-MEV1** after the 12 h diffusion study (n = 8)

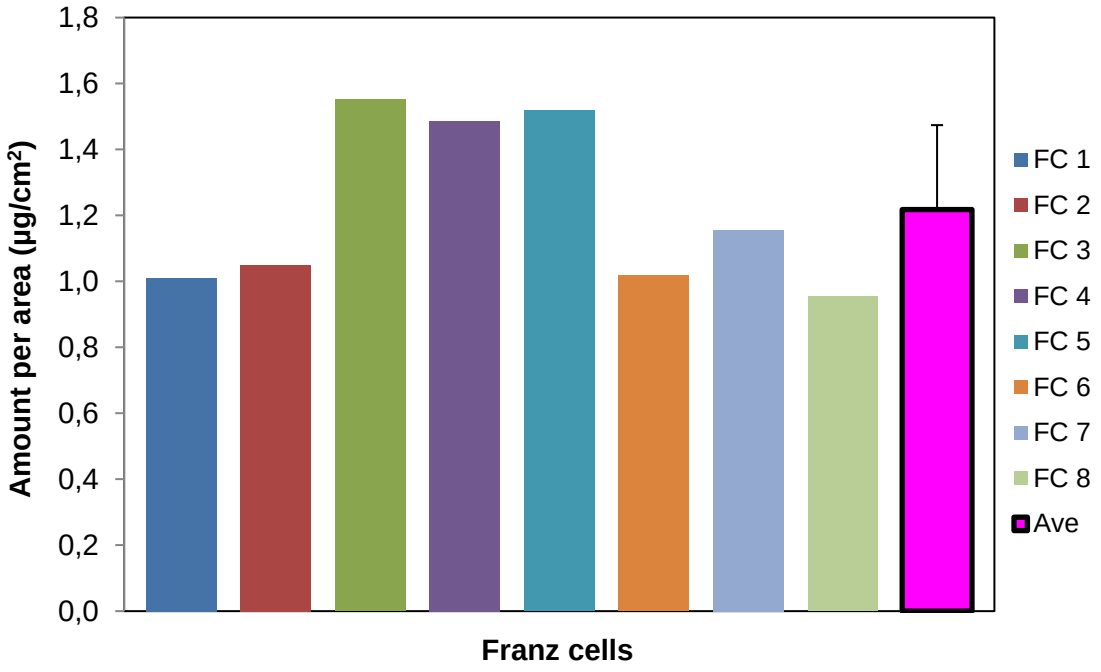


Figure D.22: The amount per area diffused (µg/cm^2) of pitavastatin from **NE-PIT1** after the 12 h diffusion study (n = 8)

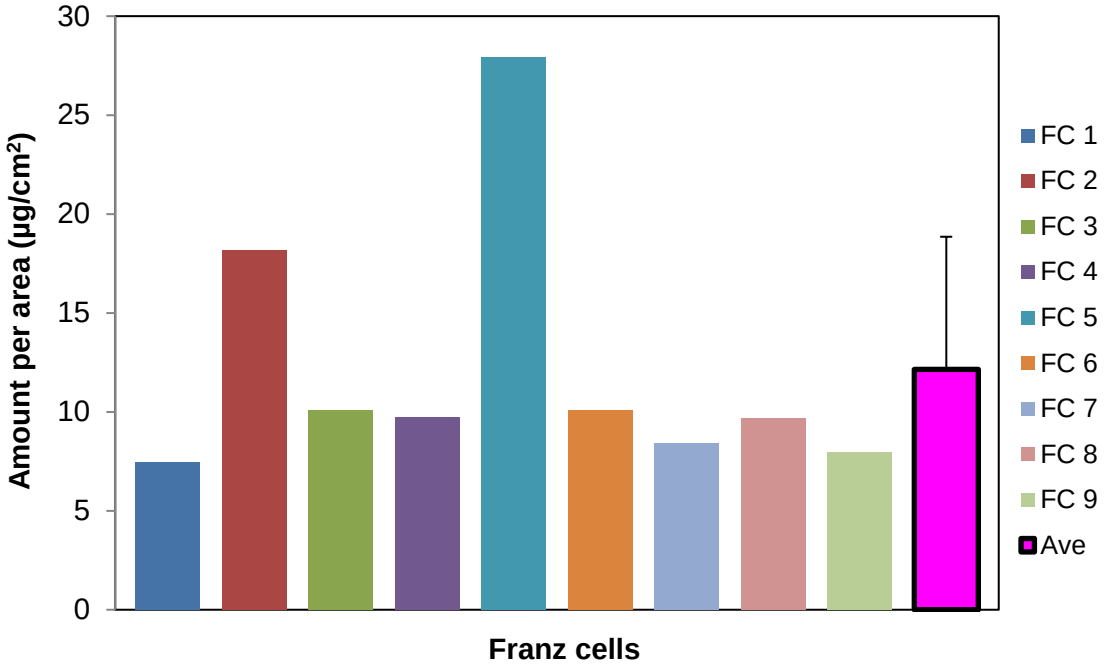


Figure D.23: The amount per area diffused (µg/cm^2) of pitavastatin from **NG-PIT1** after the 12 h diffusion study (n = 9)

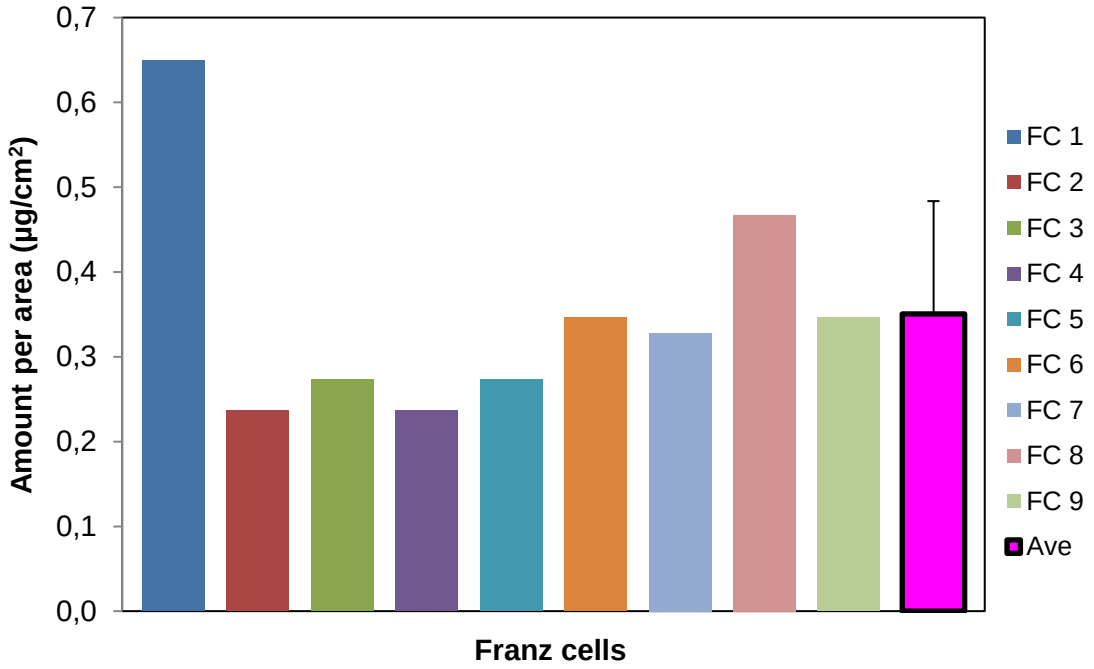


Figure D.24: The amount per area diffused (µg/cm^2) of pravastatin from **NE-PRA1** after the 12 h diffusion study (n = 9)

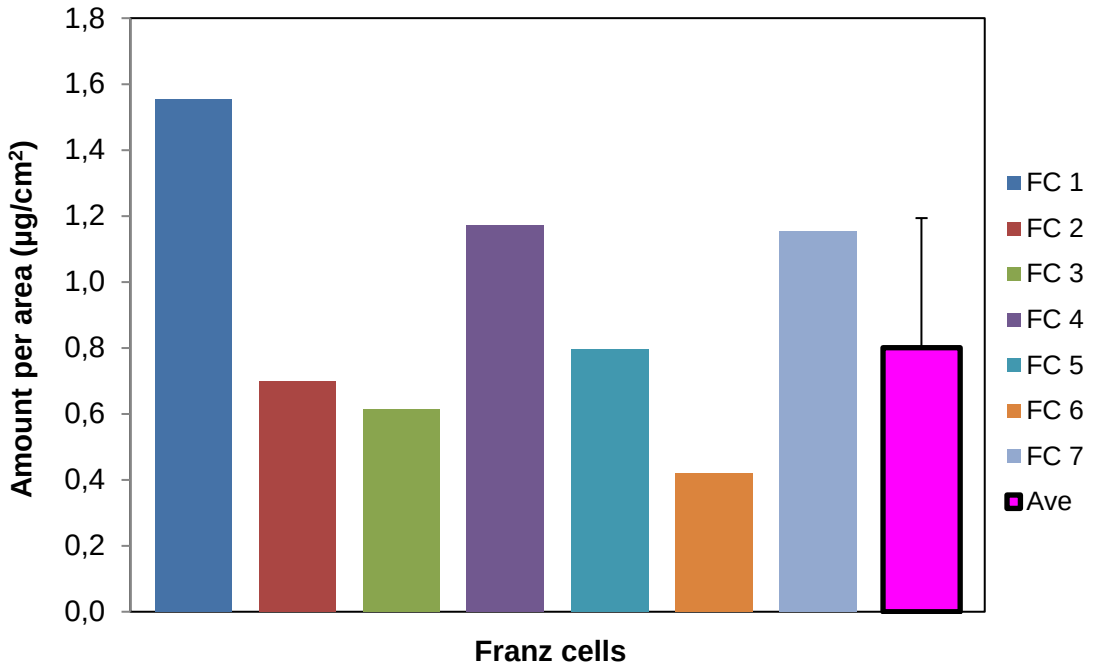


Figure D.25: The amount per area diffused (µg/cm^2) of pravastatin from **NG-PRA1** after the 12 h diffusion study (n = 7)

Table D.4: The average percentage diffused (%), average concentration diffused (µg/ml), together with the average and median amount per area diffused (µg/cm^2) for the **NE1s** and the **NG1s** (*n* = number of Franz cells used)

Formula	n	Average percentage diffused (%)	Average concentration diffused (µg/ml)	Average amount per area diffused (µg/cm²)	Median amount per area diffused (µg/cm²)
NE-ATO1	8	0.000 ± 0.000	0.179 ± 0.016	0.096 ± 0.015	0.182
NG-ATO1	8	0.003 ± 0.003	0.993 ± 1.335	0.534 ± 1.249	0.317
NE-MEV1	9	0.000 ± 0.000	0.147 ± 0.007	0.076 ± 0.007	0.140
NG-MEV1	8	0.000 ± 0.000	0.152 ± 0.015	0.081 ± 0.014	0.153
NE-PIT1	8	0.003 ± 0.002	1.218 ± 0.256	0.655 ± 0.240	1.103
NG-PIT1	9	0.031 ± 0.018	12.154 ± 6.699	6.536 ± 6.315	9.749
NE-PRA1	9	0.001 ± 0.000	0.351 ± 0.133	0.189 ± 0.125	0.328
NG-PRA	7	0.002 ± 0.001	0.915 ± 0.393	0.492 ± 0.364	0.795

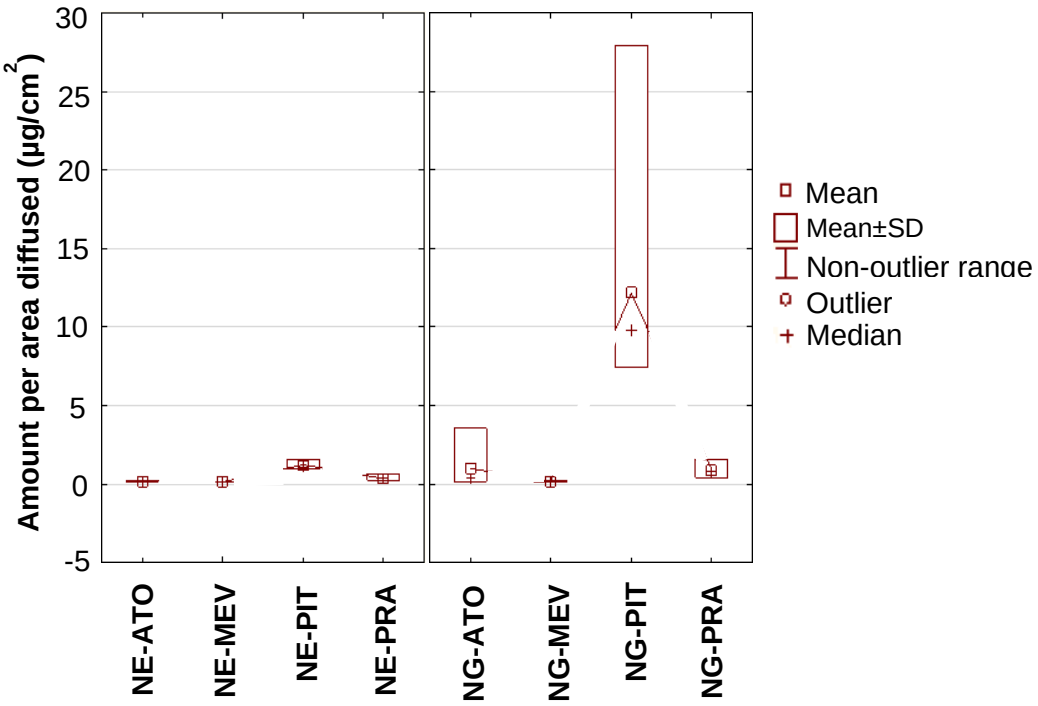


Figure D.26: Box-plots indicating the mean and median amount per area diffused (µg/cm^2) for each of the selected statins in the **NE1s** and the **NG1s**

The concentration present in the receptor phase (average concentration diffused (µg/ml)) of the statins in the **NE1s** and **NG1s** were higher than the LOD and LLOQ values (see Appendix A.3.8), with the exception of **NE-ATO1**, **NE-MEV1**, **NG-MEV1** and **NE-PRA1**, which obtained values under their LLOQ, but above their LOD and could therefore still be quantified and analysed with the rest of the formulations.

It is essential to establish whether data will be defined in terms of mean or median, for both are considered central tendencies. The mean is generally utilised to indicate data, but is extremely sensitive to outliers as mentioned. Therefore, it is not suitable to use a mean value regarding skewed distribution (Manikandan, 2011), as seen in skin diffusion data. Hence, median values will be discussed further.

Table D.5: The median amount per area diffused (µg/cm^2) ranking for the **NE1s** and **NG1s** together

1	2	3	4	5	6	7	8
NG-PIT1	**NE-PIT1**	**NG-PRA1**	**NE-PRA1**	**NG-ATO1**	**NE-ATO1**	**NG-MEV1**	**NE-MEV1**

From Table D.5, it is evident when comparing all the formulas (**NE1s** and **NG1s**) that **NG-PIT1** obtained the highest median amount per area diffused and **NE-MEV1** obtained the lowest median amount per area diffused. It was also observed that the **NG1s** (**NG-PIT1**, **NG-PRA1**, **NG-ATO1** and **NG-MEV1**) had higher median amount per area diffused values than their **NE1**-counterpart (**NE-PIT1**, **NE-PRA1**, **NE-ATO1** and **NE-MEV1**). Hence, the **NG1s** increased the transdermal diffusion of the statins more than the **NE1s**. This phenomenon can be ascribed to the higher viscosity of the **NG1s** due to their gel-like structure (Arora *et al*., 2014; Begur *et al*., 2015) that results in a slower, more regulated release. **NG1s** provide better spreadability when applied (Chellapa, 2015) and prevents formulation particle separation (Mitsui, 1997). As a result, the **NG1s** tend to further reduce interfacial and surface tension compared to **NE1s**, which may lead to increased viscosity and enhanced skin permeability (Chellapa, 2015, Eid *et al*., 2014).

When the **NE1s** and **NG1s** were compared separately, it was detected that pitavastatin had the highest median amount per area diffused, followed by pravastatin, atorvastatin and lastly, mevastatin for both formulas (**NE1s** and **NG1s**). Pitavastatin (log D of 1.50 (Fong, 2016)) and atorvastatin (log D of 1.00 – 1.25) (Fong, 2016)) met the ideal criteria of having a log D value between 1 and 3 (Naik *et al*., 2000), required for transdermal diffusion (Subedi *et al*., 2010), but atorvastatin has a very low aqueous solubility (0.19 mg/ml (Rodde *et al*., 2014)) and high molecular weight (1155.34 g/mol (Al-Adl *et al*., 2017)). Ideally the aqueous solubility should be more than 1 mg/ml and the molecular weight should be less than 500 Da to enhance skin permeation (Naik *et al*., 2000). Therefore, due to the poor physicochemical properties of atorvastatin, it may have restricted skin penetration more than pitavastatin and pravastatin (Jia *et al*., 2017). Pravastatin is very hydrophilic and even though its log D value (- 0.47 (Fong, 2016)) was not ideal for transdermal diffusion, it has a high aqueous solubility (10.000 mg/ml (Cayman chemical, 2015)), which met the ideal criteria (> 1 mg/ml) as stated by Naik *et al*. (2000). Mevastatin, on the other hand, is very lipophilic (aqueous solubility of 0.032 mg/ml (Drugbank, 2005c) and log D of 3.570 (Kim *et al*., 2011)) and therefore, it was expected that it would want to stay in a lipophilic region (stratum corneum) rather than permeate into the hydrophilic region (receptor phase); this may have contributed to mevastatin being the API with the lowest median amount per area diffused (Förster *et al*., 2009). Conclusively, the type of formula (**NG1s** more than **NE1s**) and the API utilised in this study may have affected transdermal diffusion. Finally, both the **NE1s** and **NG1s** were successful in delivering the statins transdermally.

Additionally, when the median amount per area diffused of the **NE1s** and **NG1s** of this study were compared to previous studies performed by Maree (2018) and Marais (2018) on **NEs** and **NGs** containing 2% statin and apricot oil (as natural oil used), it was found that the median amount per area diffused for the **NEs** containing apricot oil were: 0.0530 µg/cm^2 for atorvastatin (Maree, 2018), 0.478 µg/cm^2 for mevastatin (Marais, 2018), 0.387 µg/cm^2 for pitavastatin and

0.285 µg/cm^2 for pravastatin (Maree, 2018), which posed significantly lower median amount per area diffused values compared to **NE1s** containing grapeseed oil used during this study (excluding mevastatin). The median amount per area diffused for the **NGs** containing apricot oil were: 0.0735 µg/cm^2 for atorvastatin (Maree, 2018), 0.846 µg/cm^2 for mevastatin (Marais, 2018), 0.7220 µg/cm^2 for pitavastatin and 1.1350 µg/cm^2 for pravastatin (Maree, 2018), which posed significantly lower median amount per area diffused values compared to the **NG1s** containing grapeseed oil used during this study (except for mevastatin and pravastatin). Thus, in general, it can be concluded that the grapeseed oil improved transdermal delivery of the API more than the apricot oil during skin diffusion studies.

D.3.3 Tape stripping

Tape stripping was performed after completing the *in vitro* skin diffusion studies with the **NE1s** and **NG1s** containing the respective statins, to establish the average and median concentration of the statins present in the SCE ad ED after 12 h (see Table D.6).

Table D.6: The average and median concentration (µg/ml) for each of the selected statins in the **NE1s** and **NG1s** present in the SCE and ED (*n* = number of Franz cells used)

Formula	n	Average concentration in the SCE (µg/ml)	Median concentration in the SCE (µg/ml)	Average concentration in the ED (µg/ml)	Median concentration in the ED (µg/ml)
NE-ATO1	8	15.705 ± 14.890	8.013	38.919 ± 24.051	32.715
NG-ATO1	8	0.675 ± 0.330	0.561	6.060 ± 5.211	5.728
NE-MEV1	9	0.919 ± 0.369	0.916	0.817 ± 0.217	0.773
NG-MEV1	8	-	-	11.231 ± 7.945	11.757
NE-PIT1	8	6.804 ± 6.726	5.670	19.714 ± 10.851	18.677
NG-PIT1	9	2.815 ± 1.122	2.715	23.301 ± 15.504	19.164
NE-PRA1	9	1.302 ± 0.311	1.256	14.813 ± 4.698	14.369
NG-PRA	7	0.789 ± 0.332	0.819	1.649 ± 0.756	1.442

D.3.3.1 Stratum corneum-epidermis

The concentration (µg/ml) of the statins that was delivered in the SCE from the **NE1s** and **NG1s** are illustrated in Figures D.27 – D.33.

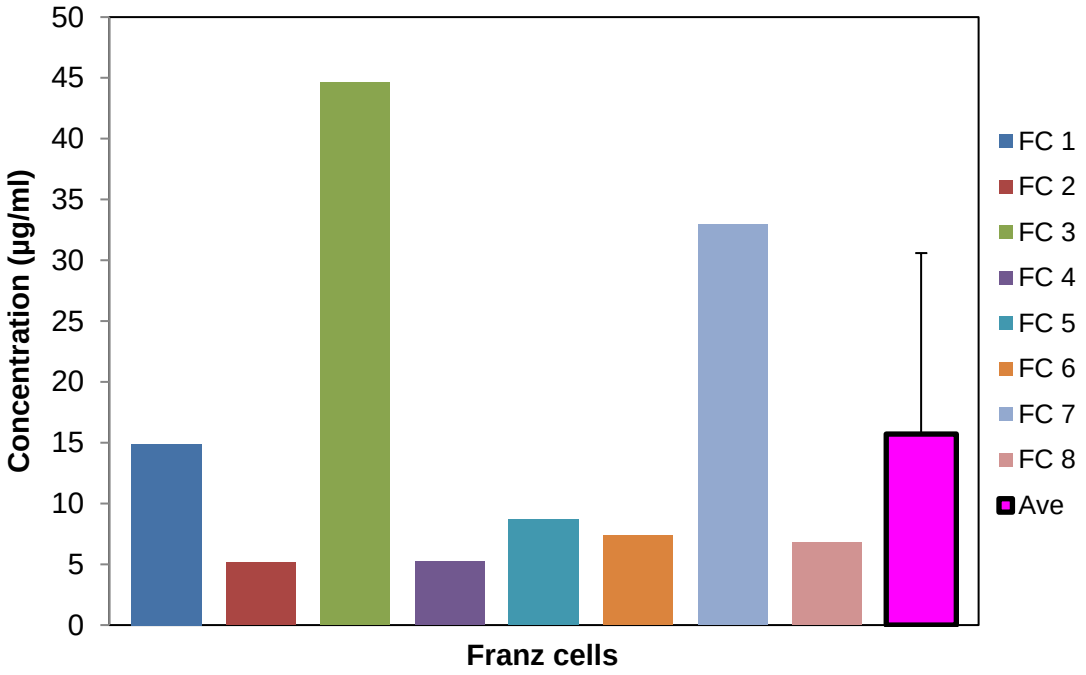


Figure D.27: Atorvastatin concentration (µg/ml) from the **NE-ATO1** present in the SCE (n = 8)

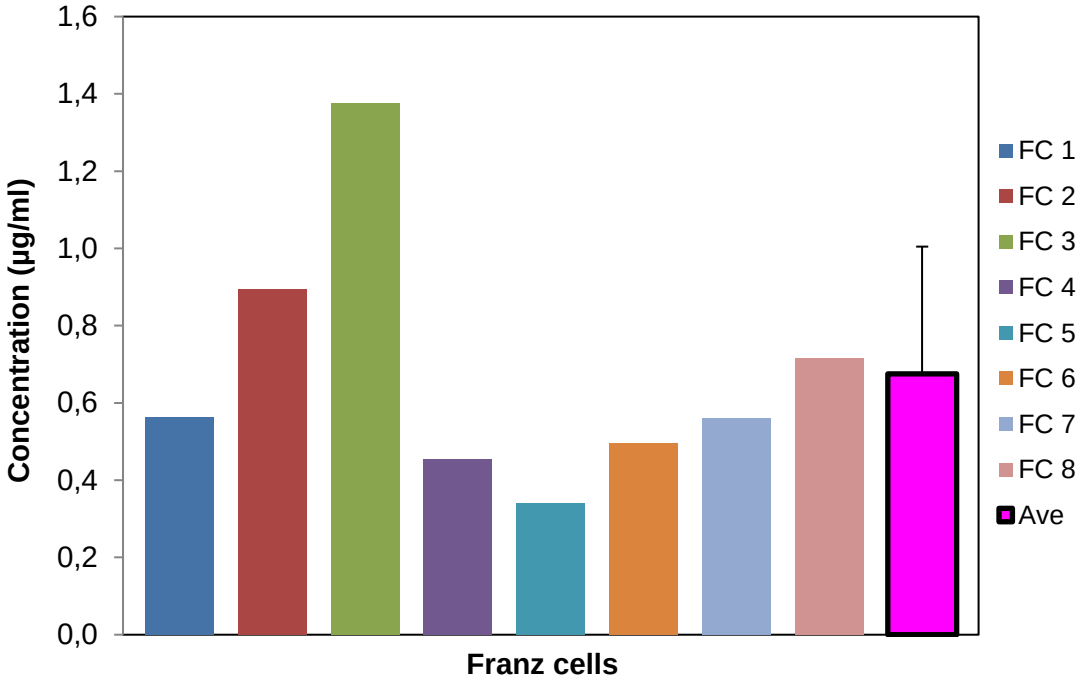


Figure D.28: Atorvastatin concentration (µg/ml) from the **NG-ATO1** present in the SCE (n = 8)

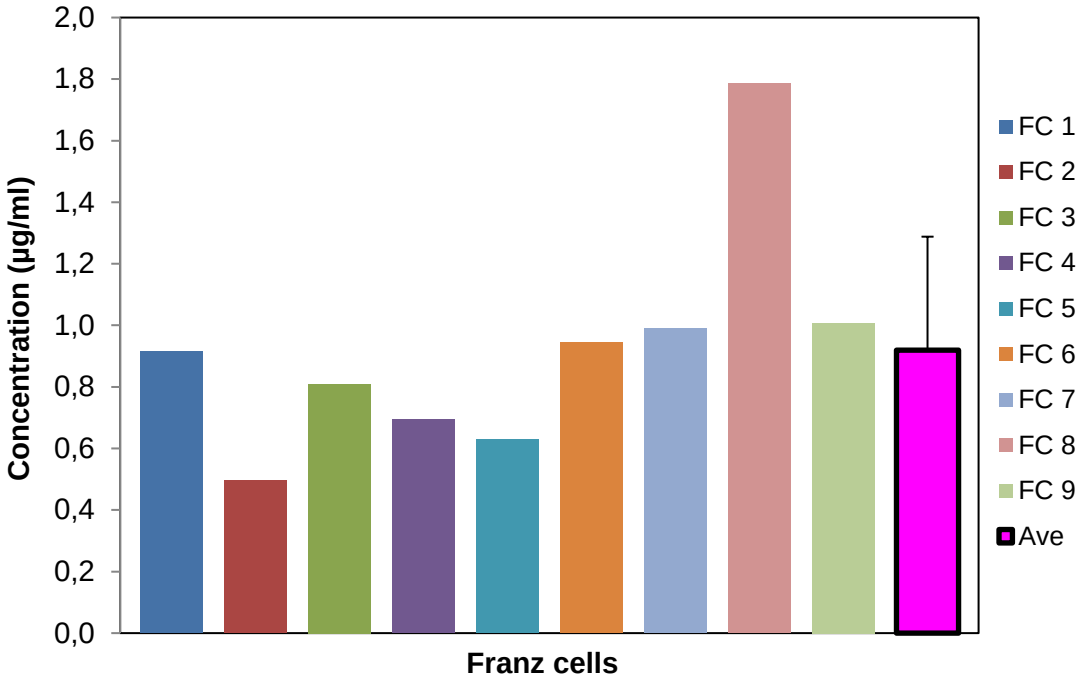


Figure D.29: Mevastatin concentration (µg/ml) from the **NE-MEV1** present in the SCE (n = 9)

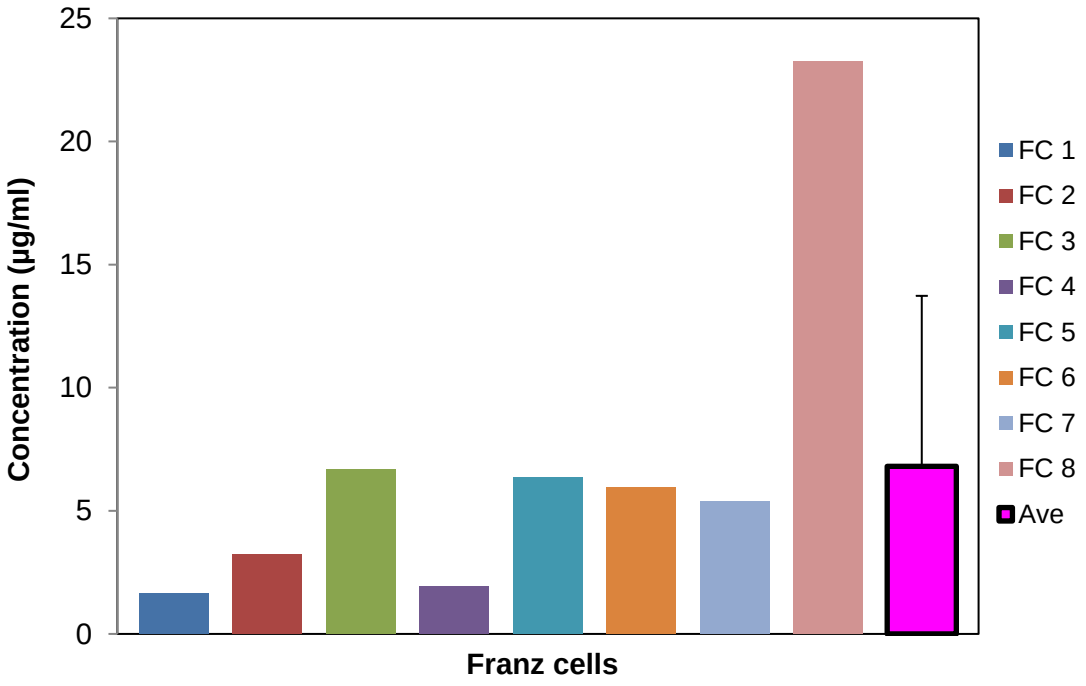


Figure D.30: Pitavastatin concentration (µg/ml) from the **NE-PIT1** present in the SCE (n = 8)

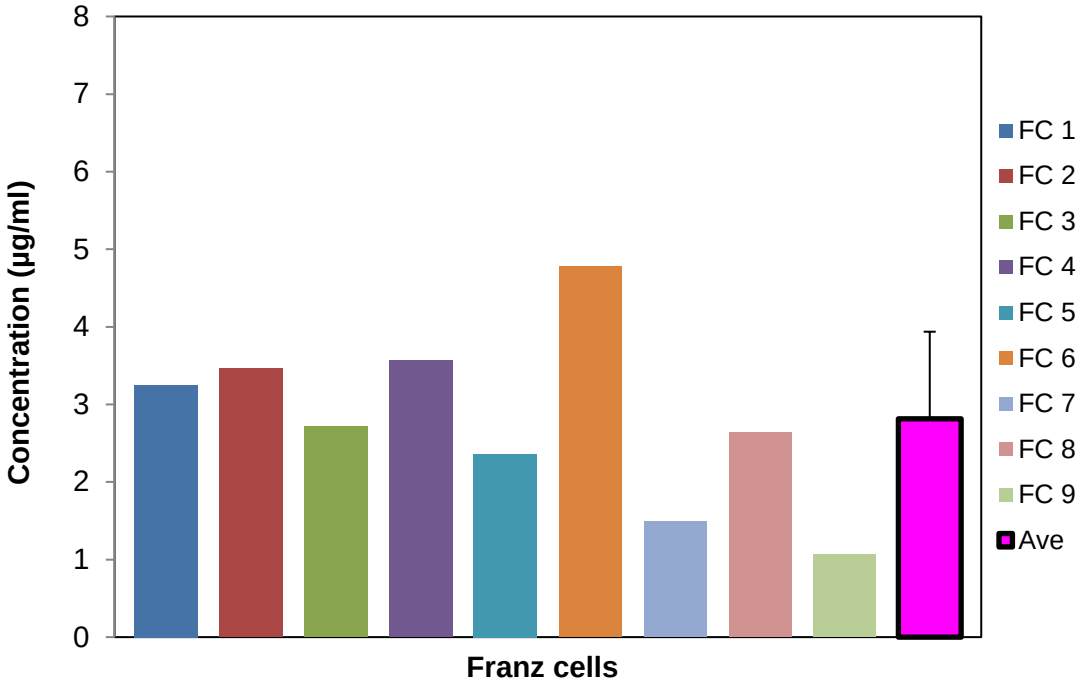


Figure D.31: Pitavastatin concentration (µg/ml) from the **NG-PIT1** present in the SCE (n = 9)

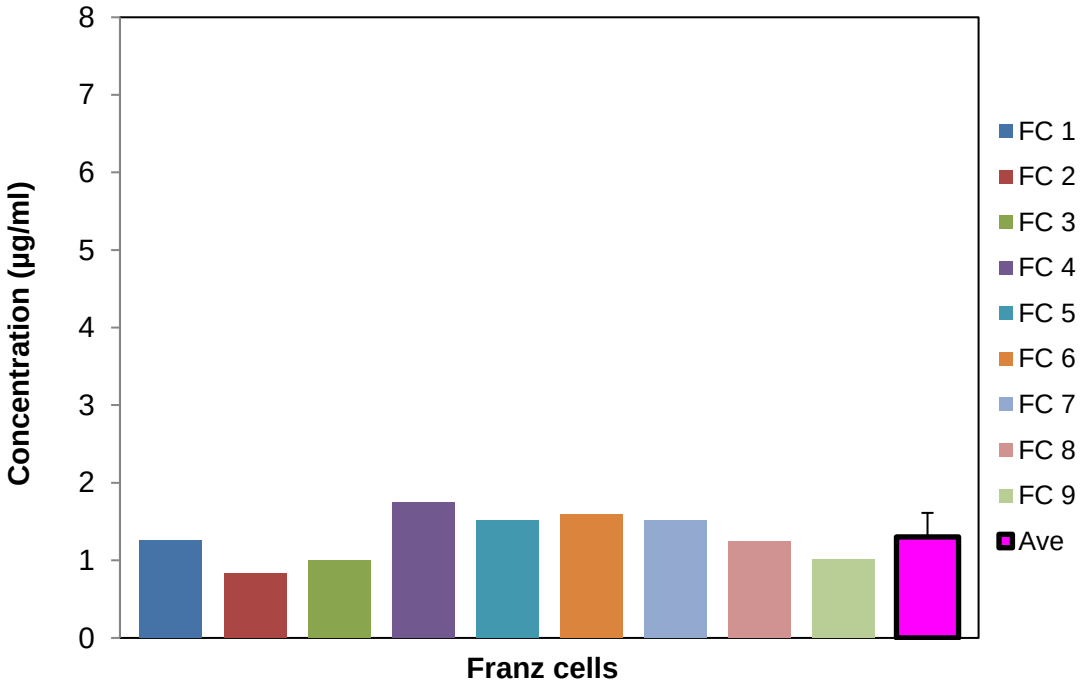


Figure D.32: Pravastatin concentration (µg/ml) from the **NE-PRA1** present in the SCE (n = 9)

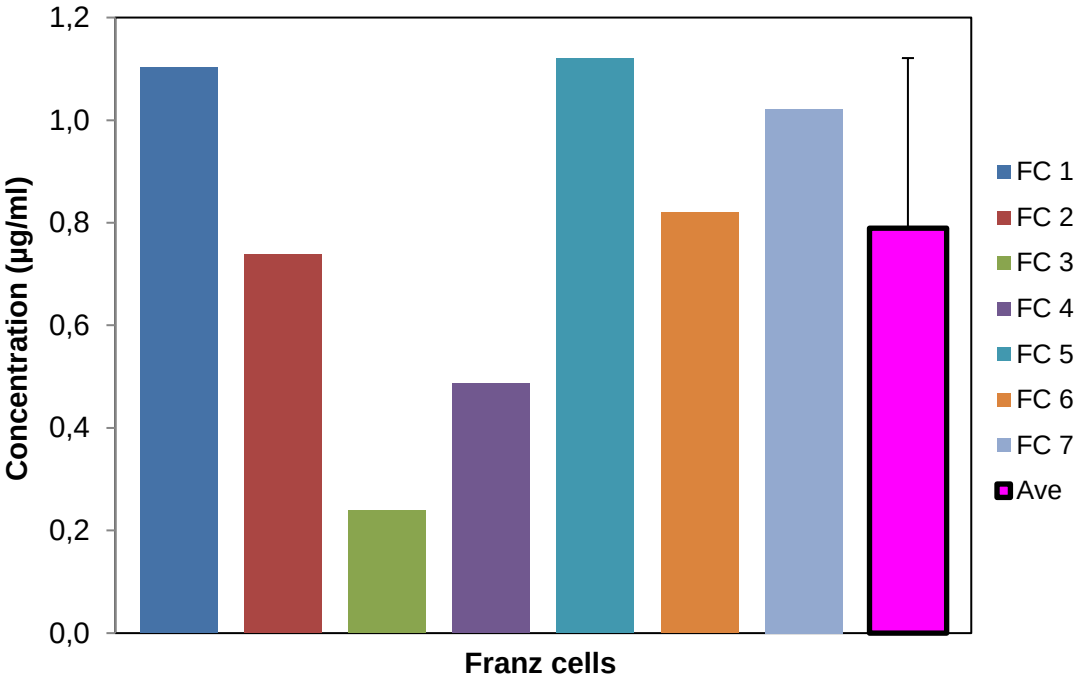


Figure D.33: Pravastatin concentration (µg/ml) from the **NG-PRA1** present in the SCE (n = 7)

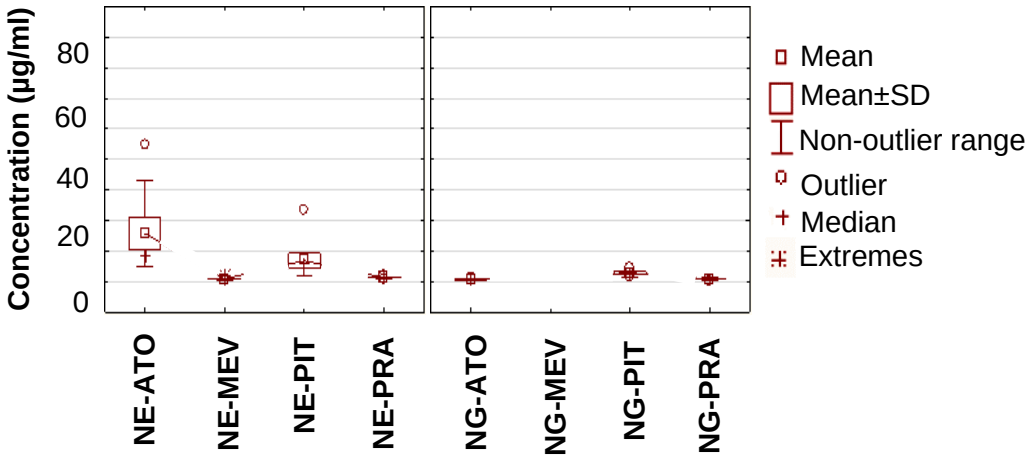


Figure D.34: Box-plots indicating the mean and median concentration (µg/ml) of the **NE1s** and the **NG1s** for each of the selected statins present in the SCE

As stated in Section D.3.1, it is not advised to utilise a mean value in the case of skewed data distribution (Manikandan, 2011). Hence, median values will be discussed further.

Table D.7: The median concentration (µg/ml) ranking for the **NE1s** and the **NG1s** in the SCE (excluding **NG-MEV1**)

1	2	3	4	5	6	7
NE-ATO1	**NE-PIT1**	**NG-PIT1**	**NE-PRA1**	**NE-MEV1**	**NG-PRA1**	**NG-ATO1**

According to Table D.7, **NE-ATO1** indicated the highest median concentration (µg/ml) in the SCE, while **NG-ATO1** obtained the lowest median concentration (µg/ml) when the formulas (**NE1s** and **NG1s**) were compare. It was also noticed the **NE1s** (**NE-PIT1**, **NE-PRA1**, **NE-ATO1** and **NE-MEV1**) had higher median concentration values than their **NG1**-counterpart (**NG-PIT1**, **NG-PRA1**, **NG-ATO1** and **NG-MEV1** (no concentration found in the SCE)). From the characterisation results (Section C.3.4), it was observed that the **NE1s** obtained smaller droplet sizes than those of the **NG1s**; hence, smaller droplets have larger surface areas, which may enhance permeation (into the stratum corneum) and can ultimately lead to the accumulation of API in that region (Arora, 2014).

It was detected that when the APIs were compared with regards to the formula (**NE1s** and **NG1s**) used, it was found that the ranking of the APIs were not the same when delivered from the **NE1s** in comparison to the **NG1s**. Hence, when the **NE1s** were investigated, it was observed that atorvastatin had the highest median concentration in the SCE, followed by pitavastatin, pravastatin and lastly, mevastatin, while it was noticed that pitavastatin obtained the highest median concentration in the SCE, followed by pravastatin and then atorvastatin (since **NG-MEV1** was not delivered into the SCE) when the **NG1s** were inspected. Hence, it can be concluded that the API used did not influence delivery into the SCE, but rather the type of formula used, of which the **NE1s** delivered higher concentrations of the statins in comparison to the **NG1s**. All formulas (except **NG-MEV1**) were successful in delivering the statins into the SCE.

After comparing the median concentration values of the **NE1s** and **NG1s** of this study with previous studies performed by Maree (2018) and Marais (2018) on **NEs** and **NGs** containing 2% statin and apricot oil (as natural oil used), it was found that the median concentration for the **NEs** containing apricot oil were: 7.087 µg/ml for atorvastatin (Maree, 2018), 1.871 µg/ml for mevastatin (Marais, 2018), 1.672 µg/ml for pitavastatin and 1.054 µg/ml for pravastatin (Maree, 2018), which posed significantly lower median concentration values compared to the **NE1s** containing grapeseed oil used during this study (excluding mevastatin). The median concentration for the **NGs** containing apricot oil were: 0.7189 µg/ml for atorvastatin (Maree, 2018), 1.416 µg/ml for mevastatin (Marais, 2018), 1.216 µg/ml for pitavastatin and 1.5076 µg/ml for pravastatin (Maree, 2018), which posed significantly higher median concentration values compared to the **NG1s** containing grapeseed oil used during this study (excluding pitavastatin). Therefore, in general, it

can be concluded that for the **NEs**, the grapeseed oil improved the delivery of the statins into the SCE more than the apricot oil. Although, the opposite was true for the **NGs**, since the apricot oil seems to improve delivery into the SCE more than the grapeseed oil.

D.3.3.2 Epidermis-dermis

The concentration (µg/ml) of the statins that was delivered in the ED from the **NE1s** and **NG1s** are illustrated in Figures D.35 – D.42.

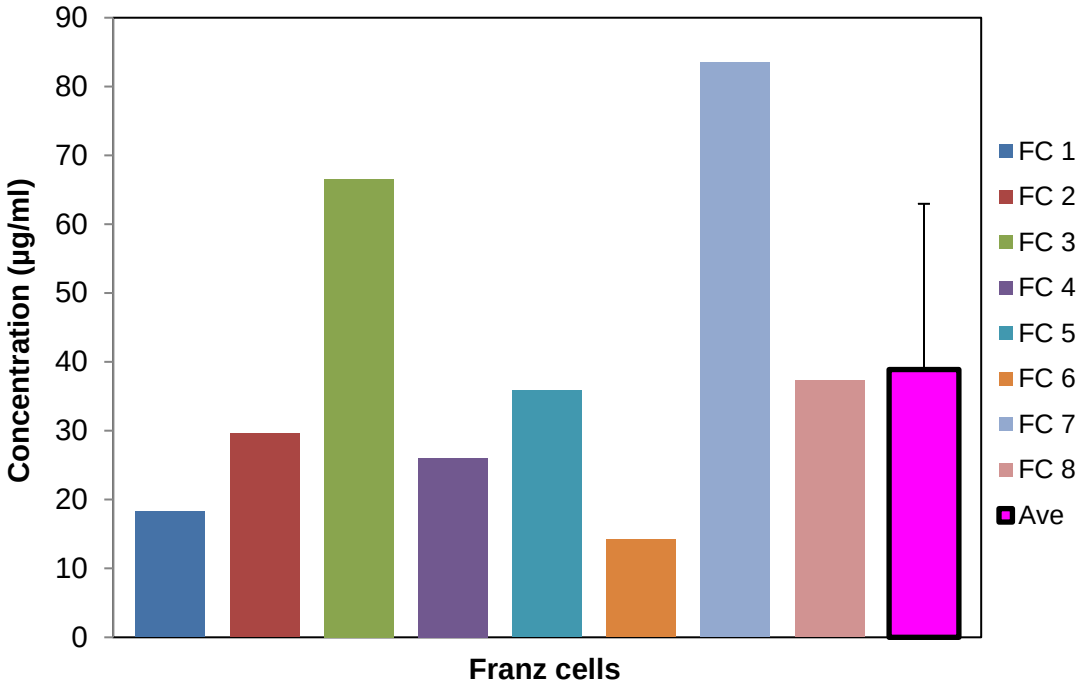


Figure D.35: Atorvastatin concentration (µg/ml) from the **NE-ATO1** present in the ED (n = 8)

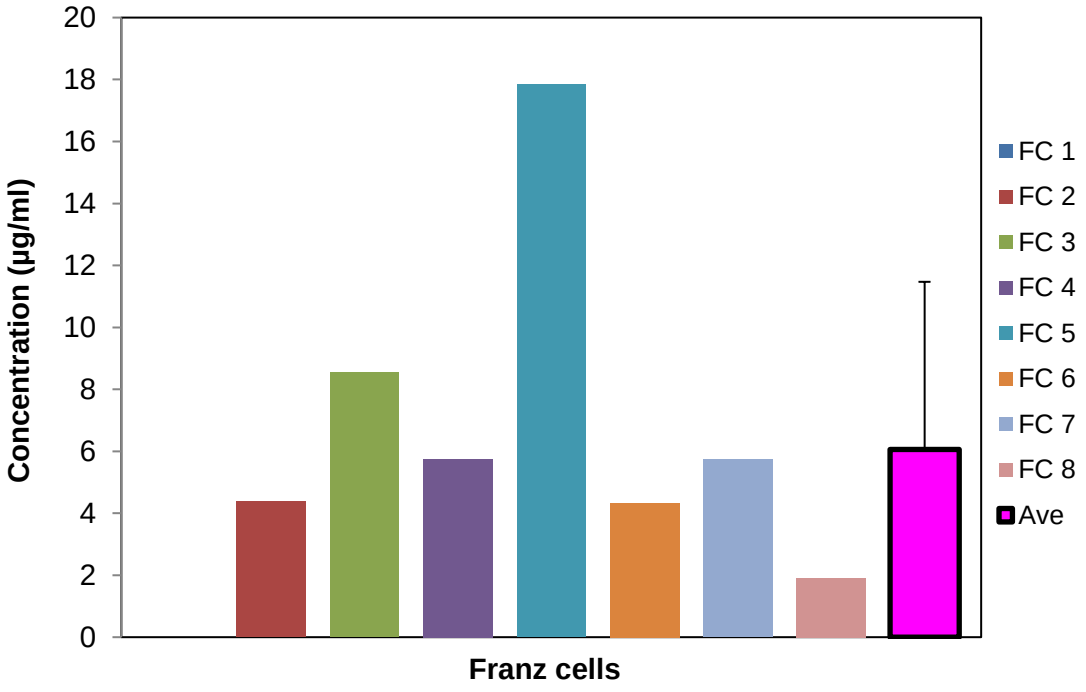


Figure D.36: Atorvastatin concentration (µg/ml) from the **NG-ATO1** present in the ED (n = 8)

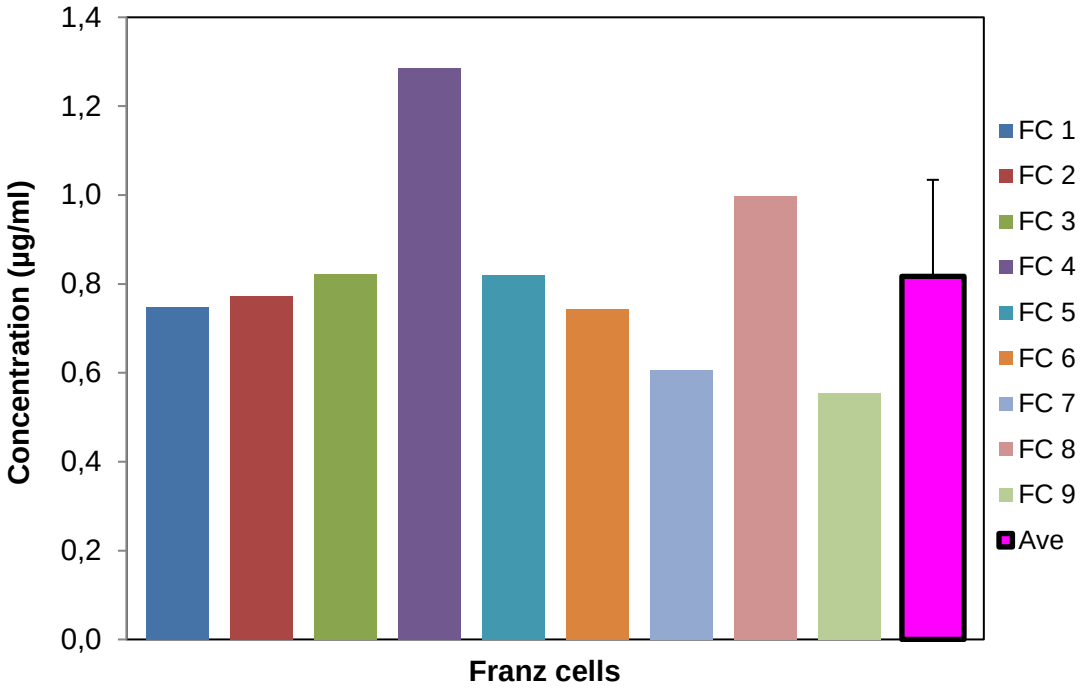


Figure D.37: Mevastatin concentration (µg/ml) from the **NE-MEV1** present in the ED (n = 9)

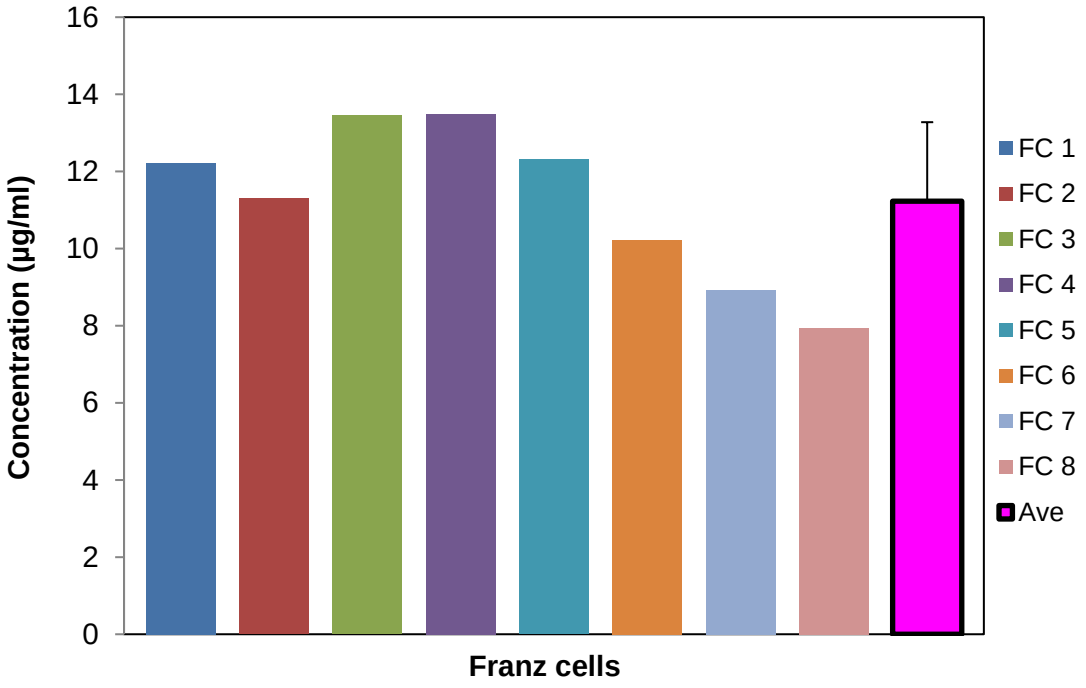


Figure D.38: Mevastatin concentration (µg/ml) from the **NG-MEV1** present in the ED (n = 8)

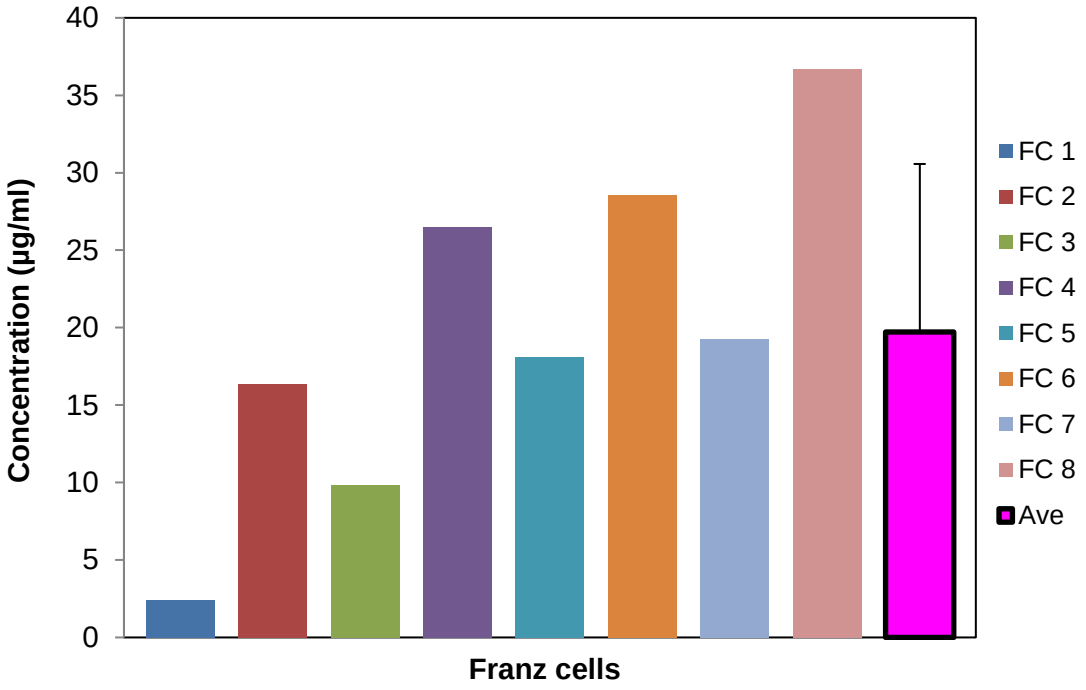


Figure D.39: Pitavastatin concentration (µg/ml) from the **NE-PIT1** present in the ED (n = 8)

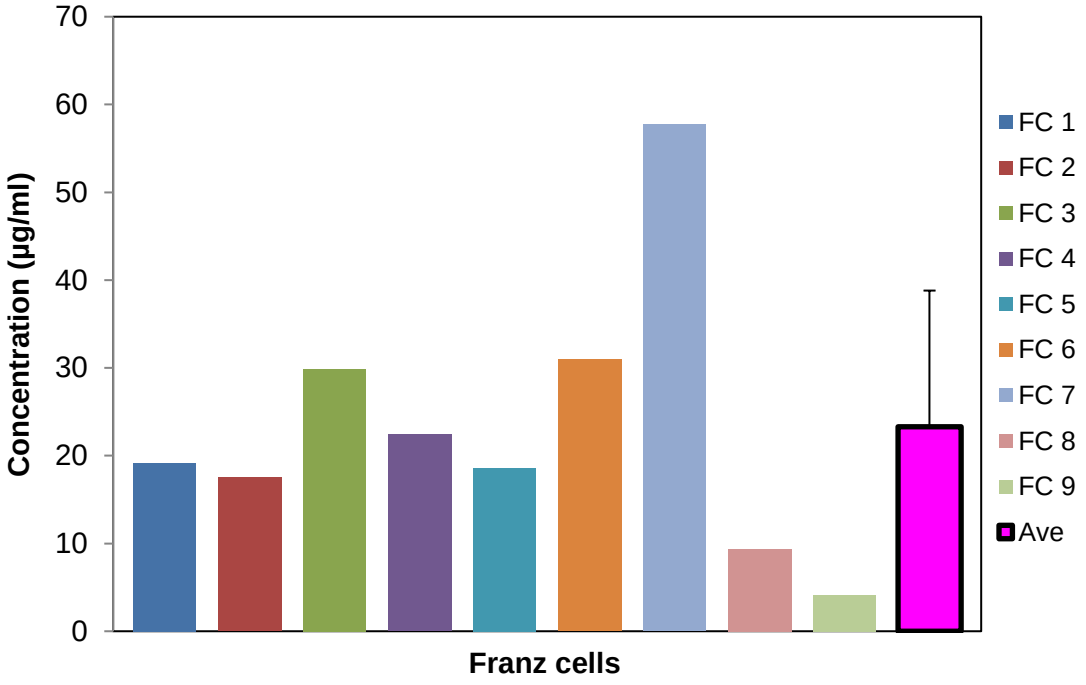


Figure D.40: Pitavastatin concentration (µg/ml) from the **NG-PIT1** present in the ED (n = 9)

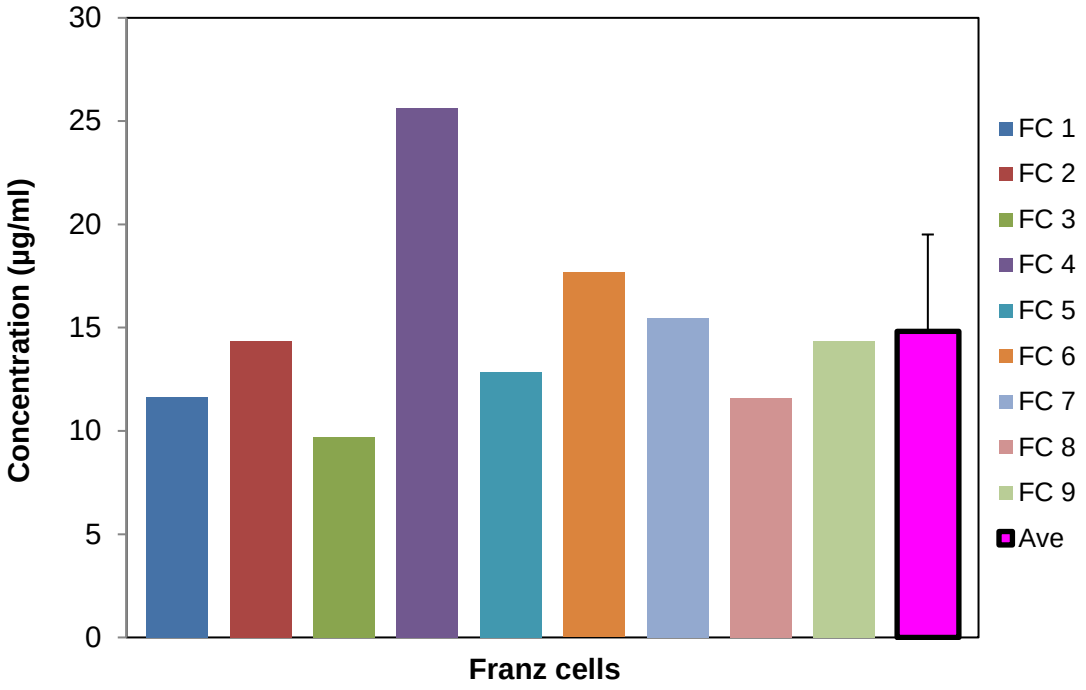


Figure D.41: Pravastatin concentration (µg/ml) from the **NE-PRA1** present in the ED (n = 9)

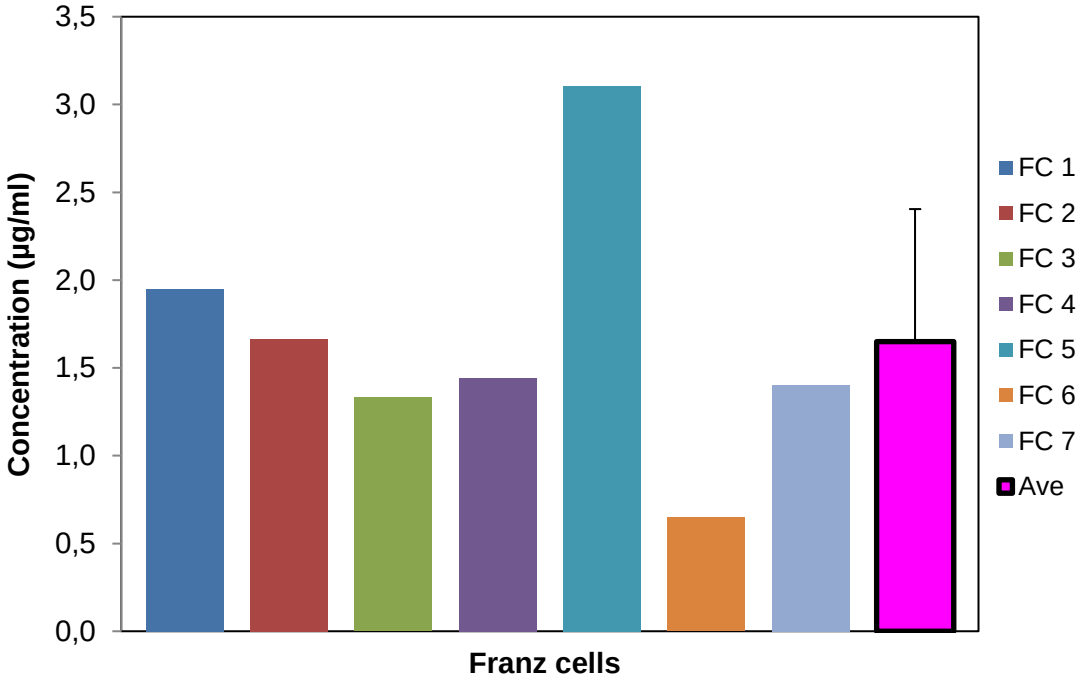


Figure D.42: Pravastatin concentration (µg/ml) from the **NG-PRA1** present in the ED (n = 7)

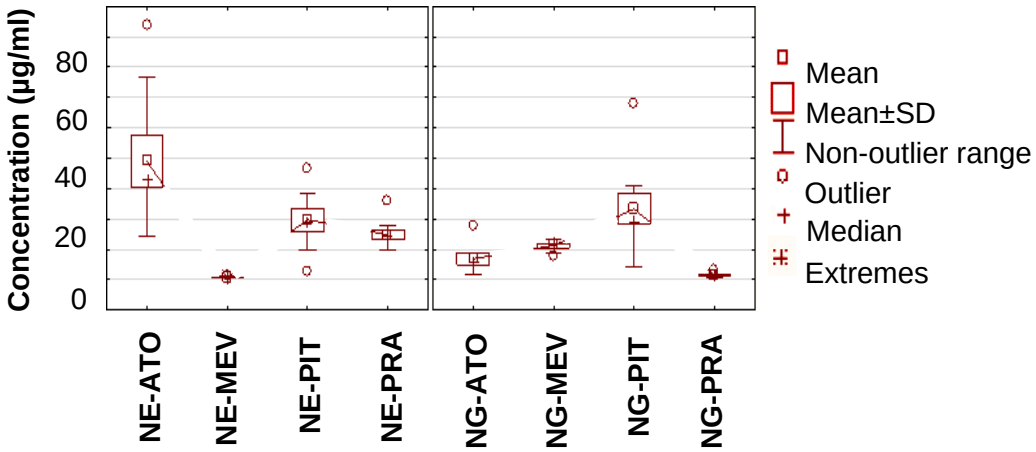


Figure D.43: Box-plots indicating the mean and median concentration (µg/ml) of the **NE1s** and the **NG1s** for each of the selected statins present in the ED

Data will be discussed according to median concentrations (as stated in Section D.3.1), since it is not affected by outliers (Manikandan, 2011).

Table D.8: The median concentration (µg/ml) ranking for the **NE1s** and the **NG1s** in the ED

1	2	3	4	5	6	7	8
NE-ATO1	**NG-PIT1**	**NE-PIT1**	**NE-PRA1**	**NG-MEV1**	**NG-ATO1**	**NG-PRA1**	**NE-MEV1**

From Table D.8, it is observed that the **NE-ATO1** obtained the highest median concentration and that the **NE-MEV1** obtained the lowest median concentration, when all the formulas (**NE1s** and **NG1s**) were compared. It was also detected that the **NE1s** containing atorvastatin and pravastatin (**NE-ATO1** and **NE-PRA1**) had higher median concentrations than their **NG1s**-counterpart. The opposite was true for pitavastatin and mevastatin, with **NG-PIT1** and **NG-MEV1** presenting with higher median concentrations than their **NE1s**-counterpart.

When the APIs were compared with regards to the formula (**NE1s** and **NG1s**) used, it was observed that the ranking of the APIs delivered to the ED from the **NE1s** in comparison to the **NG1s** were not the same. Hence, when the **NE1s** were examined, it was detected that atorvastatin had the highest median concentration, followed by pitavastatin, pravastatin and lastly, mevastatin, while for the **NG1s** it was observed that pitavastatin attained the highest median concentration in the ED, followed by mevastatin, atorvastatin and lastly, pravastatin. Hence, it can be concluded that the neither the API nor the type of formula used (**NE1s** and **NG1s**), influenced the delivery into the ED, since the ranking of the APIs delivered to the ED differed from each formula and also, both the **NE1s** and **NG1s** had delivered higher concentrations for two of

the four statins. Nevertheless, all formulas (**NE1s** or **NG1s**) were successful in delivering the statins into the ED.

When the median concentration values in the ED of previous studies performed by Maree (2018) and Marais (2018) on **NEs** and **NGs** containing 2% statin and apricot oil (as natural oil used) were compared to the median concentration values of the **NE1s** and **NG1s** of this study, it was observed that the median concentration for the **NEs** containing apricot oil were: 24.555 µg/ml for atorvastatin (Maree, 2018), 1.338 µg/ml for mevastatin (Marais, 2018), 6.481 µg/ml for pitavastatin and 2.471 µg/ml (Maree, 2018) for pravastatin, which posed significantly lower median concentration values compared to the **NE1s** containing grapeseed used during this study (except for mevastatin). The median concentration for the **NGs** containing apricot oil were: 3.819 µg/ml for atorvastatin (Maree, 2018), 3.429 µg/ml for mevastatin, 9.771 µg/ml for pitavastatin and 1.8380 µg/ml for pravastatin (Maree, 2018), which posed significantly lower median concentration values compared to the **NG1s** containing grapeseed oil used during this study (except for pravastatin). Consequently, in general, it can be concluded that the grapeseed oil improved the delivery of the statins into the ED more than the apricot oil.

D.3.4 Statistical analysis

Before initiating statistical analysis of data, a H_0 should be suggested, which relates to groups of interest, e.g. that there are no differences between group means (McCluskey & Lalkhen, 2007). Subsequently, statistical analysis can be performed and obtained results can be interpreted by relying on the consideration of the H_0, p-value, study power, concept of statistical significance, and multiple comparisons, before initiating the study (McCluskey & Lalkhen, 2007). The p-value can be described as the probability under the H_0 that a result or a more extreme can be attained. A p-value smaller or equal to 0.05 is generally regarded statistically significant (Concato & Hartigan, 2016), but it is suggested that the p-value be reported as very significant or highly significant (depending on the proximity to 0) (Dahiru, 2008).

D.3.4.1 Statistical analysis of membrane release studies

Statistical analyses were performed on membrane release studies by ANOVA, followed by the Tukey's HSD post-hoc tests, and also using non parametric Mann-Whitney U tests. A two-way ANOVA was performed on **NE1s** and **NG1s**. The interaction between the type of formula (**NE1s** or **NG1s**) and the statin indicated a p-value of < 0.001. Thus, a highly statistical significant interaction was observed. As a result, a one-way ANOVA was performed for respective **NE1s** and **NG1s** to compare the statins.

Table D.9: P-values of one-way ANOVAs that were performed on the selected statins for the **NE1s** and **NG1s**

Membrane release studies	
Comparison	**p-value**
Statin for **NE1s**	< 0.001
Statin for **NG1s**	< 0.001

Table D.9, indicates the comparison between the means (averages) of the **NE1s** and the **NG1s**. Differences between the actual means of the statins test samples were significant ($p < 0.001$, which is smaller than 0.05). It is evident that both the **NE1s** and **NG1s** displayed highly significant effects in this study. Tukey's HSD post-hoc tests were performed for the respective formulas to compare the means of the statins pairwise.

Table D.10: Tukey's HSD post-hoc test data of statins in various **NE1s** in terms of means

Tukey's HSD post-hoc test data (NE1s)	
Statin formula	**Mean flux (µg/cm².h)**
NE-MEV1	11.2818 [a]
NE-ATO1	123.0216 [b]
NE-PIT1	207.2776 [c]
NE-PRA1	902.7226 [d]

a, b, c and d indicates statistically non-significant homogeneous groups on 0.05 level, which indicates all groups are significantly different

Table D.11: Tukey's HSD post-hoc test data of statins in various **NG1s** in terms of means

Tukey's HSD post-hoc test data (NG1s)	
Statin formula	**Mean flux (µg/cm².h)**
NG-MEV1	6.1480 [a]
NG-ATO1	151.3084 [b]
NG-PIT1	257.5121 [c]
NG-PRA1	376.0044 [d]

a, b, c and d signify statistically non-significant homogeneous groups on 0.05 level, which indicates all groups are significant

It can be observed from Tables D.10 and D.11 that each statin related to the **NE1** and **NG1** differed significantly from each other during the membrane release studies (as indicated by a, b, c and d).

Table D.12: Non-parametric Mann-Whitney U test to compare data of the statins in the **NE1s** and **NG1s** according to p-values

Mann-Whitney U test data (NE1s vs NG1s)	
Statin	**p-value**
Atorvastatin	0.048
Mevastatin	0.001
Pitavastatin	0.025
Pravastatin	0.001

From Table D.12, it is observed that all the selected statins had p-values below 0.05, thus indicating that the statins in the type of formula (**NE1s** vs **NG1s**) were significantly different.

D.3.4.2 Statistical analysis of *in vitro* skin diffusion studies

Statistical analysis was carried out on the *in vitro* skin diffusion studies by ANOVAs, Mann-Whitney U tests and Kruskal-Wallis tests. Non-normal data was obtained for the **NE1s** and the **NG1s** during the skin diffusion studies; therefore, a Kruskal-Wallis multiple comparisons test and Mann-Whitney U test were performed. Results were grouped according to the statin that diffused through the skin and the statin that remained in skin layers e.g. SCE-ED.

D.3.4.2.1 Statistical analysis of the statins in the NE1s and NG1s that diffused through the skin

A two-way ANOVA was performed on the **NE1s** and **NG1s**, which contained the selected statins that diffused through the skin. An interaction was observed between the respective statin and the specific formula (**NE1s** or **NG1s**) with a determined p-value. An interaction between the type of formula (**NE1s** or **NG1s**) and the statin revealed a p-value of 0.025. Therefore, a statistical significant interaction was observed. Consequently, a one-way ANOVA was performed for each of the **NE1s** and the **NG1s** to compare the statins.

Table D.13: One-way ANOVAs with p-values on the respective statins in the **NE1s** and **NG1s** that diffused through the skin

Skin diffusion studies	
Comparison	**p-value**
Statin for **NE1s**	< 0.001
Statin for **NG1s**	< 0.001

Table D.13 illustrates that the amount per area diffused of the selected statins in the **NE1s** and **NG1s** were compared, with p-values below 0.001, thus indicative of highly statistical significant variances.

Table D.14: Kruskal-Wallis multiple comparison test for the **NE1s** that diffused through the skin categorised according to their p-values between two selected statins according to their amount per area diffused

Statins	Atorvastatin	Mevastatin	Pitavastatin	Pravastatin
Atorvastatin		0.474	0.004	0.474
Mevastatin	0.474		< 0.001	0.002

Non-normal data was obtained during the skin diffusion studies (**NE1s**); therefore, the Kruskal-Wallis test was performed. From Table D.14, it can be explained that **NE-ATO1** significantly differed from **NE-PIT1** due to the appropriate p-value, which was smaller than 0.05. Therefore, **NE-MEV1** also differed significantly from **NE-PIT1** and **NE-PRA1**, when looking at the p-values, while **NE-ATO1** did not significantly differ from **NE-PRA1** or **NE-MEV1** (and *vice versa*) (p-values > 0.05).

Table D.15: Kruskal-Wallis multiple comparison test for the **NG1s** that diffused through the skin categorised according to their p-values between two selected statins according to their amount per area diffused

Statins	Atorvastatin	Mevastatin	Pitavastatin	Pravastatin
Atorvastatin		0.257	0.014	1.000
Mevastatin	0.257		< 0.001	0.035

Non-parametric data was obtained during the skin diffusion studies (**NG1**); therefore, the Kruskal-Wallis test was performed. From Table D.15, it can be explained that **NG-ATO1** significantly differed from **NG-PIT1** due to the appropriate p-value, which was smaller than 0.05. Therefore, **NG-MEV1** also differed significantly from **NG-PIT1** and **NG-PRA1** ($p < 0.05$), while **NG-ATO1** did not significantly differ from **NG-PRA1** or **NG-MEV1** (and *vice versa*) (p-values > 0.05).

D.3.4.2.2 Statistical analysis of the statins in the NE1s and NG1s that remained in skin layers

A statistical significant interaction between the statin, type of formula and the skin layer were observed; therefore, different ANOVAs were performed to establish if there was a statistical effect

on the different combinations of the respective statin, the formula and the skin layer during tape stripping studies (as seen in Table D.16).

Table D.16: ANOVAs showing the interactions with regards to the statins, the type of formula (**NE1s** and **NG1s**) and the skin layers (SCE and ED)

SCE			**ED**		
Interaction	**Level of interaction**	**p-value**	**Interaction**	**Level of interaction**	**p-value**
Statin*Skin layers	Two-way ANOVA	0.016	Statin*Skin layers	Two-way ANOVA	< 0.001
Skin layers*Type of formula	Two-way ANOVA	< 0.001	Skin layers*Type of formula	Two-way ANOVA	< 0.001
Statin*Skin layers*Type of formula	Three-way ANOVA	0.010	Statin*Skin layers*Type of formula	Three-way ANOVA	< 0.001

As a result, all the p-values were < 0.05, indicating significant interactions. It was then decided that one-way ANOVAs followed by Tukey's HSD-tests would be implemented on all the formulas in combination with the skin layers, to establish the pairwise variance between the four statin means.

Table D.17: P-values regarding the one-way ANOVA to compare statin means with the formulas in combination with the skin layers during tape stripping

Tape stripping	
Combination	**p-value**
NE1 SCE	< 0.001
NE1 ED	< 0.001
NG1 SCE	< 0.001
NG1 ED	< 0.001

As a result, each selected formula obtained effects for the statins ($p < 0.001$, smaller than 0.05). This was an indication that there were statistical significant effects observed for the statins from each combination (formula with the skin layer). To compare the formulas within regards to the statin in combination with the skin layer (while deviations of normality were apparent), non-parametric Mann-Whitney U tests were performed instead of unpaired t-tests.

Table D.18: The non-parametric Mann-Whitney U test of statins to compare **NE1s** with **NG1s** according to p-values for the SCE

Mann-Whitney U test data (SCE)	
Statin	**p-value**
Atorvastatin	< 0.001
Pitavastatin	0.092
Pravastatin	0.020

Atorvastatin and pravastatin obtained p-values smaller than 0.05 and was regarded as statistically significantly different. It should be noted that mevastatin was excluded from the Mann-Whitney U test due to unsubstantial data.

Table D.19: The non-parametric Mann-Whitney U test of statins to compare **NE1s** with **NG1s** according to p-values for the ED

Mann-Whitney U test data (ED)	
Statin	**p-value**
Atorvastatin	0.002
Mevastatin	< 0.001
Pitavastatin	0.025
Pravastatin	0.001

All statins in the ED obtained p-values smaller than 0.05 and was regarded as a statistically significantly different.

To compare the formulas pairwise within all combinations of the statins with the skin layer, the Tukey's HSD post-hoc test was performed.

Table D.20: Tukey's HSD post-hoc test for the **NE1s**, which contains statins in combination with the layer of the skin (SCE) where the statin presented in

Tukey's HSD post-hoc test for NE1s*SCE	
Statin formula	**Mean concentration (µg/ml)**
NE-MEV1	0.919[a]
NE-ATO1	15.705[b]
NE-PIT1	6.804[ab]
NE-PRA1	1.302[a]

Homogenous groups (a, b) respectively for each combination

Table D.20 illustrates that mevastatin and pravastatin formed a homogenous group. As a result, pitavastatin was only partly homogenous with the other statins with regards to the **NE1s** in the SCE combination and therefore, non-relatable in the other three combinations.

Table D.21: Tukey's HSD post-hoc test for the **NG1s**, which contains statins in combination with the layer of the skin (SCE) where the statin presented in

Tukey's HSD post-hoc test for NG1s*SCE	
Statin formula	**Mean concentration (µg/ml)**
NG-ATO1	0.675[a]
NG-PIT1	2.815[b]
NG-PRA1	0.789[a]

Homogenous groups (a, b) respectively for each combination. Mevastatin was excluded due to unsubstantial data

Table D.21 indicates that only atorvastatin and pravastatin formed a homogenous group in the SCE concerning **NG1s**.

Table D.22: Tukey's HSD post-hoc test for the **NE1s**, which contains statins in combination with the layer of the skin (ED) where the statin presented in

Tukey's HSD post-hoc test for NE1s*ED	
Statin formula	**Mean concentration (µg/ml)**
NE-MEV1	0.817[a]
NE-ATO1	38.919[c]
NE-PIT1	19.714[b]
NE-PRA1	14.813[ab]

Homogenous groups (a, b, c) respectively for each combination

Table D.22 indicates the different homogenous groups (a, b, c) and it was observed that pravastatin was only partly homogenous with mevastatin and pitavastatin in the ED concerning **NE1s** combination.

Table D.23: Tukey's HSD post-hoc test for the **NG1s**, which contains statins in combination with the layer of the skin (ED) where the statin presented in

Tukey's HSD post-hoc test for NG1s*ED	
Statin formula	**Mean concentration (µg/ml)**
NG-MEV1	11.231[ab]
NG-ATO1	6.926[a]
NG-PIT1	23.301[b]
NG-PRA1	1.649[a]

Homogenous groups (a, b) respectively for each combination

Table D.23 indicates that only atorvastatin and pravastatin formed a homogenous group. However, it was noticed that mevastatin was only partly homogenous with the other statins in the ED concerning **NG1s** combination and therefore, non-relatable in the other three combinations.

D.4 Conclusion

Table D.24 give a summary for the ranking of the different formulas (**NE1s** and **NG1s**) with the respective statins (pitavastatin, pravastatin, atorvastatin, mevastatin) in regards to the release, transdermal diffusion and topical (SCE and ED) delivery, where 1 represents the highest median value to 8 as the lowest median value.

Table D.24: Ranking from highest (1) to lowest (8) for the median flux (µg/cm^2.h; membrane release studies), median amount per area diffused (µg/cm^2; skin diffusion studies) and the median concentration (µg/ml; SCE and ED) regarding the statins from the **NE1s** and the **NG1s**

Formula	NG-PIT1	NE-PIT1	NG-PRA1	NE-PRA1	NG-ATO1	NE-ATO1	NG-MEV1	NE-MEV1
Release (µg/cm^2.h)	3	4	2	1	5	6	8	7
Transdermal (µg/cm^2)	1	2	3	4	5	6	7	8
SCE (µg/ml)	3	2	6	4	7	1	-	5
ED (µg/ml)	2	3	7	4	6	1	5	8

When examining the membrane release studies, it is evident that all the statins were successfully released from the formulas (**NE1s** and **NG1s**) and that both formulas followed the same trend when releasing the statins (in the order of highest to lowest release: pravastatin, pitavastatin, atorvastatin and mevastatin).

It is evident when only focusing on the **NE1s** that atorvastatin was the API with the highest topical (in both the SCE and ED) drug delivery, followed by pitavastatin, pravastatin and lastly, mevastatin. However, this trend was not followed transdermally (from highest to lowest: pitavastatin, pravastatin, atorvastatin, mevastatin) for the **NE1s**, except for mevastatin (which in all cases had the lowest API delivery of all the **NE1s**). As a result, **NE-MEV1** had the smallest amount of API penetration via topical and transdermal delivery. This could possibly be due to the poor release of mevastatin from the formulation during the membrane release studies or due to the poor physicochemical properties of mevastatin (low aqueous solubility value of 0.032 mg/ml (Drugbank, 2005c) and high log D value of 3.570 (Kim *et al*., 2011), which should ideally be > 1 mg/ml and have a log D between 1 and 3, respectively for topical and transdermal delivery (Naik *et al*., 2000)). According to literature, it can be proposed that mevastatin, due to its high

lipophilicity has the potential to express a higher affinity for the stratum corneum (Förster *et al*., 2009), and should enable effortless penetration of this layer (Sugibayashi, 2017). However, this was not observed during this study and it can therefore be suggested that the lipophilic mevastatin had a greater affinity towards the oil phase (grapeseed oil) of the **NE1** rather than the proposed lipophilic stratum corneum, which could have affected the release of the API from the formulation, and subsequently reduced skin diffusion (Haque & Talukder, 2018). With that being said, the formula of the **NE1s** and **NG1s** contain 90% water and mevastatin is practically insoluble with water, which conflicted with the selected o/w formulas (Kim *et al*., 2011; Yoshida *et al*., 2011).

Although atorvastatin (**NE-ATO1**) had the best topical (SCE and ED) delivery of all the **NE1s** and also possessed an ideal log D (1.00 – 1.25 (Fong, 2016)); its molecular weight (1155.34 g/mol (Al-Adl *et al*., 2017), which should ideally be less than 500 Da to enhance skin permeation (Naik *et al*., 2000)) and aqueous solubility (0.19 mg/ml (Rodde *et al*., 2014) was very poor and could therefore impede transdermal delivery. It can also be suggested that atorvastatin accumulated in the SCE and ED (when compared to transdermal results) due to its low aqueous solubility, which delayed it from penetrating deeper into the hydrophilic regions.

It was detected that pitavastatin (**NE-PIT1**) had the best transdermal delivery and the second highest topical (SCE and ED) delivery of all the **NE1s**, which may be due to the ideal log D (1.50 (Fong, 2016)) and moderate aqueous solubility (0.500 mg/ml (Cayman chemical, 2014)) of pitavastatin.

It was observed that pravastatin (**NE-PRA1**) had the second best transdermal delivery of all the **NE1s**, but when the topical (SCE and ED) data was investigated, it was noticed that pravastatin was not one of the top two statins to be delivered topically. Hence, a reason why the aforementioned occurred may possibly be due to the effective release of pravastatin from the **NE1** and the high aqueous solubility (10.000 mg/ml (Cayman chemical, 2015)), which may have led to a high concentration gradient acting as a driving force to get the statin into the systemic circulation (Chellapa, 2015).

With regards to the **NG1s**, it was observed that pitavastatin (**NG-PIT1**) and atorvastatin (**NG-ATO1**) had the best and third best topical (SCE and ED) delivery, respectively (this trend was also followed transdermally). Hence, from the topical and transdermal data, it was also evident that the **NG1** promoted the delivery of pitavastatin not only into the systemic circulation (like the **NE1s**), but also into both the skin layers, even though pitavastatin (with an ideal log D of 1.50 (Fong, 2016)) had a less ideal aqueous solubility (0.500 mg/ml (Cayman chemical, 2014)).

It was also noted that no mevastatin (**NG-MEV1**) was delivered in the SCE. However, when looking further at the data, the **NG1** promoted the delivery of mevastatin to the ED (and it appears

that the **NG1** targeted the ED for mevastatin, as mevastatin was once again the least systemically delivered of all the respective statins for the **NG1**).

Pravastatin (**NG-PRA1**) showed good penetration for the SCE (second best delivery), but had the weakest delivery to the ED, followed by the second best transdermal delivery of all the statins. Hence, it seems that the **NG1** assisted with hydrophilic pravastatin passing through the lipophilic SCE and expressing a higher affinity for the hydrophilic region (Jankowski *et al*., 2017); especially the systemic circulation, more than the hydrophilic ED. It is proposed that higher transdermal delivery (for both the **NE-PRA1** and **NG-PRA1**) can further be attributed to the salt form being used during this study, therefore presenting with higher solubility than the specific acid or base form (Serajuddin, 2007).

After investigating the transdermal and topical data, it was evident that **NE1s** and **NG1s** improved statin delivery. The **NE1s** indicated higher statin concentrations in the SCE than their **NG1s**-counterpart, while the **NG1s** were more transdermally targeted at delivering the statins than their **NE1s**-counterpart. The occurrence of the **NG1s** resulting with lower median concentrations in the SCE can be ascribed to the addition of a gelling-agent (Carbopol® Ultrez 20). This agent causes swelling of the **NE1** to form the **NG1** (which will release an API in a more controlled manner (Chellapa *et al*., 2015) and produce formulations with higher viscosity (Mahalingam *et al*., 2008; Mitsui, 1997), which in turn increases API permeation through the skin (Eid *et al*., 2014).

In general, the formulas (**NE1s** and **NG1s**) containing grapeseed oil used during this study resulted in better topical (SCE and ED) and transdermal delivery of the statins than from the **NEs** and **NGs** containing apricot oil used during previous studies by Marais (2018) and Maree (2018). The aforementioned was not true for atorvastatin, mevastatin and pravastatin within **NGs** containing apricot oil, which obtained higher median concentrations in the SCE together with mevastatin (in the **NEs**) and pravastatin (in the **NGs**) within the apricot oil formulas that obtained higher topical (SCE and ED) and transdermal delivery than the grapeseed oil formulas (used during this study). Both grapeseed oil and apricot oil are polyunsaturated oils; however, grapeseed oil contains oleic acid (15.8%) and linoleic acid (69.6%) (AL-Bayati & Enaad, 2015) and apricot oil contains oleic acid (73.4%) and linoleic acid (31.7%) (Alpaslan & Hayta, 2006). It can be observed that grapeseed oil consists of a significant larger concentration of linoleic acid (C18:2 ω-6) (Rustan *et al*., 2001) in comparison to apricot oil, while apricot oil contains a larger concentration of oleic acid (C18:1 ω-9) (Rustan *et al*., 2001) opposed to grapeseed oil. According to literature, longer chain fatty acids have proved to enhance percutaneous drug absorption, where unsaturated C_{18} chain fatty acids are near to ideal (Cho *et al*., 2009; Williams & Barry, 2012). Conclusively, it may explain why higher linoleic concentrations have higher enhancing qualities for grapeseed oil, since it is already part of the skin structure and may have encountered

less hindrance from the skin barrier (Hopkins, 2012; McCusker *et a*l., 2010; Suhonen *et al*., 1999). A study by Choi *et al*. (2009) found that an increase in double bonds of the essential fatty acids, i.e. from linoleic- (C18:2) to linolenic acid (C18:3), proposed a significant reduction in skin permeation of a lipophilic compound. Mevastatin is considerate highly lipophilic (Kim *et al*., 2011), therefore it can be concluded that formulations that contain more oleic acid (C18:1 ω-9) will be more preferable for lipophilic APIs, like mevastatin (as seen with the **NEs** and **NGs** containing apricot oil).

Statin plasma concentrations after oral administration (found in literature) and the transdermal concentrations (obtained in study) were compared. The plasma concentrations after oral administration of the statins (mevastatin is unknown) were 0.0009 µg/ml for atorvastatin (DeGorter *et al*., 2013), 0.3000 µg/ml for pitavastatin (Therapeutic goods administration, 2013) and 0.0641 µg/ml for pravastatin (Clarke *et al*., 2011), while the transdermal concentrations (obtained during this study) was as follow for the **NE1s**: atorvastatin (0.179 ± 0.016 µg/ml), pitavastatin (1.218 ± 0.256 µg/ml) and pravastatin (0.351 ± 0.133 µg/ml) and for the **NG1s**: atorvastatin (0.993 ± 1.335 µg/ml), pitavastatin (12.154 ± 6.699 µg/ml) and pravastatin (0.915 ± 0.393 µg/ml)). It was observed that each formula (**NE1s** and **NG1s**) containing the statin presented with higher concentrations in the receptor phase when it was compared to the plasma concentrations after oral administration.

The following conclusions were reached:

- Another administration route does exist for the administration of the selected statins, as both the **NE1s** and **NG1s** succeeded in delivering the statins transdermally, confirming that the goal of the study was accomplished.
- **NG1s** were the favourable transport system to deliver statins through the skin layers to reach the systemic circulation, as delivery to the receptor phase was higher from the **NG1s** than from the **NE1s**.
- **NE1s** also succeeded in delivering the statins transdermally.
- Both the **NE1s** and **NG1s** used during this study, delivered higher concentrations of the statins transdermally when compare to those of the orally administered plasma concentrations.

References

AL-Bayati, A.J. & Enaad, D.F. 2015. Histopathological study and surgery the effect of grape seed oil on wound healing in rabbits. *International journal of science and research*, 4(6):959-962.

Alpaslan, M. & Hayta, M. 2006. The effects of flaxseed, soy and corn flours on the textural and sensory properties of a bakery product. *Journal of food quality*, 29:617-627.

Ali, S., Shabbir, M., Shahid, N. 2015. The structure of skin and transdermal drug delivery system. *Research journal of pharmacy and technology*, 8(2):103-109.

Arda, O., Göksügür, N. & Tüzün, Y. 2014. Basic histological structure and functions of facial skin. *Clinics in dermatology*, 32(1):3-13.

Arora, R., Aggarwal, G., Harikumar, S.L., Kaur, K. 2014. Nanoemulsion based hydrogel for enhanced transdermal delivery of ketoprofen. *Advanced in pharmaceutics*, 1(2):1-12.

Baert, B., Vansteelandt, S. & De Spiegeleer, B. 2011. Ion mobility spectrometry as a high - throughput technique for *in vitro* transdermal Franz diffusion cell experiments of ibuprofen. *Journal of pharmaceutical and biomedical analysis*, 55, 472-478.

Bartosova, L. & Baljar, J. 2012. Transdermal drug delivery *in vitro* using diffusion cells. *Current medicinal chemistry*, 19:4671-4677.

Begur, M., Pai, V.K., Gowda, D.V., Srivastava, A., Raghundan, H.V., Shinde, C.G., Manusri N. 2015. Enhanced permeability of Cyclosporine from a transdermally applied nanoemulgel. *Der pharmacia sinica*, 6(2):69-79.

Berardesca, E. & Maibach, H. 2003. Ethnic skin: overview of structure and function. *Journal of the american academy of dermatology*, 48(6):S139-S142.

Burger, C. 2014. Formulation, *in vitro* release and transdermal diffusion of pravastatin by the implementation of the delivery gap principle. Potchefstroom: NWU. (Dissertation-MSc).

Cabral, M.E.S., Ramos, A. N., Cabrera, C.A., Valdez, J.C., Gonzalez, S.N. 2014. Equipment and method for *in vitro* release measurements on topical dosage forms. *Pharmaceutical development and technology*, 1-7.

Cayman Chemicals. 2008. Product information sheet: https://www.caymanchem.com/pdfs/10010340.pdf. Date of access: 05 Feb 2019.

Cayman chemical. 2014. Pitavastatin calcium salt (Product information 15414) https://www.caymanchem.com/pdfs/15414.pdf Date of access: 18 Jan 2019.

Cayman chemical. 2015. Pravastatin (sodium salt) (Material safety data sheet). https://www.caymanchem.com/msdss/10010343m.pdf Date of access: 18 Feb 2019.

Chaudhary, S., Verma, N.K., Panda, P., Singh, A.P. & Alam, G. 2018. Nanoemulsion based drug delivery-a review. *European journal of pharmaceutical and medical research*, 5(6):178-191.

Chellapa, P., Mohamed, A.T., Keleb, E.I., Elmahgoubi, A., Eid, A.M., Issa, Y.S. & Elmarzugi, N.A. 2015. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR journal of pharmacy*, 5(10): 43-47.

Chime, S.A., Kenechukwu, F.C. & Attama, A.A. 2014. Nanoemulsions: advances in formulation, characterization and application in drug delivery. (In Sezer, A.D., *ed*. Application of nanotechnology in drug delivery. p. 77-126). -http://cdn.intechopen.com/pdfs-wm/47116.pdf Date of access: 20 Nov. 2019.

Choi, J., Choi, M.K., Chong, S., Chung, S.J., Shim, C.K. & Kim, D.D. 2012. Effect of fatty acids on the transdermal delivery of donepezil: *In vitro* and *in vivo* evaluation. *International journal of pharmaceutics*, 422:83-90.

Clarke, E.G.C., Moffat, A.C., Osselton, M.D. & Widdop, B. 2011. Clarke`s analysis of drugs and poisons in pharmaceuticals, body fluids and postmortem material. 4th *ed*. Pharmaceutical Press. p. 1947.

Collier, S.W. & Bronaugh, R.L. 1991. Receptor fluids. (*In* Bronaugh, R.L. & Maibach, H.I., *eds*. *In vitro* percutaneous absorption: principles, fundamentals, and applications. Boca Raton: CRC Press. p. 31-49.)

Concato, J. & Hartigan, J.A. 2016. P-values: from suggestion to superstition. *Journal of investigative medicine*, 64:1166-1171.

Consonni, D., Bertazzi, P.A. 2017. Health significance and statistical uncertainty: The value of P-value. *Medicina del lavaro*, 108(5): 327-331.

Clarke, E.G.C., Moffat, A.C., Osselton, M.D. & Widdop, B. 2011. Clarke`s analysis of drugs and poisons in pharmaceuticals, body fluids and postmortem material. 4th ed. Pharmaceutical Press. p. 1947.

Dahiru, T. 2008. P-value, a true test of statistical significance? A cautionary note. *Annals of Ibadan postgraduate medicine*, 6(1):21-26.

Dawson, B. & Trapp, R.G. 2004. Basic & clinical biostatistics. 4th ed. New York: McGraw-Hill. p. 30, 38-39.

DeGorter, M.K., Tirona, R.G., Schwarz, U.I., Choi, Y., Dresser, G.K., Suskin, N., Myers, K., Zou, G.Y., Iwuchukwu, O., Wei, W., Wilke, R.A., Hegele, R.A., Kim, R.B. 2013. Clinical and pharmacogenetic predictors of circulating atorvastatin and rosuvastatin concentrations in routine clinical care. *Circulation cardiovascular genetics*, 6:400-408.

Denda, M. & Takahashi, M. 1990. Measurement of facial skin thickness by ultrasound method. *Journal of society of cosmetic chemists of Japan*, 23(4):316-319.

Dev, A., Chodankar, R. & Shelke, O. 2015. Emulgels: a novel topical drug delivery system. *Pharmaceutical and biological evaluations*, 2(4):64-75.

Driscoll, P., Lecky, F. & Crosby, M. 2000. An introduction to everyday statistics-2. *Journal of accident & emergency medicine*, 17:274-281.

Drugbank. 2005. https://www.drugbank.ca/drugs/DB06693. Date of access: 11 May 2017.

Eid, A.M., El-Enshasy, H.A., Aziz, R. & Elmarzugi, N.A. 2014. Preparation, characterization and anti-inflammatory activity of Swietenia macrophylla nanoemulgel. *Journal of nanomedicine & nanotechnology*, 5(2):1-10.

El Maghraby, G.M., Barry, B.W. & Williams, A.C. 2008. Liposomes and skin: from drug delivery to model membranes. *European journal of pharmaceutical sciences*, 34:203-222.

Encyclopaedia Britannica. 2016. Partition coefficient. https://www.britannica.com/science/cellbiology/Transport-across-the-membrane#ref313705 Date of access: 4 Oct 2019.

Escobar-Chávez, J.J., Rodríguez-Cruz, I.M., Domínguez-Delgado, C.L., Díaz-Torres, R., Revilla-Vázquez,A.L. & Aléncaster, N.C. 2012. Nanocarrier systems for transdermal drug delivery. (In Sezer, A.D., ed. Recent advances in novel drug carrier systems. Intech, p201-240.) https://www.intechopen.com/books/recent-advances-in-novel-drug-carrier systems/nanocarriersystems- for-transdermal-drug-delivery. Date of access: 8 Feb 2019.

Fong, C. 2016. Statins in therapy: Cellular transport, side effects, drug-drug interactions and cytotoxicity –the unrecognized role of lactones https://hal.archives-ouvertes.fr/hal-01185910v2 Date of access: 12 April 2019.

Förster, M., Bolzinger, M-A., Fessi, H. & Briançon, S. 2009. Topical delivery of cosmetics and drugs. Molecular aspects of percutaneous absorption and delivery. *European journal of dermatology*, 19(4):309-23.

Gniadecka, M. & Jemec, G. 1998. Quantitative evaluation of chronological ageing and photoageing *in vivo*: studies on skin echogenicity and thickness. *The British journal of dermatology*, 139(5):815-821.

Goodwin, C. 2011. Anatomy and Physiology of the Skin. *Journal of the dermatology nurses' association*, 3(4):203-213.

Hamada, C. 2018. Statistical analysis for toxicity studies. *Journal of toxicology and pathology*, 31:15-22.

Hopkins, L. 2012. http://www.sumakhafarms.com/emu-oil-fatty-acids-and-human-skin Date of access: 20 November 2019.

Haque, T. & Taluker, M.U. 2018. Chemical Enhancer: A simplistic way to modulate barrier function of the stratum corneum. *Advanced pharmaceutical bulletin*, 8(2):169-179.

Igarashi, T., Nishino, K. & Nayar, S.K. 2007. 2 Physiology and anatomy of human skin. *Foundations and trends in computer graphics and vision*, 3(1):13-13.

Jankowski, A., Dya, R. & Sarecka-hujar, B. 2017. Dermal and transdermal delivery of active substances from semisolid bases. *Indian journal of pharmaceutical sciences*, 79(4):488-500.

Jepps, O.W., Dancik, Y., Anissimov, Y.G. & Roberts, M.S. 2013. Modelling the human skin barrier-towards a better understanding of dermal absorption. *Advanced drug delivery reviews*, 65:152-168.

Johnsen, G.K. 2010. Skin electrical properties and physical aspects of hydration of keratinized tissues. (Thesis-PhD).

Jug, M., Hafner, A., Lovrić, J., Kregar, M.L., Pepić, I., Vanić, Z., Cetina-Čižmek, B. & Filipović-Grčić, J. 2017. *In vitro* dissolution/release methods for mucosal delivery systems. ADMET (absorption, distribution, metabolisms, excretion and technology) & DMPK (pharmacokinetic oof drugs) 5(3): 173-182.

Kanfer, I., Rath, S., Purazi, P. & Mudyahoto, A. 2017. *In Vitro* Release testing of semi-solid dosage forms. *Dissolution technologies*, 24(3):52-60.

Karadzovska, D & Riviere, J.E. 2013. Assessing vehicle effects on skin absorption using artificial membrane assays. *European journal of pharmaceutical sciences*, 50:569-576.

Khandavilli, S. & Panchagnula, R. 2006. Studies of the skin permeation of lipophilic drugs: paclitaxel. *Pharmazie*, 6:471-473.

Khurana, S., Jain, N. & Bedi, P. 2013. Nanoemulsion based gel for transdermal delivery of meloxicam: physico-chemical, mechanistic investigation. *Life sciences*, 92(6-7):383-392.

Kim, M.C., Ahn, Y., Jang, S.Y., Cho, K.H., Hwang, S.H., Lee, M.G., Ko, J.S., Park, K.H., Sim, D.S., Yoon, N.S., Yoon, H.J., Kim, K.H., Hong, T.J., Park, H.W., Kim, J.H., Jeong, M.H., Cho, J.G., Park, J.C. & Kang, J.C. 2011. Comparison of clinical outcomes of hydrophilic and lipophilic statins in patients with acute myocardial infarction. *The Korean journal of internal medicine*, 26:294-303.

Klein, S. 2013. Influence of different test parameters on *in vitro* drug release from topical diclofenac formulations in a vertical diffusion cell setup. *Pharmazie*, 68:565-571.

Krzywinski, M & Altman, N. 2014. Visualizing samples with box plots. *Nature methods*, 11(2):119-120.

Kute, S.B. & Saudagar, R.B. 2013. Emulsified gel: a novel approach for delivery of hydrophobic drugs: an overview. *Journal of advanced pharmacy education and research*, 3:368-376.

Lai-Cheong, J.E. & McGrath, J.A. 2009. Structure and function of skin, hair and nails. *Medicine*, 37(5):223-226.

Lasagni, C. & Seidenari, S. 1995. Echographic assessment of age-dependent variations of skin thickness: A study on 162 subjects. *Skin research and technology*, 1(2):81-85.

Lynch, J.C., Myers, K.F., Brannon, J.M. & Delfino, J.J. 2001. Effects of ph and temperature on the aqueous solubility and dissolution rate of 2,4,6-trinitrotoluene (tnt), hexahydro-1,3,5-trinitro-1,3,5-triazine (rdx), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). *Journal of chemical and engineering data*, 46:1549-1555.

Madison, K.C. 2003. Barrier function of the skin:“la raison d'etre” of the epidermis. *Journal of investigative dermatology*, 121(2):231-241.

Mahalingam, R., Li, X. & Jasti, B.R. 2008. Semisolid dosages: ointments, creams and gels. (In Gad, S.C., ed. Pharmaceutical manufacturing handbook: production and processes. New Jersey: John Wiley & Sons. p. 267-309).

Manikandan, S. 2011. Measures of central tendency: The mean. *Journal of pharmacology and pharmacotherapeutics*, 2(2):140-142.

Marais, S. 2018. The use of apricot oil emulsions for the transdermal delivery of selected statins. Potchefstroom: NWU. (Dissertation-MSc).

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

McCluskey, A. & Lalkhen, A.G. 2007. Statistics IV: Interpreting the results of statistical tests. *Continuing education in anaesthesia, critical care & pain*, 7(6):209-212.

McCusker, M.M. & Grant-Kels, J.M. 2010. Healing of the skin: the structural and immunologic roles of the ω-6 and ω-3 fatty acids. *Clinicsl dermatology*, 28(4):440-451.

Mitsui, T. 1997. Polymers. (*In* Mitsui, T., *ed*. New cosmetic science. Netherlands: Elsevier. p. 138-140).

Modi, P.B. & Shah, N.J. 2015. Optimization of an *in vitro* release test for topical formulations containing eberconazole nitrate and mometasonefuroate. *Der pharma chemica*, 7:1-9.

Mou, D., Chen, H., Du, D., Mao, C., Wan, J., Xu, H. & Yang, X. 2008. Hydrogel-thickened nanoemulsion system for topical delivery of lipophilic drugs. *International journal of pharmaceutic*s, 353(1-2):270-276.

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

Nastiti, C.M.R.R., Ponto, T., Abd, E., Grice, J.E., Benson, H.A.E. & Roberts, M.S. 2017. Topical nano and microemulsions for skin delivery. *Pharmaceutics*, 9(37):1-25.

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

Ng, K.W. & Lau, W.M. 2015. Skin deep: the basics of human skin structure and drug penetration. (In Dragicevic-Curic, N. & Maibach, H.I., eds. Percutaneous penetration enhancers: chemical

methods in penetration enhancement: drug manipulation strategies and vehicle effects. Heidelberg: Springer. p. 3-12).

Ng, S-F., Rouse, J.J., Sanderson, D. & Eccleston, G. 2010a. A comparative study of transmembrane diffusion and permeation of ibuprofen across synthetic membranes using Franz diffusion cells. *Pharmaceutics*, 2:209-223.

Ng, S-F., Rouse, J.J., Sanderson, F.D., Meidan, V. & Eccleston, G.M. 2010b. Validation of a static franz diffusion cell system for *in vitro* permeation studies. American Association of Pharmaceutical Scientists: *Journal of the american association of pharmaceutical scientists*, 11(3):1432-1441.

Nuzzo, R.L. 2016. The box plots alternative for visualizing quantitative data. *Physical medicine and rehabilitation*, 8(3):268-272.

OECD (Organisation for Economic Co-operation and Development). 2004. Guidance document for the conduct of skin absorption studies: OECD series on testing and assessment, No. 28. https://www.oecd-ilibrary.org/docserver/9789264078796 en.pdf?expires=1541172508&id=id&accname=guest&checksum=796E77B02778DA73DA CC 9E4C1EBB306 Date of access: 1 Feb. 2019.

OECD (Organisation for economic co-operation and development). 2011. Guidance notes on dermal absorption: series on testing and assessment, No. 156. http://www.oecd.org/chemicalsafety/testing/48532204.pdf Date of access: 6 Sep. 2019.

Olsen, L.O., Takiwaki, H. & Serup, J. 1995. High-frequency ultrasound characterization of normal skin. Skin thickness and echographic density of 22 anatomical sites. *Skin research and technology*, 1(2):74-80.

O'Neil, M.J., *ed*. 2006. The Merck index: an encyclopedia of chemicals, drugs and biologicals. 14th ed. Whitehouse Station, NJ: Merck.

Rodde, M.S., Divase, G.T., Devkar, T.B., Tekade, A.R. 2014. Solubility and bioavailability enhancement of poorly aqueous soluble atorvastatin: *in vitro*, *ex vivo*, and in vivo studies. *BioMed research international*, 1-11.

Rustan, A.C. & Drevon, C.A., 2001. Fatty acids: structures and properties.

Sahebzamani, F.M., Munro, C.L., Marroquin, O.C., Diamond, D.M., Keller, E., Kip, K.E. 2014. Examination of the FDA warning for statins and cognitive dysfunction. *Journal of pharmacovigilance*, 2(4):1-9.

Serajuddin, A.T.M. 2007. Salt formation to improve drug solubility. Advanced drug delivery reviews, 59:603.

Shahzad, Y., Louw, R., Gerber, M. & Du Plessis, J. 2015. Breaching the skin barrier through temperature modulations. *Journal of controlled release*, 202:1-13.

Shakeel, F., Baboota, S., Ahuja, A., Ali, J., Aqil, M. & Shafiq, S. 2007. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. *American association of pharmaceutical scientists: pharmscitech*, 8:E1-E9.

Sheskin, D.J. 2000. Handbook of parametric and nonparametric statistical procedures. 2nd *ed*. Boca Raton: Chapman & Hall/CRC. p 982.

Simon, A., Amaro, M.I., Healy, A.M, Cabral, L.M. & De Sousa, V.P. 2016. Comparative evaluation of rivastigmine permeation from a transdermal system in the Franz cell using synthetic membranes and pig ear skin with *in vivo-in vitro* correlation. *International journal of pharmaceutics*, 512:234-241.

Subedi, R.K., Oh, S.Y., Chun, M. & Choi, H. 2010. Recent advantages in transdermal drug delivery. *Archives of pharmacal research*, 33:339-351.

Sugibayashi, K. 2017. Skin permeation and disposition of therapeutic and cosmeceutical 819 compounds: Springer. p. 428.

Suhonen, M.T., Bouwstra, J.A. & Urtti, A. 1999. Chemical enhancement of percutaneous absorption in relation to stratum corneum structural alterations. *Journal of control release,* 59(2): 149-161.

Sutradhar, K.B. & Amin, L. 2013. Nanoemulsion: increasing possibilities drug delivery. *European journal of nanomedicine*, 5(2):97-110.

Therapeutic goods administration. 2013 Australian public assessment report for pitavastatin. https://www.tga.gov.au/sites/default/files/auspar-pitavastatin-130902.docx Date of access: 15 Feb. 2019.

TIBCO STATISTICA (data analysis software system), 2018. version 13.3 .

Thomas, S., Vieira, C.S., Hass, M.A. & Lopes, L.B. 2014. Stability, cutaneous delivery and antioxidant potential of a lipoic acid and α-tocopherol co-drug incorporated in microemulsions. *Journal of pharmaceutical scienc*es, 103:2530-2538.

Van Zyl, L., Du Preez, J., Gerber, M., Du Plessis, J. & Viljoen, J. 2016. Essential Fatty Acids as Transdermal Penetration Enhancers. *Journal of pharmaceutical sciences*, 105:188-193.

Venus, M., Waterman, J. & McNab, I. 2011. Basic physiology of the skin. *Surgery* (Oxford), 29:471-474.

Weigand, D.A., Haygood, C. & Gaylor, J.R. 1974. Cell layer and density of Negro and Caucasian stratum corneum. *Journal of investigative dermatology*, 62(6):563-568.

Weiss, S.C. 2011. Conventional topical delivery systems. *Dermatologic therapy*, 24: 471-474.

Wenlock, M.C., Potter, T., Barton, P., Austin,R.P. 2011. A method for measuring the lipophilicity of compounds in mixtures. *Journal of biomolecular screening*, 16(3):348-355.

Williams, A.C. 2003. Structure and function of human skin. (In Williams, A.C., *ed*. Transdermal and topical drug delivery: from theory to clinical practice. London: Pharmaceutical Press. p. 1-26).

Williams, A.C. 2013. Topical and transdermal drug delivery. (In Aulton, M.E., *ed*. Aultons pharmaceutics: the design and manufacture of medicines. 3rd ed. London: Churchill Livingstone. p. 675-697).

Williams, A.C. & Barry, B.W., 2012. Penetration enhancers. *Advanced drug Delivery, 64*:128-137.

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

Wilbur, R.L. 2017. The Difference Between Topical and Transdermal Medications.

Wong, S., Chen, Y. & Wu, L. 2008. Development of Predictive Quantitive retention- activity relationship models of HMG-CoA reductase inhibitors by biopartitioning micellar chromatography, *Journal of pharmaceutical and biomedical analysis*, 46:243-249.

Yoshida, M., Oliveira, M., Gomes, E., Mussel, W., Castro, W. & Soares, C. 2011. Thermal characterization of lovastatin in pharmaceutical formulations. *Journal of thermal analysis and calorimetry*, 106(3):657-6.

APPENDIX E

CYTOTOXICITY STUDIES OF RESPECTIVE STATIN AND GRAPESEED OIL INFUSED O/W NANO-EMULSIONS

E.1 Introduction

During pre-clinical development of novel drug delivery systems, it is crucial to determine whether the API, and even more so, the formulation containing the API, presents with any toxic effects towards humans or human tissue (Astashkina *et al*, 2012). Various cytotoxicity studies base cell viability measurements on the integrity of the cell membrane; whether it remains intact or it is destroyed after a predetermined treatment period (Niles *et al*., 2009; Riss *et al*., 2011). Cytotoxicity studies are a crucial factor in pharmaceutical and cosmetic product development and therefore, only low toxicity levels are acceptable (McGaw *et al*., 2014). According to Riss *et al*. (2013), all the currently available cell culture-based toxicity assays aim to determine whether the test formulation presents with possible cytotoxic effects and if it would be safe to use topically on humans (McGaw *et al*., 2014). Cytotoxicity studies have become useful in the preclinical screening of new therapeutic agents (Van Tonder *et al*., 2015), seeing as unsuitable compounds can be eliminated before proceeding to expensive clinical testing.

In vitro cytotoxicity studies can be expressed as the response of a cell to its external factors (ATCC, 2011), ultimately determining the growth, viability and/or proliferation of a cell (ATCC, 2011; Li *et al*., 2015). In this study, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and NR-assays were performed to assess if the formulations presented with any cytotoxic effects.

The MTT-assay measures cellular metabolic activity as a proxy for cell viability in the post-exposure phase of investigations. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes, which reduce the MTT (yellowish aqueous solution) reagent to formazan crystals (an insoluble product with a deep purple colour) (Stockert *et al*., 2012). Dark purple solutions indicate a high number of viable, metabolically active cells (Stockert *et al*., 2012). The formazan crystals are unable to cross cell barriers; thus remaining in the undamaged cells (Fotakis & Timbrell, 2006).

The NR-assay can determine instant toxic effects of relatively high concentrations of test substances on cells (Zuang, 2001). The principle of this assay is based on the uptake of NR-dye by viable cells through active transport and the incorporation of the dye into the lysosomes (Fautz *et al*., 1991; Repetto *et al*., 2008; Zuang, 2001). The incorporated dye can be released under

acidified conditions. Thus, drug cytotoxicity can be determined by analysing the amount of released dye (Zuang, 2001).

By using more than one assay for *in vitro* cytotoxicity screening, the reliability of the results obtained are drastically increased. For example, by using the NR-assay in combination with other assays, it is a useful guideline to distinguish between cytotoxicity and organelle damage (Fotakis & Timbrell, 2006).

E.2 Study objective

The main focus of this study was to investigate the cytotoxicity effects of each selected statin (atorvastatin, mevastatin, pitavastatin and pravastatin) as a nano-emulsion (**NE1**) containing 2.0% (*w/w*) of each selected statin, 7.9% (*w/w*) Tween® 80 and Span® 60 and 7.9% (*w/w*) grapeseed oil; as well as pure statin stock solutions (**SS1s**). Since, Tween® 80 and Span® 60 excipients used in the formulations have already been tested in previous studies (Marais, 2018; Maree, 2018), it was not included in this study. Due to the viscous nature of the **NG1s**, it was not assessed. The HaCaT cell line (between passage 24 and 31, which indicates the number of times a cell culture has been subcultured) was used for the *in vitro* investigations due to its close similarities to that of normal keratinocytes (Van de Sandt *et al*., 1999), making it ideal for transdermal toxicity work. The IC_{50} of the formulations, which is expressed as the concentration of drug that inhibits 50% of the cell viability, was determined (Roesler *et al*., 2010). After considering the skin diffusion studies in Section D.3.2, it was evident that **NG-PIT1** in comparison to the other **NEs** and **NGs** obtained the highest percentage diffused (0.031%) from the 2 g per 100 ml (2%) **NG**. Therefore, it was appropriate to test different concentrations of the APIs with nano-formulations during the cytotoxicity studies.

E.3 Statistical data-analysis and processing

Statistically significant differences ($p < 0.05$) were determined between treatment groups and control groups. A Brown-Forsythe test was performed to test the homogeneity of the data (Roth, 1983). An ANOVA, followed by the Tukey's HSD test was also performed (Liu, 2015). The %cell inhibition was calculated by subtracting the concentration of viable cells from 100%. A regression analysis was then performed to calculate the concentration of each treatment (x-value), where 50% of the cells were inhibited (y-value) by using the slope (m) and intercept (c) (Kumar & Sandhya, 2014). STATISTICA® 13.3 (StatSoft, TIBCO® Software Inc., CA, U.S.A.) was utilised for the statistical data analysis of the toxicity screening results. Additionally, the cell culture assays were performed in triplicate and the experiments were done in duplicate.

E.4 *In vitro* toxicity investigation

E.4.1 Experimental procedure

In vitro toxicity experiments, such as cell cultivation, seeding and cell line treatment was performed with the assistance of Dr C Willers under aseptic conditions in the Mammalian Cell Culture Laboratory (LAMB) at the North-West University (NWU), Potchefstroom Campus (RSA).

E.4.2 Materials

Table E.1 presents the reagents, catalogue numbers and suppliers used during the cytotoxicity studies.

Table E.1: Reagents used during *in vitro* cytotoxicity studies

Reagent	Catalogue number	Supplier
DMEM (Dulbecco's Modified Eagle's Medium), high glucose (4 500 mg/l), L-glutamine (4.0 mM) and sodium pyruvate	SH30243.01	HyClone™
Non-essential amino acid (NEAA) (100X)	M7145	Sigma-Aldrich®
Penicillin/streptomycin (Pen/Strep) (10 000 U/ml)	P4333	Sigma-Aldrich®
L-glutamine (200 mM)	BE17-605E	Lonza™
Foetal Bovine Serum (FBS)	10270106	Gibco™
Trypan Blue solution (0.4%)	SV30084.01	HyClone™
Phosphate buffered saline (1X)	SH30256.01	HyClone™
MTT (methylthiazol tetrazolium)	M5655	Sigma-Aldrich®
Trypsin-Versene® (EDTA) (1X)	BE17-161E	Lonza™
DMSO (dimethyl sulfoxide)	D5879	Sigma-Aldrich®
NRS (Neutral Red solution; 0.33%)	N2889	Sigma-Aldrich®
96-Well Cell Culture Cluster plate	3597	Corning Costar®

E.4.3 Cell line selection

It is imperative to choose the most appropriate cell line with regards to transdermal drug delivery. Therefore, selection criteria that needs to be considered are cell species (type), growth conditions, characteristics, normal or transformed cell lines, etc. (Gibco®, 2016). HaCaT cells, a spontaneously immortalised human keratinocyte line, was selected for the study objectives. Keratinocytes are the main cell types present in human skin (López-García *et al*., 2014) and still possesses proliferative epidermal characteristics (López-García *et al*., 2014). Thus, the HaCaT cell line was the most appropriate for the *in vitro* cytotoxicity studies.

E.4.4 Subculturing

The first step regarding subculturing is to detach the cells from the surface of the primary culture vessel via trypsinisation (Ricardo & Phelan, 2008). Trypsinisation can be defined as the process of cell dissociation by utilising trypsin, a proteolytic enzyme, which breaks down proteins to dissociate adherent cells from the vessel in which they are being cultured (Brown *et al.*, 2007). Trypsinisation is used in this study to transfer cells to a new vessel, which allows cells to multiply in ideal conditions.

Ethanol (70%) was used during the study to sterilise the work surface in the laminar flow hood at all times, which included all the items that were placed within it as well. DMEM and phosphate buffered saline were preheated at 37 °C prior to use. HaCaT cells were retained in high glucose DMEM enriched with 10% FBS, 1% NEAA, L-glutamine (2.0 mM) and 1% Pen/Strep in flasks incubated at 37 °C with 5% CO_2 and humidified air.

A pipette was utilised to remove the growth media from the incubated HaCaT cell flask. The flask containing the HaCaT cells was then rinsed twice with 10 ml preheated phosphate buffered saline. Precisely 3 ml of the Trypsin-Versene® was carefully added to the flask to detach the cells from the surface area. The flask was closed and equal distribution of Trypsin-Versene® was ensured before it was transferred to a CO_2 humidified incubator. For approximately 9 min, the flask was incubated (37 °C with 5% CO_2), followed by a forceful, yet short and steady hit on the side to detach the cells. A volume of 6 ml preheated growth medium was added to the flask to neutralise the trypsin solution and to disperse the cells. The medium turned cloudy after aspiration of dispersed cells, which is considered normal (Ricardo & Phelan, 2008). Cells were efficiently pipetted with the use of a serological pipette. Afterwards, the cell suspension was transferred to a 15 ml centrifuge tube, placed within the centrifuge and spun at 140 *x g* for 5 min. The obtained pellet was re-suspended in 5 ml growth medium, after removing the supernatant. The suspension was divided at a ratio of 1:10 into new flasks to ensure continuous cell growth.

E.4.5 Cell viability determination

Before seeding could be initiated for the MTT- and NR-assays, the amount of viable cells within the cell culture flask had to be established. Trypan blue was used to determine the number of viable cells by utilising a haemocytometer (Figure E.1) (Cadena-Herrera *et al*., 2015). Trypan blue is not absorbed by viable cells and therefore, viable cells appear bright. On the other hand, dead cells' membranes are not intact and the dye penetrates the cell, resulting in a dark stained cell (Sigma, 2018).

For the counting solution, 25 µl of Trypan Blue solution (0.4%) was mixed with 15 µl phosphate buffered saline and 10 µl cell suspension. A small volume of 10 µl was extracted and slowly loaded (in duplicate) at the edge of the cover slip of the haemocytometer. The haemocytometer was then positioned and viewed under a microscope.

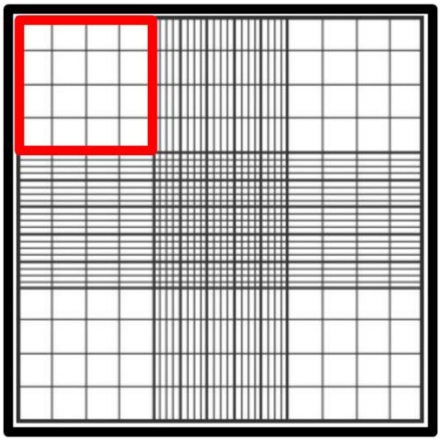


Figure E.1: A representation of a haemocytometer, indicating one of the sets of 16 squares that should be used for counting

The counting chamber is divided into nine large squares (on each side) with a total surface area of 1 mm^2 each. Viable cells in the centre square, as well as each corner square was counted under the microscope. The calculation of the total number of viable cells (per ml) in the suspension was determined as follow:

1. Only 5 squares per side of the counting chamber were counted.
2. The counted viable cells in the 10 squares were added together and divided by 2 to calculate the average of the two sides.
3. The average of the two sides were then divided by 5 to calculate the average cells per square.
4. The average cells per square was then multiplied with the dilution factor (5×10^4) to calculate the quantity of viable cells (per ml) of the cell suspension (C1).

Equation E.1 was used to determine the required dilution of the cell suspension for seeding a specific density.

$C_1V_1 = C_2V_2$ **Equation E.1**

Where:

C_1 = The initial cell concentration (viable cells/ml)

C_2 = Required cell concentration (75 000 viable cells/ml)

V_2 = Total volume of cell suspension needed

E.4.6 Seeding the experimental plates

HaCaT cells were seeded at a density of 15 000cells per well of a 96-well plate by using a reservoir and multichannel pipette. This density corresponds to 75 000 cells/ml, as a volume of 200 µl was used per well. Four 96-well plates were seeded in total for the various treatment groups for the MTT-assay, whereas three plates were seeded for the NR-assay. Table E.2 provides an example of how much cell suspension was used for seeding the various plates.

Table E.2: The volume of cell suspension diluted with growth medium for MTT- and NR-assay plates

MTT (x 4 plates)		NR (x 2 plates)	
Cell suspension (ml)	**Growth medium (ml)**	**Cell suspension (ml)**	**Growth medium (ml)**
1.127	30.273	1.436	29.964

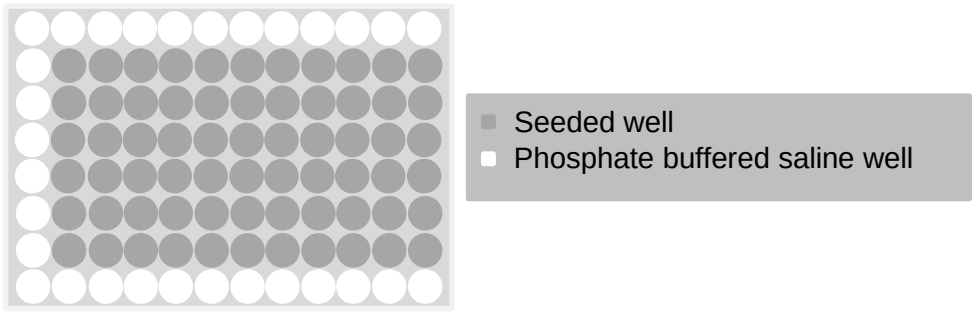


Figure E.2: A representation of a 96-well plate indicating the seeded wells

After the plates were incubated at 37 °C (5% CO_2, 95% humidity) for 24 h, exposure or treatment of cells was initiated for both MTT- and NR-assays and this was regarded as time 0 h.

E.4.7 Treatment groups

The **NE1** stock solution was formulated as discussed in Section B.5. A volume of 0.2 ml of each **NE1** was diluted with 9.8 ml supplemented DMEM and used in this study.

The statin stock solution was prepared by weighing 400 mg of each selected statin. These statins were then transferred into a volumetric flask and diluted with 100.0 ml methanol. The API was properly dissolved in methanol and 0.3 ml of each solution was made up to a volume of 10.0 ml with enriched DMEM.

Table E.3: Stock solution codes

Nano-emulsion stock solution (NE1)		Statin stock solution (SS1)	
Statin	**Code**	**Statin**	**Code**
Atorvastatin	**NE-ATO1**	Atorvastatin	**SS-ATO1**
Mevastatin	**NE-MEV1**	Mevastatin	**SS-MEV1**
Pitavastatin	**NE-PIT1**	Pitavastatin	**SS-PIT1**
Pravastatin	**NE-PRA1**	Pravastatin	**SS-PRA1**
Placebo	**NE-PLAS1**		

Five different concentrations of both the **NE1s** and **SS1s** were loaded onto the HaCaT cells. Volumes (µl) applied to the wells were calculated by means of the concentration of the APIs present in the **NE1** and **SS1**. A specific amount of supplemented DMEM was also added to each of the wells, respectively, to make up a final diluted volume of 200 µl per well as presented in Table E.4.

Table E.4: Test concentrations per well for both MTT- and NR-assays

NE1		SS1	
Volume NE1s	**Volume DMEM**	**Volume SS1s**	**Volume DMEM**
0.00250% (12.5 µl)	187.5 µl	0.00075% (12.5 µl)	187.5 µl
0.00500% (25.0 µl)	175.0 µl	0.00150% (25.0 µl)	175.0 µl
0.01000% (50.0 µl)	150.0 µl	0.00300% (50.0 µl)	150.0 µl
0.02000% (100.0 µl)	100.0 µl	0.00600% (100.0 µl)	100.0 µl
0.04000% (200.0 µl)	No extra DMEM added	0.01200% (200.0 µl)	No extra DMEM added
200 µl per well		200 µl per well	

The **NE1s** with or without statins were tested in concentrations ranging from 0.00250% (25.0 µg/ml) to 0.04000% (400.0 µg/ml) in a two-fold serial dilution range (with supplemented DMEM). Each API (atorvastatin, mevastatin, pitavastatin and pravastatin) was dissolved in 100.00000% methanol and tested alone in a two-fold serial dilution (with supplemented DMEM) ranging from 0.00075% (7.5 µg/ml) to 0.01200% (120.0 µg/ml). It can also be stated that the concentration of methanol, which the cells were exposed to, never exceeded 3.00000% (*v/v*).

The cells were exposed to the **NE1s** and **SS1s** for a 12 h period in the incubator at 37 °C (5% CO_2, 95% humidity). The 12 h period of exposure correlates with the skin diffusion studies in Section D.2.2.4.3.

E.5 *In vitro* cytotoxicity testing

E.5.1 Methylthiazol tetrazolium assay

MTT-assays determine cell viability by means of the formation of insoluble, purple formazan. The principle of this assay is the fact that the mitochondria in active cells have dehydrogenase enzymes, which convert soluble MTT into insoluble purple formazan (Stockert *et al*., 2012). Damaged cells cannot metabolise MTT to purple formazan (Fotakis & Timbrell, 2006).

Table E.5: Calculating the volume of MTT-solution required for the plates

Number of plates	Wells used in plates	Volume needed for plates (ml)	Extra volume (ml)	Total volume MTT-solution needed (ml)
4	264 wells	26.4	1.6	28.0

The calculated volume of assay media was consequently pipetted into a 50 ml tube. The amount of MTT that had to be weighed and added to the assay media was calculated, seeing as the MTT-solution should have a concentration of 0.5 mg/ml (Riss *et al*., 2016). Therefore, the amount of MTT was determined according to Equation E.2.

MTT (mg) = Total volume (ml) x 0.5 mg/ml **Equation E.2**

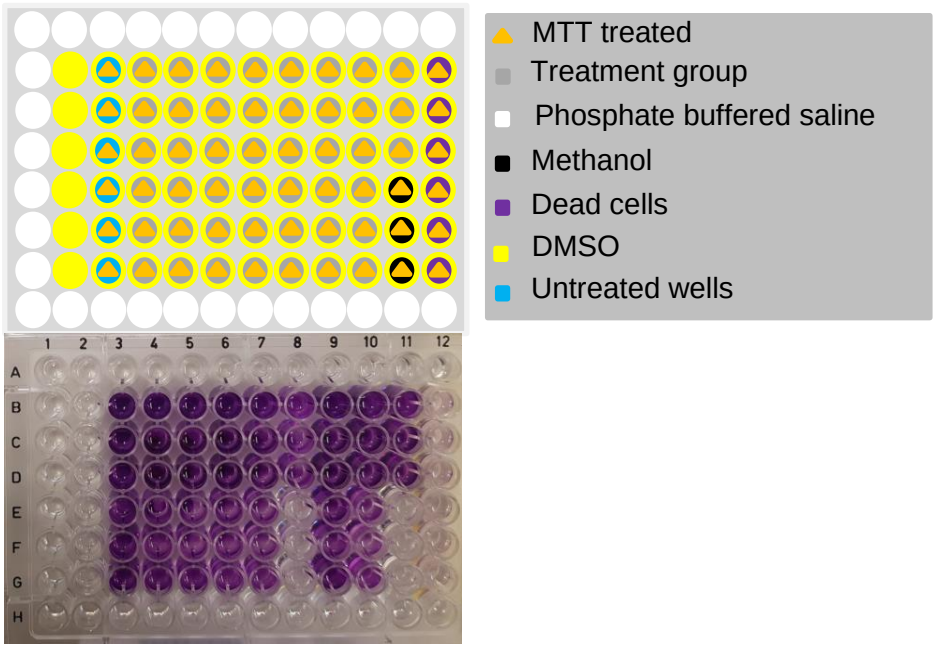


Figure E.3: The final representation of a 96-well plate layout for MTT-assays, as well as the purple formazan formation after an incubation period

An acceptable amount of 14 mg was calculated for the four 96-well plates. MTT was placed in a 50 ml centrifuge tube and dissolved in the 28 ml of non-supplemented DMEM. The MTT-solution

was covered with foil, as it is light sensitive, and kept in a water bath at 37 °C. This solution was prepared fresh. The four plates were removed from the incubator and the cells were visually inspected under a microscope. Figure E.3 displays the final layout of an MTT plate.

Growth medium from the untreated wells was removed and the dead cells were executed with 200 µl of 100% methanol for a period of ± 15 min. The growth medium was removed from the remaining wells, and cells were gently rinsed twice with 100 µl phosphate buffered saline. The phosphate buffered saline was removed from the wells and a volume of 100 µl MTT-solution was added into each well, including the untreated wells. The 96-well plates containing MTT-solution were incubated for 2 h in the CO_2 incubator under standard growth conditions. The plates were covered with aluminium foil to prevent light exposure. Afterwards, the 96-well plates were removed from the incubator and the cells were visually inspected under a microscope to establish whether formazan crystals had formed. The MTT-solution was aspirated from the 96-well plates and a volume of 200 µl DMSO was added to each well. The plates were placed on a shaker for 15 – 30 min to dissolve the purple formazan crystals. Lastly, the absorbance was measured at 560 nm with a background signal of 630 nm with a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) analysed with SoftMax® Pro 6.2.1 Software. Equation E.3 was used to calculate the %viable cells.

$$\%\text{viable cells} = \frac{((\text{sample absorbance 560 nm - 630 nm}) - (\text{blank absorbance 560 nm - 630 nm})}{(\text{Untreated absorbance 560 nm - 630 nm - blank absorbance 560 nm - 630 nm})} \times 100$$ **Equation E.3**

E.5.1.1 MTT-colorimetric assay results and discussion

The cytotoxic effect of a specific treatment can be categorised according to cell viability percentages (López-García *et al*., 2014) as illustrated in Table E.6.

Table E.6: %Cell viability used to classify treatment cytotoxicity in this study (López-García *et al*., 2014)

%Cell viability	Cytotoxicity
< 40	Strong
40 – 60	Moderate
60 – 80	Weak
> 80	None

Tables E.7 – E.8, along with Figures E.4 – E.5, illustrated the obtained results after exposure of each statin (**SS1s**) at the five respective concentration ranges, together with the **NE1s** of the respective statins. Control wells were left untreated to serve as the control group. The MTT %cell viability of the HaCaT cell line after 12 h of treatment was measured and will be discussed in Sections E.5.1.1.1 to E.5.1.1.2.

E.5.1.1.1 Cell viability of HaCaT cells after treatment with SS1s determined with MTT-assay

Table E.7: The cell viability (%) of the HaCaT cells treated with **SS1s** at different concentrations during the MTT-assay

%Cell viability (%)						
Concentration utilised for exposure	**SS-ATO1**	**SS-MEV1**	**SS-PIT1**	**SS-PRA1**	**5% Methanol**	**Control**
0.00075% (0.0075 mg/ml)	89.720 (± 2.165)	110.795 (± 7.987)	88.074 (± 19.516)	102.980 (±6.643)	113.111 (± 9.977)	100.000
0.00150% (0.0150 mg/ml)	99.067 (± 3.887)	90.967 (± 0.498)	88.458 (± 0.952)	98.608 (±4.733)		
0.00300% (0.0300 mg/ml)	94.489 (± 7.621)	75.721 (± 13.321)	84.031 (± 5.934)	95.960 (±8.378)		
0.00600% (0.0600 mg/ml)	90.301 (± 3.452)	56.664 (± 11.474)	85.660 (± 5.087)	102.917 (±4.466)		
0.01200% (0.1200 mg/ml)	63.236 (± 3.518)	51.436 (± 12.520)	71.483 (± 5.120)	91.166 (±5.006)		

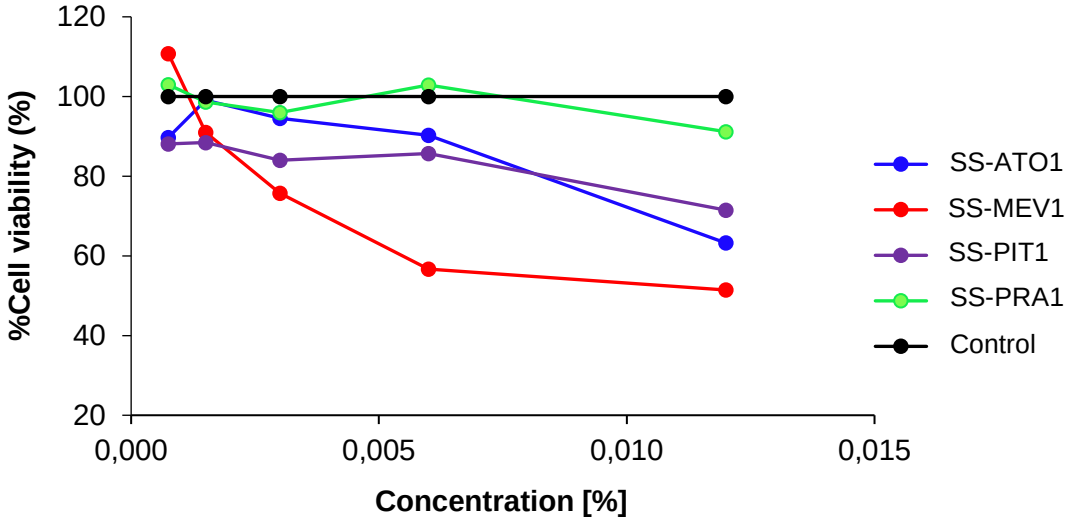


Figure E.4: The %cell viability after treatment with the five respective concentrations of **SS-ATO1**, **SS-MEV1**, **SS-PIT1** and **SS-PRA1** during the MTT-assay

From Table E.7 and Figure E.4, it was evident that higher concentrations of the selected statins decreased the cell viability. There were no significant differences between the **SS1s** groups at each specific concentration. Nonetheless, the following was observed:

- **SS-ATO1** at concentrations of 0.0075 – 0.0600 mg/ml had a non-cytotoxic effect on the cells, as the cell viability was higher than 80%, but when the concentrations were higher (0.1200 mg/ml), the cytotoxic effect was weak, as the cell viability was between 60 – 80%.
- **SS-MEV1** at concentrations of 0.0075 – 0.0150 mg/ml were non-cytotoxic (> 80%); when concentrations were increased to 0.0300 mg/ml, the cytotoxic effect was weak (60 – 80%). However, when concentrations were increased (0.0600 – 0.1200 mg/ml), the cytotoxic effect was moderate (40 – 60%).
- **SS-PIT1** at concentrations of 0.0075 – 0.0600 mg/ml posed as non-cytotoxic (> 80%); however, when the concentrations were higher (0.01200 mg/ml), the cytotoxic effect was weak, as the cell viability (71.483%) was still above 60%.
- **SS-PRA1** at concentrations of 0.0075 – 0.1200 mg/ml was non-cytotoxic (> 80%) throughout the experiment.

E.5.1.1.2 Cell viability of HaCaT cells after treatment with NE1s determined with MTT-assay

The **NE1s** and **NE-PLAS1** were applied at different concentrations than the **SS1** during the MTT-assay, and presented with the following cell viability (%) results as seen in Table E.8 and Figure E.5.

Table E.8: Cell viability (%) of the HaCaT cells treated with the **NE1s** and **NE-PLAS1** concentration ranges during the MTT-assay

Cell viability (%)						
Concentration utilised for exposure	**NE-ATO1**	**NE-MEV1**	**NE-PIT1**	**NE-PRA1**	**NE-PLAS1**	**Control**
0.0025% (0.025 mg/ml)	65.351 (±19.016)★	96.317 (±14.150)	106.815 (±9.480)	100.477 (±8.123)	98.028 (±15.889)	100.000
0.0050% (0.050 mg/ml)	43.681 (±3.499)★	70.316 (±13.403)★	58.209 (±4.822)★	87.060 (±6.610)	88.121 (±4.547)	
0.0100% (0.100 mg/ml)	23.767 (±13.842)★	39.874 (±5.815)★	56.256 (±2.863)★	44.936 (±1.089)★	55.111 (±5.185)	
0.0200% (0.200 mg/ml)	18.531 (±6.732)★	22.903 (±4.564)★	21.239 (±2.412)★	19.763 (±2.392)★	40.023 (±1.634)	
0.0400% (0.400 mg/ml)	4.191 (±3.840)★	8.043 (±3.161)★	1.050 (±0.100)★	11.389 (± 0.456)★	23.800 (±1.180)	

★ (**NE1**) Concentrations that differ significantly from the control group, as determined with Tukey's HSD test ($p < 0.05$)

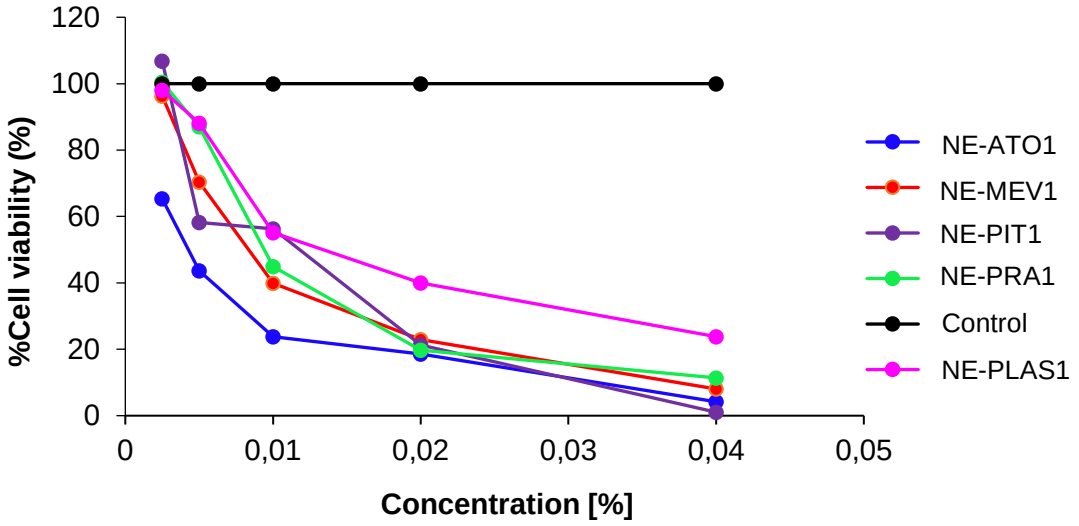


Figure E.5: The %cell viability after treatment with the five respective concentrations of **NE-ATO1**, **NE-MEV1**, **NE-PIT1**, **NE-PRA1** and **NE-PLAS1** during the MTT-assay

Table E.8 and Figure E.5 clearly indicated the 12 h exposure of the **NE1s** and **NE-PLAS1**, and managed to obtain a concentration-dependant effect on HaCaT cells. It was evident that higher concentrations of the selected statins also reduced cell viability. The following was observed regarding MTT-treatment:

- **NE-ATO1** at a concentration of 0.025 mg/ml had a weak cytotoxic effect on the cells, as the cell viability was between 60 and 80%, when the concentration was elevated to 0.050 mg/ml, the cytotoxic effect was moderate (40 – 60%). The cytotoxicity was strong (< 40%) at concentrations of 0.100 – 0.400 mg/ml, as the cell viability ranged between 4.191 – 23.767%.
- **NE-MEV1** at a concentration of 0.025 mg/ml was non-cytotoxic (> 80%); however, when the concentration was increased to 0.050 mg/ml, the cytotoxic effect was weak (60 – 80%). At concentrations of 0.100 – 0.400 mg/ml, the cytotoxic effect was strong, since the cell viability (8.043 – 39.874%) was below 40%.
- **NE-PIT1** at a concentration of 0.025 mg/ml were non-cytotoxic (> 80%), but when the concentration was higher (0.050 – 0.100 mg/ml), the cytotoxic effect was moderate (40 – 60%). It became strong cytotoxic to cells at concentrations of 0.200 – 0.400 mg/ml due to the cell viability (1.050 – 21.239%) being < 40%.
- **NE-PRA1** at concentrations of 0.025 – 0.050 mg/ml were non-cytotoxic (> 80%), although, during higher concentrations (0.100 mg/ml) the cytotoxic effect posed as moderate (40 – 60%) and at a concentration of 0.200 – 0.400 mg/ml, the cytotoxic effect on the cell viability (11.389 – 19.763%) was strong, as it was below 40%.

- **NE-PLAS1** at concentrations of 0.025 – 0.050 mg/ml posed as non-cytotoxic, as the cell viability was greater than 80%, while concentrations at 0.100 – 0.200 mg/ml had a moderate (40 – 60%) cytotoxic effect. At the concentrations of 0.400 mg/ml, it had a strong cytotoxic effect (< 40%) on the cell viability (23.800%).

Figure E.5 indicated that **NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1** had cytotoxic effects on the cells at the strongest concentrations.

E.5.1.1.3 IC_{50} values obtained from the MTT-results

The IC_{50} value describes the concentration of a drug or substance that leads to a 50% inhibition in cell viability (Koba *et al*., 2009). In this study, the IC_{50} values were determined via regression analysis (Shaikh *et al*., 2012). Regression analysis was performed from the %inhibited cells (y-value) and the concentration range (x-value) to obtain the slope and intercept, which was utilised to determine the IC_{50} values. The IC_{50} values of **SS1s**, **NE1s** and **NE-PLAS1** were calculated by firstly subtracting the %viable cells from 100 (initial concentration viable cells). This calculation was applied to each treatment concentration. A linear line graph was plotted, where (y) was set as 50 (50% inhibition), (m) represented the slope, (c) the intercept to calculate (x), the IC_{50} value (Kumar & Sandhya, 2014). Table E.9 represent the IC_{50} values obtained after a 12 h exposure to the **NE1s** and **SS1s** samples.

$$y = mx + c \qquad \textbf{Equation E.4}$$

Table E.9: Calculated IC_{50} values for **SS1s** and **NE1** determined from the MTT-assay results

Stock solutions	Compounds	IC_{50} values (mg/ml)
SS1s	**SS-ATO1**	0.182
	SS-MEV1	0.105
	SS-PIT1	0.281
	SS-PRA1	0.704
NE1s	**NE-ATO1**	0.015
	NE-MEV1	0.143
	NE-PIT1	0.150
	NE-PRA1	0.167
	NE-PLAS1	0.214

From Table E.9, the IC_{50} values (mg/ml) for **SS1s** indicated that **SS-MEV1** inhibited the HaCaT cells at the lowest concentration (0.105 mg/ml); therefore, it was the most toxic API to the cells, in comparison to the other statins. **SS-PRA1** showed the lowest toxicity with an IC_{50} value of 0.704 mg/ml.

When the **NE1s** and **NE-PLAS1** were compared, the **NE-PLAS1** provided the lowest toxicity, as it presented with the highest IC_{50} value (0.214 mg/ml). The **NE-ATO1** displayed the lowest IC_{50} value (0.015 mg/ml), representing the highest cytotoxicity; followed by **NE-MEV1** (0.143 mg/ml), **NE-PIT1** (0.150 mg/ml) and lastly, **NE-PRA1** (0.167 mg/ml), which displayed with the highest IC_{50} value among the statins and thus, the lowest toxicity. The order of IC_{50} values of statins formulated in the **NE1s** differed from the **SS1s**; therefore, Tween® 80, Span® 60 and grapeseed oil could have played a role. This may be due to the viscosity level of these constituents, as cells cannot fulfil proper gas diffusion through an oil-based layer.

E.5.2 Neutral red

The NR-assay is uncomplicated, precise and the outcomes are reproducible. The main principle of NR can be described by the fact that viable cells absorb the red dye via active transport into the lysosomes (Miller *et al*., 2015). This phenomenon will not occur in non-viable cells; therefore, they will not incorporate any red dye (Mamaca *et al*., 2005). In this study, it can be concluded that the amount of red dye included in the cells specifies the level of cytotoxicity produced by the added samples.

Table E.10: Calculating the volume of NRS required for the plates

Number of plates	Wells used in plates	Volume needed for plates (ml)	Extra volume (ml)	Total volume NRS needed (ml)
3	198 wells	19.8	0.2	20.0

The 0.33% NR-concentrate was diluted to obtain a 10% dilution to the final volume needed (Sigma, 2018). The total volume NRS (20 ml) was calculated by using Equation E.5. Therefore, the amount of NRS required to obtain the final 20 ml assay solution to be used on the three plates could be determined with Equation E.5.

Amount NRS = Total amount x 10% **Equation E.5**

From Equation E.5, it can be determined that the total amount of NRS (20 ml) should contain 2 ml NR-stock and 18 ml DMEM (non-additive). The NRS was prepared a day before the study commenced. The three 96-well plates were incubated overnight at 37 °C and removed to inspect the cells visually under a microscope.

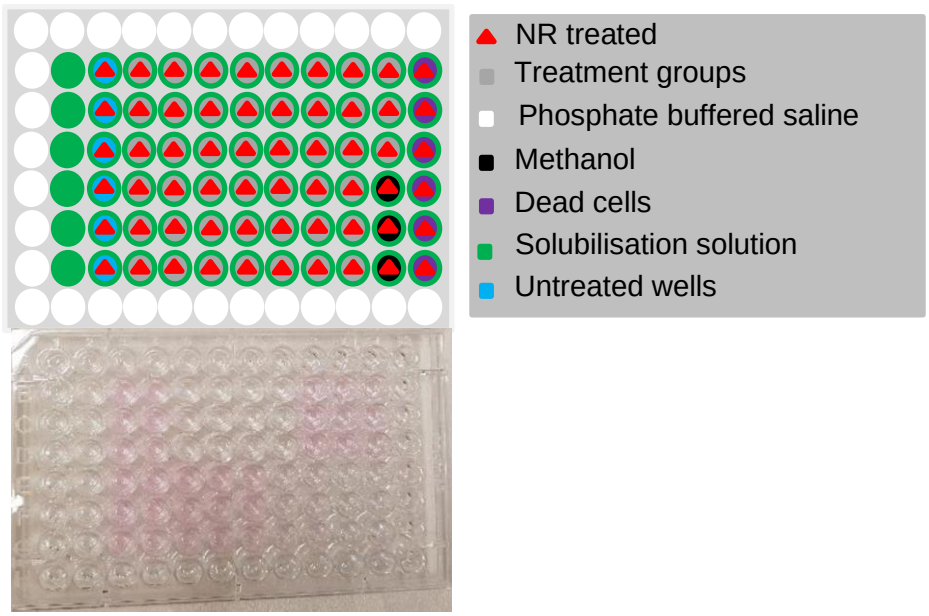


Figure E.6: The final representation of a 96-well plate intended for the NR-assay

The growth medium was extracted from the dead control wells and executed with 200 µl of 100% methanol for a period of ± 15 min. Spent growth medium was then aspirated from the treatment groups and gently rinsed with 150 µl phosphate buffered saline. NRS was added to the wells containing the HaCaT cells. The plates were then incubated for 2 h in the incubator at 37 °C (5% CO_2, 95% humidity) under standard growing conditions. They were then removed from the incubator and NRS aspirated. The cells were then fixed with fixative (0.1% $CaCl_2$ in 0.5% formaldehyde), which were also aspirated. Then 200 µl of NR-assay solubilisation solution (1% acetic acid in 50% ethanol) was added to each well to solubilise the incorporated dye. The 96-well plates were then placed on a gyratory shaker at room temperature for 15 min to ensure proper mixing. Finally, absorbance was measured at 540 nm with a background of 690 nm with a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) analysed with SoftMax® Pro 6.2.1 Software. The cell viability was calculated by Equations E.2 and E.3 in (Section E.5.1).

E.5.2.1 NR-colorimetric assay results and discussion

The cytotoxic effect of the treatments was categorised according to the %cell viability parameters (see Section E.5.1.1) set out by López-García *et al*. (2014). To establish NR %cell viability of the HaCaT cell line, a SpectraMax® Paradigm® Multi-Mode Microplate reader was utilised. The intensity of the dye is directly proportional to the amount of living cells (Roesler *et al*., 2010).

E.5.2.1.1 Cell viability of HaCaT cells after treatment with SS1s determined with NR-assay

SS1s were dosed in five different concentrations concerning the NR-assay and displayed the following cell viability (%) results as seen in Table E.11 and Figure E.7.

Table E.11: The cell viability (%) of the HaCaT cells treated with the **SS1** concentrations ranges during the NR-assay

%Cell viability (%)						
Concentration utilised for exposure	**SS-ATO1**	**SS-MEV1**	**SS-PIT1**	**SS-PRA1**	**5% Methanol**	**Control**
0.00075% (0.0075 mg/ml)	101.695 (±8.339)	89.632 (±5.754)	95.745 (±6.234)	101.670 (±6.093)	76.666 (±3.855)	100.000
0.00150% (0.0150 mg/ml)	99.794 (±5.467)	88.040 (±4.351)	90.626 (±4.769)	103.460 (±3.597)		
0.00300% (0.0300 mg/ml)	77.365 (±9.659)★	81.697 (±3.502)★	87.402 (±5.372)	102.685 (±6.246)		
0.00600% (0.0600 mg/ml)	59.404 (±5.021)★	54.313 (±8.899)★	73.448 (±3.545)★	97.870 (±1.025)		
0.01200% (0.1200 mg/ml)	42.291 (±9.778)★	12.650 (±7.331)★	55.004 (±6.158)★	62.771 (±7.925)★		

★ (**NE1**) Concentrations that differ significantly from the control group determined with Tukey's HSD test ($p < 0.05$)

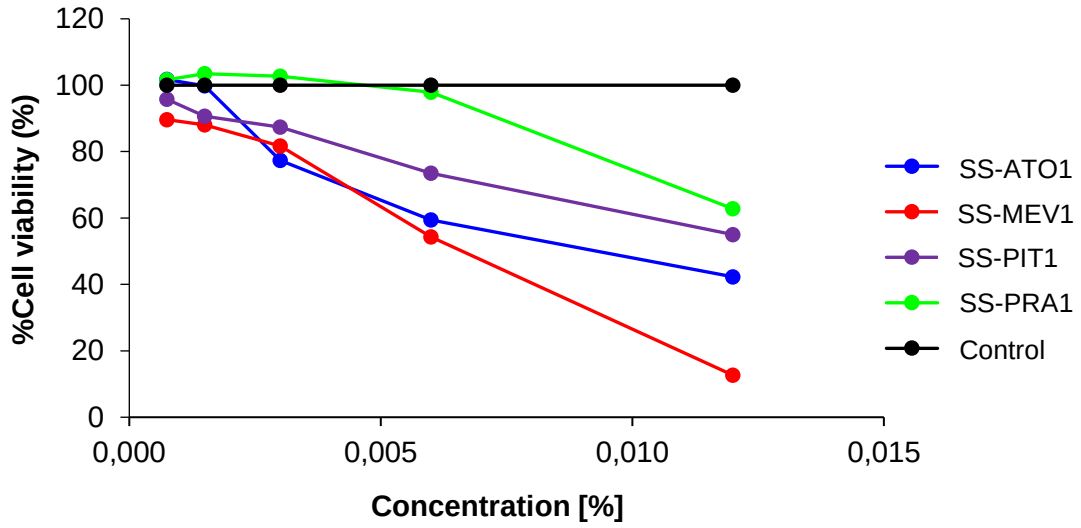


Figure E.7: The %cell viability after treatment with the five respective concentrations of **SS-ATO1**, **SS-MEV1**, **SS-PIT1** and **SS-PRA1** during the NR-assay

From Table E.11 and Figure E.7, the cell viability (%) of the **SS1s** dosed in different concentrations was compared to the parameters of cell viability (López-García *et al*., 2014) when treated with NR and the following was revealed:

- **SS-ATO1** at concentrations of 0.0075 – 0.0150 mg/ml had a non-cytotoxic effect on the cells, as the cell viability was greater than 80%; although, when the concentration was higher (0.0300 mg/ml), the cytotoxic effect was weak (60 – 80%), as the cell viability was 77.365%. However, at a higher concentration (0.0600 – 0.1200 mg/ml), the cytotoxic effect was moderate (40 – 60%).
- **SS-MEV1** at concentrations of 0.0075 – 0.0300 mg/ml were non-cytotoxic (> 80%); hence, when the concentrations were increased to 0.0600 mg/ml, the cytotoxic effect was moderate, since the cell viability ranged between 40 and 60%. As the concentration increased to 0.1200 mg/ml, the cytotoxic effect was strong cytotoxic (< 40%).
- **SS-PIT1** at concentrations of 0.0075 – 0.0300 mg/ml were non-cytotoxic (> 80%); however, when concentrations were higher (0.0600 mg/ml), the cytotoxic effect was weak, as cell viability (73.448%) was between 60 and 80%. As the concentration increased (0.1200 mg/ml), the cytotoxic effect became moderate cytotoxic (40 – 60%).
- **SS-PRA1** at concentrations of 0.0075 – 0.0600 mg/ml were non-cytotoxic (> 80%); however, when concentrations were higher (0.1200 mg/ml), the cytotoxic effect was weak, as cell viability (62.771%) was between 60 and 80%.

E.5.2.1.2 Cell viability of HaCaT cells after treatment with NE1s determined with NR-assay

NE1s and **NE-PLAS1** were dosed in five different concentrations during the NR-assay and presented with the following cell viability (%) results as seen in Table E.12 and Figure E.8.

Table E.12: Cell viability (%) of the HaCaT cells treated with the **NE1s** and **NE-PLAS1** concentration ranges during the NR-assay

%Cell viability (%)						
Concentration utilised for exposure	**NE-ATO1**	**NE-MEV1**	**NE-PIT1**	**NE-PRA1**	**NE-PLAS1**	**Control**
0.0025% (0.025 mg/ml)	73.910 (± 3.920)	98.190 (± 8.827)	54.589 (± 4.224)★	73.542 (± 13.752)	76.014 (± 7.143)	100.000
0.0050% (0.050 mg/ml)	71.511 (±20.054)	85.109 (±18.943)	44.377 (± 2.727)★	66.019 (± 2.021)★	70.859 (± 9.004)	
0.0100% (0.100 mg/ml)	30.935 (±5.138)★	37.526 (±5.637)★	43.448 (± 10.038)★	27.183 (± 6.738)★	58.855 (± 3.203)★	
0.0200% (0.200 mg/ml)	20.859 (±5.933)★	14.914 (±4.048)★	11.217 (± 0.435)★	7.688 (± 5.450)★	30.905 (± 5.062)★	
0.0400% (0.400 mg/ml)	2.166 (±45.764)★	19.290 (±28.441)★	0.396 (± 7.577)★	0.706 (± 11.781)★	14.479 (± 18.401)	

★ (**NE1**) Concentrations that differ significantly from the control group determined with Tukey's HSD test ($p < 0.05$)

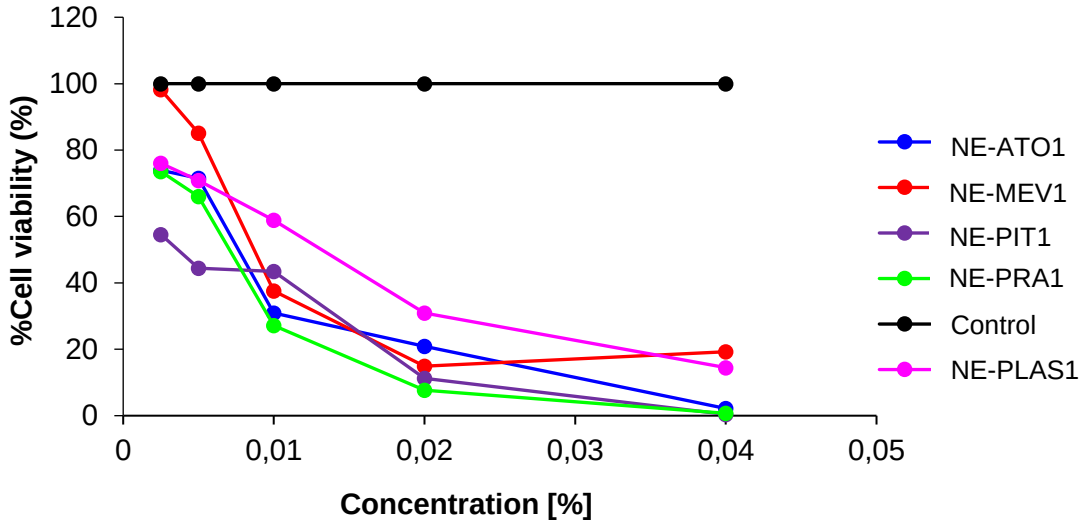


Figure E.8: The %cell viability after treatment with the five respective concentrations of **NE-ATO1**, **NE-MEV1**, **NE-PIT1**, **NE-PRA1** and **NE-PLAS1** during the NR-assay

From Table E.12 and Figure E.8, the cell viability (%) of the **NE1s** and the **NE-PLAS1** were dosed in five different concentrations that were compared to the parameters of cell viability (López-García *et al*., 2014). The following was identified when treated with NR:

- **NE-ATO1** at concentrations of 0.025 – 0.050 mg/ml, the cell viability was between 60 and 80% and therefore had a weak cytotoxic effect on the cells; but when the concentration was elevated to 0.100 – 0.400 mg/ml, the cytotoxic effect was strong (< 40%) and became cytotoxic, since the cell viability ranged between 2.166 and 30.935%.
- **NE-MEV1** at concentrations of 0.025 – 0.050 mg/ml was non-cytotoxic (> 80%); although, when the concentration was increased to 0.100 – 0.400 mg/ml, the cytotoxic effect became strongly (< 40%) cytotoxic (cell viability was 19.290 – 37.526%).
- **NE-PIT1** at concentrations of 0.025 – 0.100 mg/ml posed as moderate-cytotoxic effect (40 – 60%), when the concentration was increased (0.200 – 0.400 mg/ml), the cytotoxic effect had a strong cytotoxic effect on the cell viability (0.396 – 11.217%), as it was below 40%.
- **NE-PRA1** at concentrations of 0.025 – 0.050 mg/ml presented as weak-cytotoxic effect (60 – 80%); hence, during higher concentrations (0.100 – 0.400 mg/ml), the cytotoxic effect on the cell viability (0.706 – 27.183%) was strong, since it was below 40%.
- **NE-PLAS1** at concentrations of 0.025 – 0.050 mg/ml, the cytotoxic effect was weak, as cell viability (70.859 – 76.014%) was between 60 and 80%. As the concentration increased (0.100 mg/ml) the cytotoxic effect became moderate cytotoxic (40 – 60%).

However, during higher concentrations (0.200 – 0.400 mg/ml), the cytotoxic effect on the cell viability (18.401 – 30.905%) was strong, since it was below 40%.

Figure E.6 indicated that the damaged cells did not incorporate NR-dye. The viable cells that were treated with lower concentrations remained pink due to NR-dye being incorporated by the viable cells. However, **NE-ATO1**, **NE-MEV1**, **NE-PIT1** and **NE-PRA1** had cytotoxic effects on the cells at the most extreme concentrations.

E.5.2.1.3 IC_{50} values obtained from the NR-results

During the determination of IC_{50} values for the NR-assays, the calculation method utilised in NR is identical to the method used for the MTT-assays (Section E.5.1.1.3). Since, the IC_{50} value was utilised to establish the concentration of the drug that leads to 50% of inhibition of cells (Koba *et al*., 2009).

Table E.13: Calculated IC_{50} values for **SS1s** and **NE1** determined from the NR-assay results

Stock solutions	Compounds	IC_{50} values (mg/ml)
SS1s	**SS-ATO1**	0.095
	SS-MEV1	0.068
	SS-PIT1	0.132
	SS-PRA1	0.169
NE1s	**NE-ATO1**	0.075
	NE-MEV1	0.023
	NE-PIT1	0.101
	NE-PRA1	0.160
	NE-PLAS1	0.156

From Table E.13, the IC_{50} values (µg/ml) for **SS1s** indicated that **SS-MEV1** (0.068 mg/ml) and **SS-ATO1** (0.095 mg/ml) inhibited HaCaT cells at lower concentrations; therefore, it is evident that they presented as the most toxic to the cells compared to **SS-PIT1** (0.132 mg/ml) and **SS-PRA1** (0.169 mg/ml), which had the lowest toxicity and highest IC_{50} value.

The IC_{50} values (µg/ml) for **NE1s** indicated that **NE-PLAS1** (0.156 mg/ml) and **NE-PRA1** (0.160 mg/ml) obtained the lowest toxicity (0.156 mg/ml), since they presented with the highest IC_{50} value. The **NE1s**, **NE-MEV1** (0.023 mg/ml) was the most inhibitory towards cells and indicated the lowest IC_{50} value compared to **NE-PIT1** (0.101 mg/ml), **NE-ATO1** (0.075 mg/ml) and **NE-PRA1** (0.160 mg/ml). This data correlates with the IC_{50} values of the MTT-results, where **NE-PRA1** was also the lowest cytotoxic with the highest IC_{50} value.

E.6 Conclusion

The MTT- and NR-assays, together with the IC_{50} values, offered data as to whether the **SS1s**, **NE1s** and **NE-PLAS1** had cytotoxic effects on HaCaT cells (epidermal keratinocytes). The treatments were performed at five different concentrations, to establish whether there were any cytotoxic effects at these concentrations.

The MTT-assay is a secure and simple colorimetric method, where the mitochondria are proposed to be the main site of MTT reduction, numerous other reductases namely, intercellular reductases, has been overlooked by MTT reduction. In addition, as literature proposes that intracellularly reduced nicotinamide adenine dinucleotide (NADH) is associated to the major electron donor in MTT reduction. The enhanced concentrations of NADH may lead to inaccurate results (Jo *et al*., 2015). Therefore, as it is proposed to obtain more accurate and reliable cytotoxic results, other assays can be utilised to accompany the MTT-assay. Thus, MTT- and NR-assays were performed in this study, as the most general application of cytotoxicity is based on cell viability by measuring staining with these assays and the results of these are often comparable (Chiba *et al*., 1998). In this study, most results obtained from the MTT- and NR-assay were comparable.

The IC_{50} values from the MTT-results of **SS1s** revealed that **SS-MEV1** presented as the most toxic to the cells compared to **SS-ATO1**, **SS-PIT1** and **SS-PRA1**. This data correlated with the IC_{50} values obtained from the NR-assay for **SS1s**, which indicated that **SS-MEV1**, once again, presented with higher toxicity towards the cells than **SS-ATO1**, **SS-PIT1** and **SS-PRA1**. Both the MTT- and NR-assays indicated that the **NE1s** and **NE-PLAS1** revealed cytotoxic effects on the cells at high treatment concentrations; however, the **NE-PLAS1** indicated the lowest toxicity. The **NE-ATO1** had the lowest IC_{50} value opposed to **NE-MEV1**, **NE-PIT1** and **NE-PRA1**, which was indicative of high toxicity towards the cells. **SS-PRA1** was found the least cytotoxic by both the MTT- and NR-assay. The MTT- and NR-assays were greatly affected by the lipophilic formulations (Stockert *et al*., 2012); therefore, lipophilic statins, dispersions (**NE1** and **NE-PLAS1**) and lipophilic excipients could interfere with data and counter accurate results, especially in higher concentrations. These findings are in accordance with those of Marais (2018) and Maree (2018).

Mevastatin, the first statin to be developed (Endo, 2010), was discontinued in 1980 (Endo, 2010), following observations that it caused lymphoma in dogs that received very high doses of 100 and 200 mg/kg/day for 2 years. Importantly, no abnormalities were observed in the group receiving 20 mg/kg/day. Interestingly, mevastatin has been shown to be effective in patients with severe hypercholesterolemia at less than 1 mg/kg/day (Endo, 2010). However, to avoid any possible sequelae related to lymphoma, pravastatin was subsequently marketed at a significantly reduced dose of 25 mg/kg/day, its relative weak potency taken into account (Endo, 2010).

It can be concluded that the cytotoxic concentration of the statins was considerably higher than the concentration actually released from the formulation or that permeated into and through the skin during the membrane release and skin diffusion studies (Appendix D), respectively. Thus, these statins will have little to no side-effects on the site of application and toxicity would be kept to a minimum.

However, future *in vitro* studies using *in vitro* models with a better translation to *in vivo* models should be used to refine these findings. 3D cell cultures-based models, which mimic functions seen in human tissues are significantly better than cells grown using classical two-dimensional (2D) culture systems (Wrzesinski *et al.*, 2015).

References

American Type Culture Collection (ATCC). 2011. MTT cell proliferation assay. https://www.atcc.org/~/media/DA5285A1F52C414E864C966FD78C9A79.ashx Date of access: 10 Oct. 2019.

Astashkina, A., Mann, B. & Grainger, D.W. 2012. A critical evaluation of *in vitro* cell culture models for high-throughput drug screening and toxicity. *Pharmacology and therapeutics*, 134:82-106.

Bonnier, F., Keating, M.E., Wrobel, T.P., Majzner, K., Baranska, M., Garcia-Munoz, A., Blanco, A. & Byrne, H.J. 2015. Cell viability assessment using the Alamar blue assay: a comparison of 2D and 3D cell culture models. *Toxicology in vitro*, 29(1):124-131.

Brown, M.A., Wallace, C.S., Anamelechi, C.C., Clermont, E., Reichert, W.M. & Truskey, G.A.J.B. 2007. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials*, 28(27):3928-3935.

Elliott, A.C., Hynan, L.S.J.C.m. & biomedicine, p.i. 2011. A SAS® macro implementation of a multiple comparison post hoc test for a Kruskal–Wallis analysis. *Computer methods and programs in biomedicine*, 102(1):75-80.

Endo, A. 2010. A historical perspective on the discovery of statins. *Proceedings of the japan academy, series B*, 86(5):484-493.

Fautz, R., Husein, B. & Hechenberger, C. 1991. Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). *Mutation research/environmental mutagenesis and related subjects*, 253(2):173-179.

Fotakis, G. & Timbrell, J.A. 2006. *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology letters*, 160:171-177.

Fröhlich, E. 2013. Cellular targets and mechanisms in the cytotoxic action of nonbiodegradable engineered nanoparticles. *Current drug metabolism*, 14(9):976-988.

Galluzzi, L., Maiuri, M.C. Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., Kroemer, G. 2007. Cell death modalities: classification and pathophysiological implications. *Cell death and differentiation*, 14:1237-1266.

Gibco®. 2016. Cell culture basics. http://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf Date of access: 5 Aug. 2019.

Karakaş, D., Ari, F. & Ulukaya, E. 2017. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. *Turkish journal of biology*, 41: 919-925.

Koba, K., Sanda, K., Guyon, C., Raynaud, C., Chaumont, J.P. & Nicod, L. 2009. *In vitro* cytotoxic activity of Cymbopogon citratus L. and Cymbopogon nardus L. essential oils from Togo. *Bangladesh journal of pharmacology*, 4:29-34.

Krippendorff, B.F., Neuhaus, R., Lienau, P., Reichel, A. & Huisinga, W. 2009. Mechanism-Based Inhibition: Deriving K_1 and k_{intact} directly from time-dependent IC_{50} values. *Journal of biomolecular screening*, 14(8):913-923.

Kumar, G.V.S. & Sandhya, G.V. 2014. Preliminary phytochemical screening, total phenol content and *in vitro* antoxidant activity of Caralluma Umbellata Haw. *Journal of global trends in pharmaceutical sciences*, 5(2):1603-1611.

Li, W., Zhou, J., & Xu, Y.I. 2015. Study of the *in vitro* cytotoxicity testing of medical devices (review). *Biomedical reports*, 3:617-620.

Liu, H. 2015. Comparing Welch's ANOVA, a Kruskal-Wallis test and traditional ANOVA in case of heterogeneity of variance. Virginia: Virginia Commonwealth University. (Thesis-MSc).

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

Mamaca, E., Bechmann, R.K., Torgrimsen, S., Aas, E., Bjørnstad, A., Baussant, T. & Le Floch, S.L. 2005. The neutral red lysosomal retention assay and Comet assay on haemolymph cells from mussels (Mytilus edulis) and fish (Symphodus melops) exposed to styrene. *Aquatic toxicology*, 75:191-201.

Marais, S. 2018. The use of apricot oil emulsions for the transdermal delivery of selected statins. Potchefstroom: NWU. (Dissertation-MSc).

Maree, SM. 2018. Transdermal delivery of selected statins formulated in apricot kernel oil emulsions. Potchefstroom: NWU. (Dissertation-MSc).

Miller, M.A., Bankie, C., Al-Shaeri, M.A.M. & Hartl, M.G.J. 2015. Neutral red cytotoxicity assays for assessing *in vivo* carbon nanotube ecotoxicity in mussels – Comparing microscope and microplate methods. *Marine pollution bulletin*, 101:903-907.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, 65:55-63.

Niles, A.L., Moravec, R.A. & Riss, T.L. 2009. *In vitro* viability and cytotoxicity testing and same–well multi–parametric combinations for high throughput screening. *Current chemical genomics*, 3:33-41.

Ricardo, R. & Phelan, K.J.J. 2008. Trypsinizing and subculturing mammalian cells. *Journal of visualized experiments*, (16):e755.

Riss, T.L., Moravec, R.A., Niles, A.L., Benink, H.A., Worzella, T.J. & Minor, L. 2013. Assay guidance manual: cell viability assays. http://europepmc.org/books/NBK144065;jsessionid=nsYziiJZlQeP5FICGHoq.7. Date of access: 3 Sept. 2018.

Riss, T.L., Moravex, R.A. & Niles, A.L. 2011. Cytotoxicity testing: measuring viable cells, dead cell, and detecting mechanism of cell death, *In* Stoddart, M.J., (*Ed*), Mammalian cell viability: methods and protocols. New York: Humana Press. p. 103-114.

Repetto, G., Sanz, P. 1993. Neutral red uptake, cellular growth and lysosomal function: *in vitro* effects of 24 metals. *Alternative to laboratory animals*, 21:501-507.

Repetto, G., Del Peso, A., Zurita, J.L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols*, 3:1125-1131.

Roesler, R., Lorencini, M., Pastore, G. 2010. Brazilian cerrado antioxidants sources: cytotoxicity and phototoxicity *in vitro*. *Ciencia e tecnologia de alimentos*, 30(3):814-821.

Roth, A.J.J.J.o.t.A.S.A. 1983. Robust trend tests derived and simulated: Analogs of the Welch and Brown-Forsythe tests. *Journal of the american statistical association*, 78(384):972-980.

Saravanan, B.C., Sreekumar, C., Bansal, G.C., Ray, D., Rao, J.R. & Mishra, A.K. 2003. A rapid MTT colorimetric assay to assess the proliferative index of two Indian strains of Theileria annulate. *Veterinary Parasitology*, 113:211-216.

Shaikh, K., Pawar, A., Aphale, S. & Moghe, A. 2012. Effect of vesicular encapsulation on *in-vitro* cytotoxicity of ciclopirox olamine. *International journal of drug delivery*, 4:139-146.

Sigma. Trypan blue*. https://www.researchgate.net/.../Sigma+trypan+blue+cell+counting.pdf* Date of access: 5 Aug 2019.

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

Stoddart, M.J. Mammalian cell viability. http://file.zuma.ac.ir/ebook/023-Mammalian%20Cell%20viability%20-%20Methods%20and%20Protocols%20(Methods%20in%20Molecular%20Biology)-Martin%20J.%20Sto.pdf Date of access: 9 Oct. 2019.

Touboul, D., Maillard, L., Grasslin, A., Moumne, R., Seitz, M., Robinson, J. & Zenobi. R. 2009. How to deal with weak Interactions in noncovalent complexes analyzed by electrospray mass spectrometry: cyclopeptidic inhibitors of the nuclear receptor coactivator 1-STAT6. *Journal of the american society for mass spectrometry*, 20:303-311.

Van de Sandt, J., Roquet, R., Cohen, C., Esdaile, D., Ponec, M., Corsini, E., Barker, C., Fusenig, N., Liebsch, M., Bedford, D., De Brugerolle de Fraissinette, A. & Fartasch, M. 1999. The use of human keratinocytes and human skin models for predicting skin irritation. *Alternatives to laboratory animals*, 27:723-743.

Van Tonder, A., Joubert, A.M. & Cromarty, A.D. 2015. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes*, 8:47-57.

Wang, P., Henning, S.M. & Heber, D. 2010. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *Public library of science*, 5(4):1-10.

Wrzesinski, K. & J Fey, S. 2015. From 2D to 3D-a new dimension for modelling the effect of natural products on human tissue. *Current pharmaceutical design*, 21(38):5605-5616.

Xu, J.J., Diaz, D. & O'Brien, P.J. 2004. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chemico-biological interactions*, 150:115-128.

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

Zuang, V. 2001. The neutral red release assay: a review. *Alternatives to laboratory animals*, 29(5):575-60.

APPENDIX F

DIE PHARMAZIE: GUIDE FOR AUTHORS

Aim

The journal DiePharmazie publishes reviews, experimental studies, letters to the editor, as well as book reviews.

The following fields of pharmacy are covered:

- Pharmaceutical and medicinal chemistry,
- pharmaceutical analysis and drug control,
- pharmaceutical technology, biopharmacy (biopharmaceutics, pharmacokinetics, biotransformation),
- experimental and clinical pharmacology,
- pharmaceutical biology (pharmacognosy), and
- history of pharmacy.

Articles are published in English (preferred) or German and are classified as:

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Short communications

Brief publications about the fields mentioned above (see Preparation of manuscripts)

Book reviews

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If each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

Books/Book chapters

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

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APPENDIX G

THE INTERNATIONAL JOURNAL OF PHARMACEUTICS: GUIDE FOR AUTHORS

Introduction

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

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Study Leader/Supervisor (Principal Investigator)/Researcher: Prof M Gerber
Student: JR van Jaarsveld

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- Please provide the HREC with the registration letter from the Ethics Office indicating thatthe cell line registration has been performed for the student, when it has been obtained.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The study leader/supervisor (principle investigator)/researcher must report in the prescribed format to the NWU-HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, whereby a letter of continuation will be provided, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*

- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the study leader/researcher must apply for approval of these amendments at the NWU-HREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-RERC and NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the annual (or otherwise stipulated) monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information or any report templates via Ethics-HRECApply@nwu.ac.za or 018 299 1206.*

The NWU-HREC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the NWU-HREC or the NWU-RERC for any further enquiries or requests for assistance.

Yours sincerely



Digitally signed by Wayne Towers
Date: 2018.12.05 14:19:22 +02'00'

Prof Wayne Towers
Chair NWU Health Research Ethics Committee

Current details: (22351930) M:\DSS1\8533\Monitoring and Reporting Cluster\Ethics\Certificates\Templates\Research Ethics Approval Letters\9.1.5.4.2 HREC Ethical Approval Letter.docm
3 December 2018

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