

Transdermal delivery of selected statins formulated in grapeseed oil emulsions

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Abstract

Hypercholesterolemia can be described as high levels of low-density lipoproteins (LDL) that accumulate in the cells of the body including the liver, spleen and the intestines. Cholesterol is eliminated by increasing high-density lipoproteins (HDL) through the use of statins. Although statins are seen as the first-line treatment of hypercholesterolemia, patient compliance is decreased by the side effects statins possess after oral administration. These side-effects can be reduced by utilising alternative routes of administration, such as the transdermal route.

For a statin or any other pharmaceutical active ingredient (API) to pass through the skin, they must exert the ideal physicochemical properties. When the statins were investigated, it was observed they did not possess ideal physicochemical properties for transdermal drug delivery. Therefore, to enhance the penetration of the non-ideal statins through the stratum corneum, an oil-in-water (o/w) nano-emulsion containing 2% (w/w) of the respective statin and 8% (w/w) grapeseed oil was formulated.

After the o/w nano-emulsions were formulated and characterised, a nano-emulgel was formulated and subsequently characterised. For each of these formulations with the respective statins, membrane release studies, skin diffusion studies and tape stripping were performed to evaluate if the APIs were released from the dosage form, diffused systemically (transdermally) and permeated the skin (topically). Statistical analysis was then performed to analyse the variances between the means of the membrane release studies, skin diffusion studies and tape stripping to determine whether topical or transdermal delivery was achieved.

After skin diffusion studies, *in vitro* cytotoxicity studies were performed on normal immortalised human keratinocytes (HaCaT) cells. Both the statins (alone, dissolved in methanol) and the nano-emulsions with the different APIs were tested by means of methylthiazol tetrazolium (MTT) assay and neutral red (NR) assay. This was done to determine if the selected statins in their formulations were safe for application on the human skin. After results were determined of the MTT and NR, the IC₅₀ (concentration at which 50% of the cell growth is inhibited) values were established. It was evident that lovastatin and simvastatin were the most cytotoxic and rosuvastatin was the least cytotoxic of all the statins. It must however be mentioned that the concentrations tested on the HaCaT cells during the cytotoxic testing were much higher than the amounts that diffused through the skin during the *in vitro* diffusion study and that these 2% formulations would be safe for transdermal application.

Keyword: hypercholesterolemia, statins, nano-emulsion, nano-emulgel, transdermal drug delivery, cytotoxicity

Hipercholesterolemie kan beskryf word as hoë vlakke van lae-digtheid lipoproteïene (LDL) wat ophoop in die selle van die liggaam, insluitend die lewer, milt en die ingewande. Cholesterol word verminder deur statiene te gebruik wat hoë-digtheid lipoproteïene (HDL) verhoog. Alhoewel statiene beskou word as die eerstelinie behandeling vir hipercholesterolemie, neem pasiëntmeewerkendheid af as gevolg van statiene se nuwe-effekte na orale toediening. Deur van alternatiewe toedieningsroetes (soos die transdermale roete) gebruik te maak, kan die nuwe-effekte verminder word.

Statiene of enige ander aktiewe farmaseutiese bestanddeel (AFB) moet ideale fisiese-chemiese eienskappe besit om deur die vel te beweeg. Tydens die ondersoek van die statiene, was dit waargeneem dat die statiene nie ideale fisiese-chemiese eienskappe besit het om geneesmiddels transdermaal af te lewer nie. Dus, om die deurlaatbaarheid van die nie-ideale statiene deur die stratum korneum te verbeter, was 'n olie-in water (o/w) nano-emulsie geformuleer wat 2% (w/w) van die onderskeie statien en 8% (w/w) druïwesaadolie bevat.

Nadat die o/w nano-emulsies geformuleer en gekarakteriseer is, is 'n nano-emuljel geformuleer en vervolgens gekarakteriseer. Membraanvrystellingsstudies, veldiffusiestudies en kleefbandstropping was respektiewelik uitgevoer op elke formulering wat die onderskeie statiene bevat om te evalueer of die AFB vrygestel is vanuit die doseervorm, sistemies (transdermaal) gediffundeer het en die vel (topikaal) binnegedring het. Om te bepaal of topikale en transdermale aflewering bereik is, was statistiese analyses uitgevoer om die variansies op die gemiddelde data wat tydens die membraanvrystellingstudies, veldiffusiestudies en kleefbandstropping verkry is, te ontleed.

In vitro sitotoksiteitstudies is op normale geïmmortaliseerde menslike keratinosiet (HaCaT) selle uitgevoer na die veldiffusiestudies plaasgevind het. Beide die statiene (alleen, opgelos in metanol) en die nano-emulsies met die verskillende AFB is getoets met behulp van metieltiasoltetrasolium (MTT) toets en neutraalrooi (NR) toets om vas te stel of die geselekteerde statiene in hul formulering veilig aangewend kan word op menslike vel. Die IK_{50} -waardes (konsentrasie waarteen 50% van die selgroei geïnhibeer is) is na die resultate van MTT en NR verkry is, bepaal. Dit was duidelik dat lovastatin en simvastatin die mees sitotoksies en rosuvastatin die minste sitotoksies van al die statiene was. Dit moet egter genoem word dat die konsentrasies tydens die sitotoksiesetoetsing op die HaCaT-selle baie hoër is as die hoeveelhede wat deur die vel gediffundeer het tydens die *in vitro* diffusiestudie en dat hierdie 2% formulering dus veilig is vir transdermale toediening.

Sleutelwoorde: hipercholesterolemie, statiene, nano-emulsie, nano-emuljel, transdermale geneesmiddelaflewing, sitotoksiteit

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Abbreviations

%EE	Entrapment efficacy
%RSD	Percentage relative standard deviation
A549	Adenocarcinomic human alveolar basal epithelial cells
ACN	Acetonitrile
AFB	Aktiewe farmaseutiese bestanddeel
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
API	Active Pharmaceutical Ingredient
Apo B	Apolipoprotein B
APVMA	Australian Pesticides and Veterinary Medicines Authority
AS	APIs (statins) alone
ATCC	American Type Culture Collection
ATL	Analytical Technology Laboratory
ATP	Adenosine Triphosphate synthases
B-CPAP	Thyroid cells
CH ₃ OH	Methanol
CO ₂	Carbon dioxide
CoQ10	Coenzyme Q10
cP	Centipose
CVD	Cardiovascular disease
CYP	Cytochrome P (Hepatic enzyme)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ED	Epidermis dermis
EDTA	Trypsin-Versene®

FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FH	Familial hypercholesterolemia
FS	Fluvastatin alone
H ⁺	Concentration of hydrogen ions
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
H ₃ PO ₄	Orthophosphoric acid
HaCaT	Human immortalised keratinocytes cells
HCl	Hydrochloric acid
HDL	High-density lipoproteins
HEL	Human erythroleukemia cell line
HeLa	Human epithelial cells
Hep-2	Human epithelial type 2 cells
HepG2	Human liver cancer cell line
HLB	Hydrophilic-lipophilic balance
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HPLC	High-performance liquid chromatography
HREC	Health Research Ethics Committee
HRTEM	High-resolution transmission electron microscope
HSD	Honest Significant Difference
IC ₅₀	Concentration to which compounds inhibited 50% of the cell growth
ICH	International Conference of Harmonisation
LDL	Low-density lipoproteins
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	Lower limit of quantification
LS	Lovastatin alone
MTT	Methylthiazol tetrazolium
NaOH	Sodium hydroxide
NE1	Nano-emulsions contained Tween [®] 80:Span [®] 60 in 3:3 ratio
NE2	Nano-emulsions contained Tween [®] 80:Span [®] 60 in 3:2 ratio
NE3	Nano-emulsions contained Tween [®] 80:Span [®] 60 in 2:3 ratio
NEAA	Non-essential Amino Acids
NEF1	2% fluvastatin in nano-emulsion formula 1
NEF2	2% fluvastatin in nano-emulsion formula 2
NEF3	2% fluvastatin in nano-emulsion formula 3
NEG	Nano-emulgel
NEGF	Fluvastatin nano-emulgel
NEGL	Lovastatin nano-emulgel
NEGR	Rosuvastatin nano-emulgel
NEGS	Simvastatin nano-emulgel
NEL1	2% lovastatin in nano-emulsion formula 1
NEL2	2% lovastatin in nano-emulsion formula 2
NEL3	2% lovastatin in nano-emulsion formula 3
NEP1	Placebo of nano-emulsion formula 1
NEP2	Placebo of nano-emulsion formula 2
NEP3	Placebo of nano-emulsion formula 3
NER1	2% rosuvastatin in nano-emulsion formula 1
NER2	2% rosuvastatin in nano-emulsion formula 2
NER3	2% rosuvastatin in nano-emulsion formula 3
NR	Neutral red
NRF	National Research Foundation
NRS	Neutral red solution
NES1	2% simvastatin in nano-emulsion formula 1

NES2	2% simvastatin in nano-emulsion formula 2
NES3	2% simvastatin in nano-emulsion formula 3
NWU	North-West University
OH ⁻	Hydroxide ion concentration
o/w	Oil in water
PBS	Phosphate buffer solution
PCS	Photon correlation spectroscopy
PdI	Polydispersity index
Pen/Strep	Penicillin/Streptomycin
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
R ²	Coefficient of determination
RS	Rosuvastatin alone
SCE	Stratum corneum epidermis
SS	Simvastatin alone
TDD	Transdermal drug delivery
TEM	Transmission electron microscope
TEWL	Trans-epidermal water loss
THF	Tetrahydrofuran
UNODC	United Nations Office on Drugs and Crime
USP	United States Pharmacopeia
UV	Ultraviolet light
VLDL	Very low-density lipoproteins
w/v	Weight per volume
w/w	Weight per weight

Chapter 1

INTRODUCTION, RESEARCH PROBLEM AND AIMS

1.1 Introduction

Cardiovascular disease (CVD) can be identified by increased levels of atherogenic cholesterol, and can be a precursor to hypercholesterolemia (Vinson *et al.*, 2019:50). Hypercholesterolemia consists of different types of cholesterol, of which the most important are high-density lipoproteins (HDL), very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). With hypercholesterolemia, the LDL levels are elevated and can cause the risk of atherosclerosis, which can be characterised by the impaired function of vasodilatation, impaired integrity of the walls of the vessels and blood coagulation (Nägele *et al.*, 2018:1524). If VLDL levels are elevated, because of increased plasma, it will result in lower HDL levels (Ginsberg *et al.*, 2005:232). Familial hypercholesterolemia is identified in 1 in 250 individuals and <1% is identified in the United States (Nordestgaard *et al.*, 2013:3479). Life style intervention (like low fat diets and increased physical activity) is the most recommended route before pharmacological treatment is considered (Cuchel *et al.*, 2014:2146). Therapies available for hypercholesterolemia include statin therapy (also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) that lowers the lipids (Bays, 2005). Other treatments include ezetimibe, which inhibits the intestinal dietary uptake, and biliary cholesterol (Raal *et al.*, 2018:485). During this study, four statins (fluvastatin, lovastatin, rosuvastatin and simvastatin) were selected for investigation.

Statins can be seen as the most effective active pharmaceutical ingredient (API) against cardiovascular disease (dyslipidaemia). Statins inhibit HMG-CoA in such a way that they reduce the synthesis of cholesterol (du Souich *et al.*, 2017:2). According to Gendle (2016:54), lipid levels and neurobiological functions can be indirectly linked with one another. Statins exhibit anti-atherosclerotic effects and inhibit the endogenous cholesterol levels (Stancu & Sima, 2001:379). Mevalonate is the product formed by HMG-CoA reductase reaction and is a precursor for cholesterol, which inhibits cholesterol and increases the LDL uptake. This mechanism of action can lead to increased stability of atherosclerotic plaques and enhanced coagulation processes (Stancu & Sima, 2001:379). Statins work in a way that inhibit the HMG-CoA enzyme, thus inhibiting the mevalonic pathway and reducing cholesterol.

Statin therapy is generally well tolerated in association with a decrease in cardiovascular effects and cholesterol levels (Rosenbaum *et al.*, 2013:872; Thompson *et al.*, 2016:2395). Rhabdomyolysis occurs when myopathy progresses for a longer period of time (Furberg & Pitt, 2001:206); myopathy can be seen as the most common complication with use of statins (Ahmad, 2014:1765). Myalgia can be defined as increased muscle pain or cramps and aching of muscles

associated with exercise (Ahmad, 2014:1768). Statins reduce the amount of coenzyme Q10 (CoQ10) in plasma levels, therefore CoQ10 supplementation can prevent the severity of pain. Rhabdomyolysis can occur in long-term statin treatment, where it induces necrosis and rupturing of muscle fibres, rupturing can lead to leakage in muscle contents such as electrolytes, myoglobin and enzymes (Al-Azzawi *et al.*, 2018:1). HMG-CoA reductase inhibition can exert pleiotropic effect and in turn can affect energy metabolism (Tournadre, 2019:1).

Long term statin treatment can cause liver necrosis, liver toxicity, kidney damage or liver injury (Liu *et al.*, 2019:54). Statins increase alanine aminotransferase (ALT), which is released by the liver for pathological functions. When ALT levels increase, it can be associated with hepatic and cardiovascular risks (Harada *et al.*, 2016:57). Liver enzymes are present in skeletal muscle (aspartate aminotransferase and ALT), therefore increased toxicity in the muscles can lead to increased liver enzymes (Pinal-Fernandez *et al.*, 2018:401). These side effects are currently experienced with oral dosage forms of statins; other routes of administrations will be considered due to the first-pass metabolism with oral dosage forms.

Statins have skin side effects, such as alopecia, rashes and chronic urticaria, and gastrointestinal adverse effects, including nausea, dyspepsia, flatulence, abdominal pain and diarrhoea or constipation. The effects are not that severe, therefore patients continue to use the medication (Kiortsis *et al.*, 2007:8). It can be suggested that insulin resistance, type 2 diabetes and increased body mass can be associated with the adverse effects of statins (Mancini *et al.*, 2013:1557). Each statin produces different side effects due to the differences between them.

First-pass metabolism is carried out by the cytochrome P450 (CYP450) enzyme for metabolism (Velazquez *et al.*, 2019:134). Simvastatin is exposed to cytochrome P3A (CYP3A) in the liver and the gut (Fathi *et al.*, 2018:236). The transdermal route of delivery focuses on drug delivery for both systemic and local therapeutic action (Zhou *et al.*, 2018:1713). The transdermal route of delivery will be considered, since the first-pass metabolism is avoided when utilising this route for the selected statins (Geethu *et al.*, 2014:1809).

The skin has a surface area of 1.5 – 2.0 m² and is the largest organ of the human body (Kathe & Kathpalia, 2017:487; Zhou *et al.*, 2018:1713). Delivery through the skin delivers painless and sustained drug delivery (Lee *et al.*, 2018:35). The skin comprises different layers, the stratum corneum (outer layer of the skin), which contains the keratin filled corneocytes (Iachina *et al.*, 2019:1226), the epidermis, dermis and the hypodermis (Pathan & Setty, 2009:174).

The stratum corneum forms a protective barrier and helps prevent substance delivery through the skin (Gelker *et al.*, 2018:34). The lipid lamellae in the stratum corneum consists of bilayers, cholesterol and free fatty acids and is an effective mode of transport for small pharmaceutical particles or APIs (Gelker *et al.*, 2018:35). The stratum corneum can be described as “flat bricks”

or as a “brick and mortar” structure (Menon *et al.*, 2012:4) held together by adhesive junctions (Guo *et al.*, 2019:11). The lamellar bodies are secreted into the stratum corneum and the stratum granulosum, giving rise to the lipid lamellae, and interspersed with enzymes and anti-microbial peptides. When the stratum corneum is modified, its functional properties are weakened and substances can easily pass through (Menon *et al.*, 2012:4).

For an API to cross the stratum corneum, molecules must fall within specific parameters for physiochemical properties to enable the API to reach the site of action. Smaller molecules can permeate the skin barrier easily and more rapidly, thus a molecular mass of < 500 Da can be ideal for permeation (Naik *et al.*, 2000: 319). It is important that the selected API have a lipophilic/hydrophilic balance with an octanol-water partition coefficient (log P) value of ≤ 2 (Beetge *et al.*, 2000:262; Chandrashekar & Shobha Rani, 2008: 95). The melting point of an API ideal for transdermal delivery should be < 200°C (Alexander *et al.*, 2012:29; Chandrashekar & Shobha Rani, 2008:95; Naik *et al.*, 2000:319)

For a nano-emulsion to penetrate through the stratum corneum, the use of a permeation enhancer is advised (Chen *et al.*, 2014:51). The skin consists of a lipid bilayer structure and executes the barrier function; permeation enhancers can act on the intercellular lipids of the stratum corneum (Karande & Mitragotri, 2009:2364). For this study, grapeseed oil was used as a permeation enhancer, since it is high in oleic and linoleic acid (Göktürk Baydar *et al.*, 2007:29). Grapeseed oil contains fatty acids, which mostly consist of C₁₈-fatty acid chains (Williams & Barry, 2012:132). Fatty acids are optimal in formulations for penetration enhancers as they are nontoxic, non-irritant and can enhance penetration of both hydrophilic and lipophilic compounds by opening the lipid bilayers of the skin, thus enhancing the permeation of the selected statins through the stratum corneum into the bloodstream (Pham *et al.*, 2016:180; Van Zyl *et al.*, 2016:188).

1.2 Research problem

When statins are administered in an oral daily dose, they can lead to gastrointestinal and other side effects. Hepatic disease and toxicity may also occur due to the first-pass metabolic process (Mancini *et al.*, 2013; McKenny *et al.*, 2006).

Another research problem that occurs with transdermal delivery of statins is that they do not consist of the ideal physiochemical properties for ideal delivery. The barrier function of the skin prevents the API from passing through and permeating into the systemic circulation (Foldvari, 2000).

1.3 Aims and objectives

The aim of this study was to incorporate 2% of the selected statins (fluvastatin, lovastatin, rosuvastatin and simvastatin separately) into four nano-emulsions and four nano-emulgel

formulations; these nano-emulsions and nano-emulgels contained grapeseed oil as the oil phase. After formulation, the permeation through the skin was investigated together with the cytotoxic effects.

The objectives of this study were to:

- * develop and validate the analytical method used to determine the concentrations of the selected APIs, i.e. high-performance liquid chromatography (HPLC);
- * formulate four nano-emulsions and four nano-emulgels containing the APIs separately (the APIs will be encapsulated in the oil phase (grape seed oil));
- * characterise the emulsions (both nano-emulsions and nano-emulgels) with regard to entrapment efficacy (EE%), droplet size, zeta-potential, pH, viscosity, morphology and visual examination;
- * use membrane release studies to determine the release of the selected APIs from both the four nano-emulsions and the four nano-emulgels;
- * perform Franz cell diffusion studies on the skin and tape stripping techniques to determine the extent of transdermal and topical delivery of the APIs from the nano-emulsions and nano-emulgels;
- * evaluate the cytotoxic effects of the APIs (alone) and the combined formulation on human immortalised keratinocytes (HaCaT) cells by means of the concentration to which compounds inhibited 50% of the cell growth (IC_{50}) values for both methylthiazol tetrazolium (MTT) and neutral red (NR).

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Chapter 2

TRANSDERMAL DELIVERY OF NANO-EMULSIONS CONTAINING SELECTED STATINS IN GRAPESEED OIL

2.1 Introduction

Patients with elevated serum cholesterol levels, which may be produced by endogenous cells in the body or diet (animal derived cholesterol and/or plant sterols) can be at risk for coronary heart disease (animal-derived cholesterol and/or plant sterols) (Lütjohann *et al.*, 2019:111). These sterols are connected to cholesterol, through a hydrophobic molecule, which has a higher affinity for micelles than cholesterol. Cholesterol is subsequently displaced from the micelles, which causes cholesterol to be limitedly available for absorption, consequently causing an accumulation of cholesterol (Cofán *et al.*, 2011:652).

Cholesterol can accumulate in the liver, spleen and intestines. The liver plays an important role in the elimination of the cholesterol through bile, and the cholesterol-binding protein is important in the intracellular division of cholesterol (Klein *et al.*, 2006:126). HDL is a risk factor for cardiovascular disease (Kodama *et al.*, 2007:999), where circulating HDL protein can act as a protection barrier against atherosclerosis (Cuchel *et al.*, 2010:526).

Therapies for lowering cholesterol levels may include statins, ezetimibe and niacin. The use of statin therapy alone can be insufficient in lowering the LDL levels (Raal *et al.*, 2018:485). Statins are used as HMG-CoA inhibitors, while HMG-CoA serves as a precursor of cholesterol (DeBose-Boyd, 2008:609). Statins compete with the binding site of HMG-CoA and alter the conformation of the enzyme when they try to bind to the site (Stancu & Sima, 2001:380). HMG-CoA inhibitors can be seen as vasculature protectors and can have anti-inflammatory actions by protecting the vascular endothelium from inflammatory processes (Stalker *et al.*, 2001:406).

The use of statins can cause side-effects such as myotoxicity, which is dosage dependent and may result in reactions ranging from myalgia to rhabdomyolysis. Myositis and rhabdomyolysis with renal failure can occur when statins are combined with niacin or gemfibrozil (Liu *et al.*, 2019:55). These muscle symptoms can range from mild muscle weakness or pain of the muscles to acute necrosis, where statins damage the skeletal myofibers (Liu *et al.*, 2019:56). These adverse effects of statins are brought forward after gastrointestinal absorption. Statins are transported into the cells by an anion transporter and some can be excreted by a multi-drug resistant protein. These absorption variances have a great effect on the side-effects obtained in the tissue between the specific APIs. Gastrointestinal disturbances caused by statin usage can include bloating, diarrhoea and in some cases constipation, which is caused by disturbances in the cholesterol-bile excretion (Wierzbicki *et al.*, 2003:98). When comparing the oral

administration to transdermal delivery, the transdermal delivery is a more effective way of delivering APIs, when the side-effects of statins are considered, it is evident that the main cause of side-effects caused by oral administration is experienced due to the elimination by the liver. Elimination of substances takes place in the bile and this can be the cause of side-effects experienced (Merle *et al.*, 2005:380). Transdermal delivery avoids the first-pass elimination in the liver and intestine (Wang *et al.*, 2018:143).

Due to the aforementioned side-effects of statins after oral administration, the transdermal route was considered. This route of delivery ensures improved bioavailability, uniform plasma levels and longer duration of action (Ahad *et al.*, 2015:588). The skin consists of three layers, namely the epidermal layer, the dermal layer and the hypodermal layer. The epidermal layer contains the keratinocytes and the hypodermis consists of the fat layer (Mikesh *et al.*, 2013:191). The outermost layer of the epidermis contains the stratum corneum, which enhances the protection against environmental factors. This layer consists of the intercellular spaces between lipid matrix and the keratinocytes (Venus *et al.*, 2011:472). To penetrate through the layers of the stratum corneum, the selected API must make its way through the lipid bilayers and the keratin-rich cells, therefore only an API with the ideal physicochemical properties can penetrate through the skin (Kumar *et al.*, 2015:169).

One challenge associated with transdermal delivery is that the molecules of the API must be small enough to transfer through the stratum corneum. APIs that are hydrophilic have limited penetration, thus substances must be more lipophilic (Ahad *et al.*, 2015:588). The skin consists of low permeation characteristics thus for effective delivery, an API must have a low molecular weight and moderate lipophilicity (Paudel *et al.*, 2010:112). Other physicochemical properties statins must adhere to are ideal log P, octanol-buffer distribution coefficient (log D), melting point and aqueous solubility (Rastogi & Yadav, 2012:165). However, some APIs do not exert these properties and must therefore be formulated into a delivery system containing a penetration enhancer (Paudel *et al.*, 2010:113).

Penetration enhancement strategies and enhancing methods such as chemical enhancers, can improve passive methods. Most penetration enhancers work on the lipid region of the stratum corneum (Kumar *et al.*, 2015:168). The stratum corneum of the skin consists of tight junctions similar to epithelial cells; for transdermal penetration enhancement, penetration enhancers move between these junctions to obtain the desired permeation (He *et al.*, 2009:234). Grapeseed oil (*Vitis vinifera*) was chosen as the penetration enhancer for this study, as it has a more lipophilic nature (Mbah, 2006:353), it is high in linoleic acid, vitamin E and phenolic compounds and provides antioxidant and anti-inflammatory properties (Lin *et al.*, 2018:10). Due to the barrier function of the stratum corneum and the lipid-layers thereof, penetration enhancers can be

formulated within a delivery vehicle, i.e. nano-emulsions and nano-emulgels (Venus *et al.*, 2011:472).

A topical delivery system is needed that can be modified for penetration through the skin, and these can either be nano-emulsions or micro-emulsions. Nano-emulsions have been shown to enhance skin penetration when compared to conventional formulations for topical use (Shakeel *et al.*, 2007:E1). Nano-emulsions consist of a liquid vehicle that can be either hydrophilic or lipophilic and has a very small droplet size, providing close contact to the skin (Kong *et al.*, 2011:838). In this study, nano-emulsions were used and compared to the nano-emulgels. Both formulations contained one of the four selected statins. A gelling agent (Carbopol® Ultrez 20) was incorporated into the nano-emulsion to form the nano-emulgel (Jeengar *et al.*, 2016:222; Sengupta & Chatterjee, 2017:353). Nano-emulgels can enhance delivery through the transdermal route by maintaining a controlled release system with entrapped API particles in the formulation (Ajazuddin *et al.*, 2013:123).

2.2 Hypercholesterolemia

Cholesterol is the building blocks of plasma membranes and forms the lipid and molar basis where steroid hormones, bile acids and vitamin D is synthesised by cholesterol (Zerbinati *et al.*, 2017:151). Hypercholesterolemia is defined as high plasma cholesterol levels in the blood due to high triglyceride levels and elevated LDL levels (Martinez-Hervas *et al* 2008:385).

Cholesterol is categorised into VLDL, LDL and HDL and is packed into apolipoprotein B (apo B), which contains the VLDL that serves as an intravascular precursor of LDL. VLDL and LDL are responsible for the transport of cholesterol from the liver to peripheral tissue (Nordestgaard *et al.*, 2013:3481-3482). Cholesterol regulates the structural components, rigidity, thickness, stability and fluidity of membranes (Peetla *et al.*, 2013:1686, Kaddah *et al.*, 2018:40).

Hypercholesterolemia is characterised by high levels of LDL (Bell *et al.*, 2012:652). The shortage of LDL receptors causes LDL levels to rise in the blood, since removal of LDL cannot take place (Bilheimer *et al.*, 1983:4124). Hypercholesterolemia is a common cause of coronary heart disease and can be classified under myocardial infarction and angina pectoris, if the LDL levels stay elevated and cholesterol levels are 8 – 15 mmol/l (Nordestgaard *et al.*, 2013:3481-3482), therefore chronic high levels of cholesterol leads to atherosclerosis.

Higher HDL-cholesterol levels are needed for protection against heart disease (Wakabayashi. 2018:45). Dyslipidaemia is an imbalance of cholesterol-levels, meaning LDL levels are high, HDL levels are too low and triglycerides show an increase in the body (Leiva *et al.*, 2017:26). Statins increase the creatine kinase levels, which are produced during exercise (Noyes & Thompson, 2017:1134).

Hypercholesterolemia is the reason for endothelial dysfunction because of the lipids that accumulate in the vascular wall. When platelets form one with the endothelial cells, the matrix degrading enzymes start to secrete, which causes a degradation in the proteins of the matrix. As a result, inflammation is promoted and the inflamed tissue is destructed (Becher *et al.*, 2017:860).

2.2.1 Epidemiology of hypercholesterolemia

Investigation of hypercholesterolemia brought the following factors to light. LDL receptors and apolipoprotein B (primary component of protein in the LDL particle) are involved in degradation of the LDL-receptor during cellular recycling (Guglielmi *et al.*, 2016:701). Adenosine, which acts on the adenosine subtype receptor, inhibits the activity of lipolysis (Leiva *et al.*, 2017:26). Lipolysis is a catabolic pathway where triglycerides are hydrolysed by enzymes to form non-esterified fatty acids and glycerols (Johansson *et al.*, 2008; MacPherson *et al.*, 2016:C270).

Inhibition of lipolysis leads to the reduction of fatty acids in the blood levels. Adenosine can also lead to development of atherosclerosis. An increase of plasma cholesterol levels forms dysfunction of the endothelial cells, which leads to atherosclerosis. When such a process occurs, it promotes an inflammatory response and then macrophages and monocytes are formed. This enhances the uptake of LDL that leads to a pro-inflammatory environment (Leiva *et al.*, 2017:26). According to Lippi and Favalo (2011:541), more than 33.6 million US adults, older than 20 years of age, have a serum cholesterol level higher than 240 mg/dl, where LDL-cholesterol levels are more than 130 mg/dl and HDL-cholesterol levels are more than 40 mg/dl.

Causes of hypercholesterolemia can be susceptibility to a diet, obesity, sedentary lifestyle or genetic factors (familial hypercholesterolemia) and smoking (Lippi & Favalo, 2011:542). Assessment of cardiovascular risk factors include reducing cigarette smoking, maintaining a healthy body weight, healthy blood pressure and blood glucose; therefore, cholesterol lowering agents, such as HMG-CoA, are required. HMG-CoA reductase inhibitors can exhibit adverse side-effects in persons with genetic disposition (Cervellin *et al.*, 2010:749).

2.3 Hypercholesterolemia treatment

To treat hypercholesterolemia, the build-up of cholesterol in the vasculature and coronary arteries must be reduced. Treatment of hypercholesterolemia is divided into three categories, ezetimibe as the first step, then high intensity statin treatment and as a third option, bile acid sequestrants or niacin (Gupta *et al.*, 2018:199). Lowering LDL levels is the main treatment for high cholesterol levels. When LDL-cholesterol levels are reduced, the mortality of cardiovascular effects are decreased (Dobrzynski *et al.*, 2018:728).

Ezetimibe absorbs the cholesterol in the intestine from dietary intake and reduces lipoprotein levels (Hernandez-Mijares *et al.*, 2016:41). Ezetimibe inhibits the transport protein and is used in

combination with statins or in patients with statin intolerance. Ezetimibe can enhance mitochondrial function by increasing the oxygen consumption in the mitochondria and decreasing the risk of cardiovascular disease.

Treatment with statins are the leading medication prescribed for patients with high cholesterol levels, where statins inhibit HMG-CoA reductase. In this manner, the statins inhibit cell-mediated cholesterol synthesis, subsequently reducing LDL levels and increasing the level of HDL-cholesterol. HDL-levels are increased by preventing plaque forming in the walls of the arteries (Madasamy *et al.*, 2016:1187).

Nicotinic acid and nicotinamide, also known as niacin, are bioactive molecules and nutritional precursors (Kirkland *et al.*, 2018:83). Niacin is used for treatment of dyslipidaemia by reduction of cholesterol and triglycerides (Kamanna & Kashyap, 2008:S21), triglycerides are stored and synthesised in the liver and are in the form of free fatty acids and glycerol.

2.3.1 Statins

HMG-CoA reductase inhibitors are from fungal origin and have low molecular weights, which enables them to be stable molecules (Ruan *et al.*, 2012:511). They undergo first-pass metabolism in the liver, which reduces their bioavailability between 2 and 4% due to elimination (Bellosta *et al.*, 2000:164).

Statins are metabolised in the liver by the CYP450 enzymes and polymorphism in the CYP genes contributes to the loss of function. After the liver metabolises the statin, the effect on the response and the adverse effects can be observed, because most of the statins' function is lost (Gelissen & McLachlan, 2014:102). HMG-CoA reduces the lipid concentration in the blood and inhibits the sterol biosynthetic pathway in this manner, lowering the cholesterol levels and consequently lowering the potential of atherosclerotic coronary artery disease (Bays, 2006:6). When HMG-CoA is inhibited, the LDL-receptors in the liver are raised and in turn, this promotes clearance of LDL-cholesterol circulating from the blood.

Statins protect the myocardium during injury, increase the nitric oxide, form superoxide radicals, improve vascular function and reduce vascular inflammation. Preservation of mitochondrial permeability and prevention of mitochondrial apoptosis is done by the upregulation of the mammalian target of rapamycin and saving cardiac function. This enhancement causes the mitochondrial functioning to increase the cardio protection through stabilisation. When upregulating of nitric oxide synthase takes place, myocardial perfusion and coronary plaque stabilisation improves and vascular inflammation decreases (Mihos *et al.*, 2014:14).

Except for the function statins have on lowering the lipid concentration in the blood, they can also increase endothelial function in the endothelial cells and in turn improve vasodilatation. Statins

improve cardiovascular disease by reducing accumulation of cholesterol and plaque in the arteries, and in this manner also reduce inflammatory processes. Cholesterol causes inflammation in the arteries, periodontal inflammation and bone loss in a manner of bone resorption (Pradeep & Thorat, 2010:215).

Administration of statins as a chronic therapy can result in pre-ischemic conditioning, which protects the myocardium when ischemic injury occurs (Mihos *et al.*, 2014:14). Benefits of statins therapy include the reduced chances of heart failure, arrhythmias and vascular disease. Disorders extend to rheumatologic, gastrointestinal, neurologic and oncologic disorders (Mihos *et al.*, 2014:12).

HMG-CoA reductase inhibitors include simvastatin and pravastatin, while synthetically derived statins include atorvastatin, cerivastatin, fluvastatin, pitavastatin and rosuvastatin (Grover *et al.*, 2014:895). Simvastatin differs from lovastatin, since it has an additional methyl group meaning; thus, simvastatin is more lipophilic and has more potent cholesterol inhibiting capacity (Sirtori, 1990:550). Lipophilic statins include fluvastatin, lovastatin and simvastatin. Hydrophilic statins include rosuvastatin, which is more hydrophilic and undergoes less metabolic handling.

2.3.1.1 Fluvastatin

Fluvastatin is a synthetic HMG-CoA reductase inhibitor. This molecule is lipophilic and absorbed from the gastrointestinal tract (Scripture & Pieper, 2001:263). The lipophilic nature of fluvastatin and volume of distribution is minimal. Fluvastatin, 7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid, has a molecular weight of 433 g/mol and is a weak acid with a pKa of 5.5 and log P of 2.0.

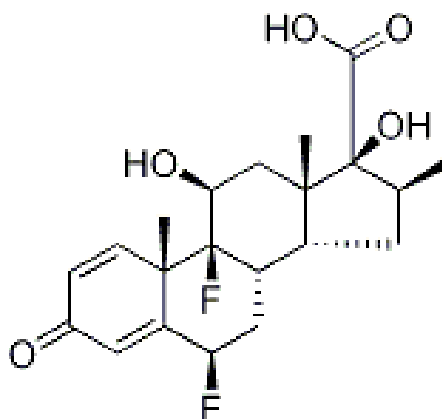


Figure 2.1: Chemical structure of fluvastatin (Ruan *et al.*, 2014:512).

Fluvastatin consists of a bicyclic ring system with an indole fluorophenyl group. The side chain of fluvastatin is an open β -hydroxy acid. The side chain and the bicyclic ring system of the molecule is needed to anchor the drug molecule into the active binding site of the enzyme (Scripture & Pieper, 2001:266).

2.3.1.2 Lovastatin

Lovastatin ($C_{24}H_{36}O_5$) can be characterised as white crystalline powder, has a melting point of 447.5 K and can absorb ultraviolet light (UV) at 231, 238 and 247 nm in acetonitrile (ACN). This substance is insoluble in water and consists of a log P of 4.26 (De Wolff & De Wolff, 2011; Yoshida *et al.*, 2011:658). Lovastatin has a molecular weight of 404.5 g/mol and lipophilic tissue selectivity (Ruan *et al.*, 2012:512). The lipophilicity of lovastatin makes it susceptible to metabolism in the CYP450 enzyme system. Lovastatin is metabolised by the CYP3A5 and CYP2C8 enzymes (Sirtori, 1990:555).

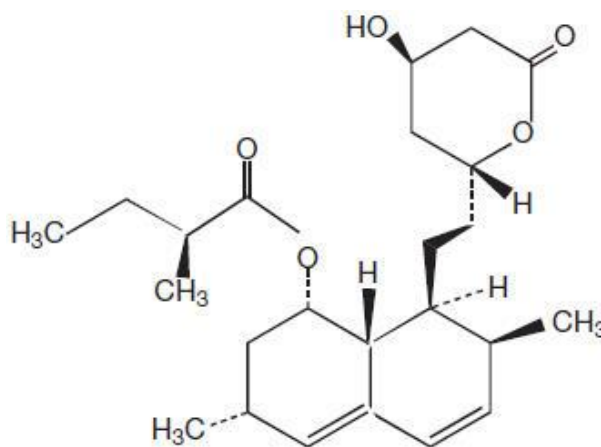


Figure 2.2: Chemical structure of lovastatin (Ruan *et al.*, 2014:512).

The lactone ring of lovastatin undergoes hydrolysis in physiological conditions, generates a δ -hydroxy acid, binds to metal-ion dependent adhesion site of the α -L-domain and represents the inhibitory activity of lovastatin on lymphocyte function-associated antigen (LFA-1) (Kallen *et al.*, 1999:2).

2.3.1.3 Rosuvastatin

Rosuvastatin has a high affinity for the HMG-CoA enzyme. This statin is formulated as either a calcium salt or a hydroxy acid. Rosuvastatin consists of a dihydroxy heptanoic acid part that binds to the site of HMG-CoA target. This statin is hydrophilic and has limited access into hepatic cells, which consequently leads to better water solubility and the reduced need for CYP450 metabolism (Chapman & McTaggart, 2002:33). Rosuvastatin is a white powder that is moderately soluble in water and ethanol (Ahmad *et al.*, 2012:2).

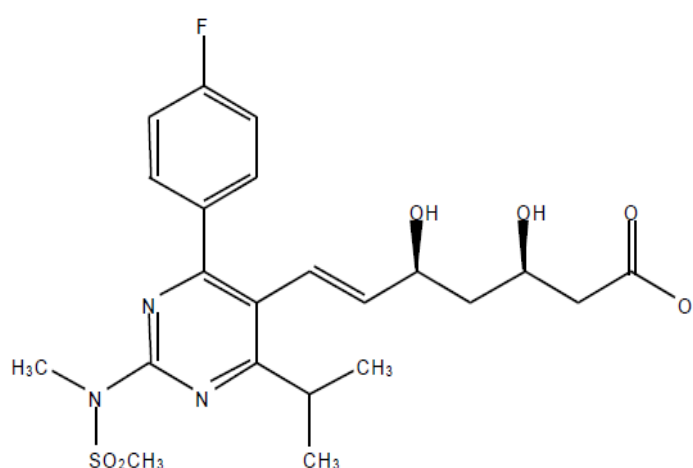


Figure 2.3: Chemical structure of rosuvastatin (Macwan, 2013:25).

2.3.1.4 Simvastatin

The chemical structure of simvastatin consists of (1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-2H-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a hexahydronaphthalen-1-yl 2,2 dimethyl butanoate. The transformation from this form to the active form of simvastatin takes place by opening the lactone ring reaction, a process that takes place in the liver (Guzik *et al.*, 2010:371).

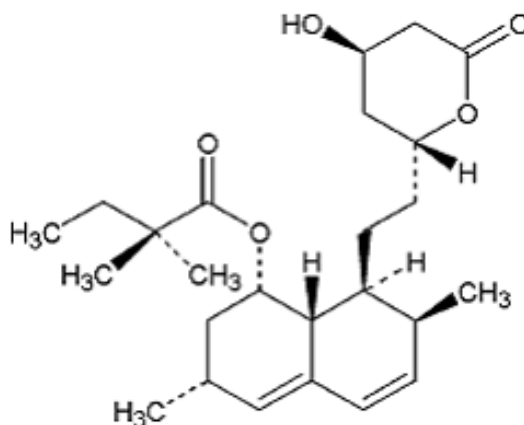


Figure 2.4: Chemical structure of simvastatin (Schachter, 2004:119).

2.3.2 Side-effects of statin use

The major organ for clearance and elimination in the body is the liver, and an inactivation mechanism is enabled for oral administration. This can be the cause of side-effects experienced when statins are taken orally (Watanabe *et al.*, 2009:5). Oral statin usage can cause side-effects such as myopathy, hepatotoxicity, nephrotoxicity, diabetes mellitus, manifestations that can be neurological, pomological and ophthalmological, and it causes a pro-inflammatory response. The extent of side-effects depends on the ability to reach the hepatocytes, where it will inhibit the HMG-CoA. This response will not only lower cholesterol levels, but also ubiquinone, steroids, bile acids, vitamin D and geranylgeranyl pyrophosphate (Du Souich *et al.*, 2017:2).

Statins can induce myotoxicity that includes manifestations such as muscle weakness and muscle pain or aching (myalgia, stiffness and muscle tenderness or cramps) (Du Souich *et al.*, 2017:2). The side-effects that relate to muscular disorders may be linked to the genetic variation in statin uptake (Ghatak *et al.*, 2010:337).

Statins alter the Adenosine Triphosphate synthases (ATP) synthesis and the mitochondrial respiratory chain, genes in the proteasome system gets upregulated and lipid stores are increased. When HMG-CoA is inhibited, craters form along the sarcolemma and cause swelling of the mitochondria. Statins can induce muscle atrophy and proteolysis. Fluvastatin causes a decrease in mitochondrial respiration. Atorvastatin and simvastatin therapy cause mitochondrial

swelling, since cytochrome is released into the sarcoplasm. When statins access the mitochondria, the respiratory chain is disrupted and a decrease in production of ATP takes place (Du Souich *et al.*, 2017:11).

Statins can cause caspase activation and are responsible for apoptosis, cell death of hepatocytes, degradation of CoQ10 levels and oxidative stress that happens in the liver. When degradation of CoQ10 takes place, no anti-oxidation, anti-inflammatory or anti-apoptotic effects can be achieved by the liver. When the liver is injured, the symptoms normally include loss of appetite, urine that is dark in colour and the skin and white of the eyes become yellow (Grover *et al.*, 2014:894).

2.4 Transdermal drug delivery

Transdermal and topical drug delivery can be used to treat anti-inflammatory disorders and cutaneous infections, but also different illnesses and dermatological conditions (Sala *et al.*, 2018:1). This route of administration consists of two routes of permeation; the first permeation route can be through the skin channels for hydrophilic substances with a diameter of a few microns. The second route of permeation is penetration through the epidermis, stratum corneum and into the deeper epidermis to reach the capillaries of the body's circulation. Drugs need to be both hydrophilic and hydrophobic to pass through keratinocytes and intercellular lipids. Most drugs do not exert these properties, thus the intercellular route is taken. Permeability of the skin barrier needs to be enhanced with permeability enhancers.

2.4.1 Advantages of transdermal drug delivery

Transdermal drug delivery has the advantages of painless drug delivery, better patient compliance and it ensures sustained and controlled drug release. This route of administration requires a lower dosage of medication and reaches the vasculature in a short diffusive pathway (Lee *et al.*, 2018:36). The transdermal route bypasses the first-pass metabolism and reduces the drug side-effects by having a more predictable pharmacological response and extended duration of action (Sala *et al.*, 2018:1).

2.4.2 Disadvantages of transdermal drug delivery

The transdermal route of delivery does not only have advantages, the skin is also an obstacle for drug delivery. The outermost layer of the skin consists of the stratum corneum, which blocks any materials from penetrating through to the deeper layers of the skin (Lee *et al.*, 2018:36). Molecules must consist of a small particle size and have a high lipophilicity to permeate through the barrier, where the majority of molecules are hydrophilic (Sala *et al.*, 2018:8). After the molecules permeate through the stratum corneum, the next obstacle is premature degradation by epidermal enzymes. The onset of action and lack of pharmacokinetic control can be another obstacle to effective drug delivery (Lee *et al.*, 2018:36).

2.5 The skin

The skin serves as a temperature regulating organ and an immunity barrier against micro-organisms. The skin plays an important role in the absorption of drugs and their efficacy (Zhou *et al.*, 2018:1713). Each one of the skin's layers has a unique composition and function.

The skin makes up about 15% of the body weight of the human body and comprises a surface area of 1.5 to 2.0 m², making it the largest organ in the body (Lai-Cheong & McGrath, 2017:347). The skin's thickness varies from 10 to 20 µm depending on the body size (Soetens *et al.*, 2018:423). The structure of the skin consists of the multi-layered epidermis, hair follicles, sweat glands and sebaceous glands, but is made up out of three main layers namely the epidermis, dermis and hypodermis (Lai-Cheong & McGrath, 2013:87). Figure 2.1 illustrates the layers of the skin.

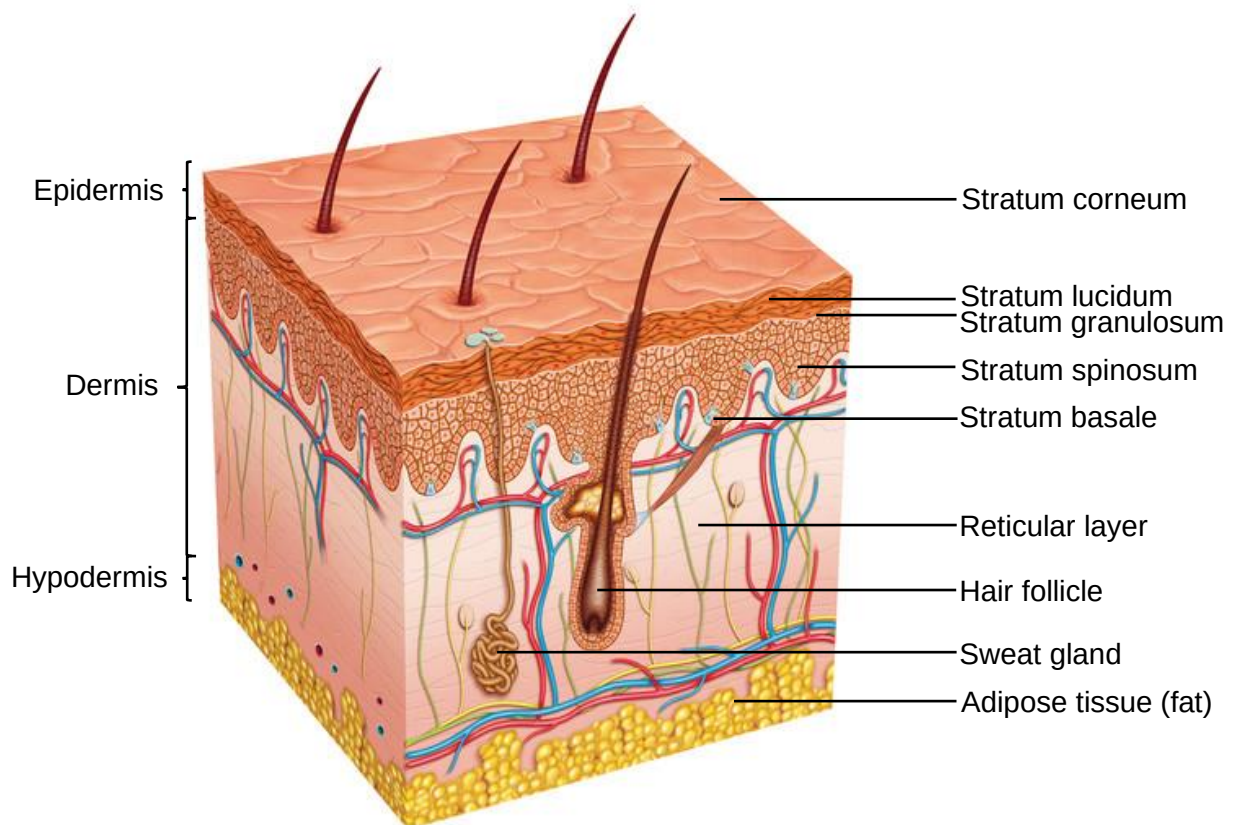


Figure 2.5: Illustration of the major layers of the skin (adapted from Wilken & Gray-Wilson, 2017).

2.5.1 The epidermis

The main function of the epidermis is the protection of the body against the external environment and regulation of body temperature (Capallere *et al.*, 2018:45). The epidermis can be categorised into two groups, namely the stratum corneum and the viable epidermis. The viable epidermis contains the *stratum granulosum* that releases lipids from lamellar bodies within corneal-granular interface, *stratum spinosum* that contains the prickle cells and *stratum basale* containing proliferative columnar keratinocytes (Capallere *et al.*, 2018:45). The stratum corneum consists of terminally differentiated non-viable keratinocytes (corneocytes) and has a thickness of 10 to 20 μm . The viable epidermis consists of differentiated keratinocytes (Soetens *et al.*, 2018:423), and has keratinocytes that are activated by temperature (Denda, 2015:70). According to Denda (2015:71), the keratinocytes produce cytokines for the anti-inflammatory response, and neurotransmitters that influence the peripheral nervous system.

Ceramides is the metabolite in sphingolipid metabolism and the intermediate for glycosphingolipid and sphingomyelin synthesis in all mammalian tissue (Rabionet *et al.*, 2014:422). Sphingolipids are responsible for cellular functions and ceramides for humidifying skin and protection against

microbial infections. They also play an important role in proliferation of the cell, differentiation and apoptosis in the epidermis (Rabionet *et al.*, 2014:422).

The barrier function of the epidermis depends on the mixture of lipids within the skin (Kendall *et al.*, 2017:1680). Lamellar bodies in the epidermis provides a function of free fatty acids and antimicrobial peptides. Antimicrobial peptides express acceleration of excretion if the permeation barrier was disrupted (Elias & Wakefield. 2015:551). When the epidermis is damaged the underlying layers restore permeability homeostasis with mechanical signal.

2.5.2 Dermis

The dermis can be divided into two parts, namely the upper dermis that consists of a high cell density and the reticular dermis that has a low cell density (Korosec *et al.*, 2019:342). According to Driskell *et al.* (2014:629), the reticular dermis covers the subcutaneous white adipose tissue and contains the fibroblasts, which play an important role in aging.

The epidermis is attached to the dermis with junctions and subcutaneous fat, and the main function of the dermis is to protect the body against mechanical injury. The upper layer of the dermis is also known as the papillary dermis and contains cells and fibres to connect with the epidermis. The lower part of the dermis consists of polysaccharides and proteins. The proteins interact to produce hygroscopic proteoglycan macromolecules (Venus *et al.*, 2011:471).

2.5.3 Hypodermis

This part of the skin is mainly loose connective tissue, which forms the layers of large pockets of adipose tissue that protects the skin and serves as insulation. These tissues are rich in proteoglycan and glycosaminoglycans, which gives the tissue mucous-like properties by attracting fluids into the tissue. Cells found in the hypodermis include fibroblasts, adipose cells and macrophages, which play a role in obesity (Wong *et al.*, 2016:94). The hypodermis is seen as the deepest lying tissue that contains adipocytes where protein can be expected (Boury-Jamot *et al.*, 2006:1035) and where fat storage takes place for additional energy (Yagi & Yonei, 2018:51). The average thickness of the hypodermis is different in every person, but varies between 4 and 9 mm (Ali *et al.*, 2015:105).

2.6 Drug transport through the skin

2.6.1 Transdermal delivery

Transdermal delivery of APIs possesses limitations compared to the oral and hypodermic delivery routes. The stratum corneum serves as the main barrier for transport for transdermal drug delivery. In order to do this, chemical penetration enhancers, or lipid nanocarriers, need to be

implemented (Hirakawa *et al.*, 2019:3). Before transdermal delivery of an API can be considered, the permeation into the skin and mobility of the selected drug in the vehicle needs to be determined. Micro-emulsions are a better choice in permeation of the skin barrier in comparison to conventional emulsions and gels (Peltola *et al.*, 2003:99). Dosage forms within the micro/nano-particle size range, enables controlled release in transdermal drug delivery mechanism (Kurniawan *et al.*, 2019:708).

There are three ways in which a molecule can enter through the skin, namely:

- * the intercellular mode of entry, which is the pathway through the lipid matrix and/or intercellular spaces of keratinocytes;
- * the transcellular pathway is where the molecule moves through the keratinocytes;
- * the transappendageal pathway is the pathway through hair follicles, sebaceous glands and sweat glands (Desai *et al.*, 2010:247).

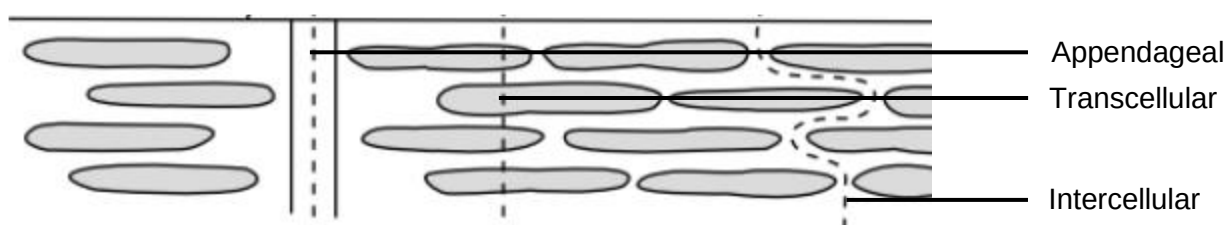


Figure 2.6: Illustration of transport pathways (adapted from Ng & Lau, 2015:6).

2.6.1.1 Transcellular route

Corneocytes in the skin are vertically organised and can add up to 30 layers, when a substance follows the transcellular route, it includes the path it followed through the keratin-packed corneocytes and diffusion through the aqueous interior consisting of a keratin fibre matrix (Schwöbel & Klamt, 2019:53). This route is important for hydrophilic solutes, where diffusion and the partitioning steps enhance the mechanism for an API to pass through. The physicochemical properties of the API can determine whether a drug can go through the transcellular route (Marrow *et al.*, 2007:38). Penetration of small polar molecules can penetrate via the transcellular route (Sahoo *et al.*, 2013:35).

2.6.1.2 Intercellular route

The intercellular route depends on the “brick and mortar” structure that lies within the stratum corneum (Hostynek *et al.*, 2001:8); this has a longer pathway for molecules to permeate through (Marrow *et al.*, 2007:39; Sahoo *et al.*, 2013:36). Normally small uncharged molecules that must penetrate the skin can move through this pathway (Marrow *et al.*, 2007:39). Lipophilic substances

will prefer penetration through the intercellular lipid bilayer, thus enhancing penetration can be a primary function (El-Maghraby *et al.*, 2008:205).

2.6.1.3 Transappendageal route

The shunts in the skin provided by the hair follicles (transfollicular route), sweat glands and sebaceous glands make the appendageal route for permeation of molecules possible (Tsakovska *et al.*, 2017:27). According to Kielhorn *et al.* (2006:192), this route of penetration can only be significant on skin that is of high density, which can act as a reservoir. As only 0.1% of the surface of the skin's features is appendageal, the physicochemical properties will play a big role in partitioning. Lipophilic drugs can be retained in the stratum corneum (lipophilic) and prevent the ingredients from moving to the hydrophilic epidermis (Ng & Lau, 2015:6).

2.7 Physicochemical properties influencing transdermal and topical drug delivery

The ideal physicochemical properties required for transdermal delivery, together with the physicochemical properties of the statins are shown in Table 2.1.

Table 2.1: The physicochemical properties of the selected statins in comparison to the ideal properties required for transdermal delivery

	Fluvastatin	Lovastatin	Rosuvastatin	Simvastatin	Ideal properties
Aqueous solubility (mg/ml)	0.2 ^a	0.0004 ^b	7.800 ^c	0.030 ^d	> 1.0000 ^{-1e}
Log D at pH 7.4	> 1 and < 2 ^f	1.18 ^g	-0.33 ^f	1.60 ^h	1.00 < 3.00 ^e
Log P	3.5 ⁱ	4.07 ^h	0.42 ^h	4.42 ^h	1 – 3 ^e
Melting point (°C)	195 °C ^j	171.8 °C ^b	122 °C ^k	139.3 °C ^l	< 200 ^e
Molecular weight (Da)	411.47 ^m	404.540 ⁿ	481.538 ^o	418.600 ^p	< 500.000 ^q

a) Cayman chemical, 2010; b) Karolewicz *et al.*, 2015:2283; c) Crestor, 2009; d) Margulis-Goshen & Magdassi, 2009:275; e) Naik *et al.*, 2000:319; f) Chapman & McTaggart, 2002:33; g) Joshi *et al.*, 1999:270; h) Wong *et al.*, 2008:245; i) Greupink *et al.*, 2011:491; j) Chu & Yalkowsky, 2009:30; k) Ahmad *et al.* 2012:2; l) Sharma *et al.*, 2009:45; m) Ruan *et al.*, 2012:512; n) O'neil, 2006:968; o) Tabassum *et al.*, 2017:105; p) Bhagat & Sakhare, 2014:1051; q) Benson, 2005:26.

For a substance to penetrate the skin, diffusion through the lipophilic stratum corneum must take place. For hydrophilic substances this can become a rate limiting step, but for lipophilic substances the hydrophilic epidermis and dermis can be rate limiting (Bartosova & Bajgar, 2012:4672). Ideal Log P values for penetration through the stratum corneum can range from - 1.66 to 4.05 and values smaller than - 2.50 are considered to be too hydrophilic (Roberts &

Aptula, 2008:380). Each of the selected statins' physicochemical characteristics will be discussed in the following section.

2.7.1 Molecular weight

Molecular weight plays a role in penetration through the skin, the smaller the molecule the better the intracellular penetration will be (Lombry *et al.*, 2000:32). According to Denet *et al.* (2004:665) the physicochemical properties of a molecule such as the molecular weight can influence the intracellular penetration. Due to the barrier of the skin, the delivery of macromolecules is not a realistic option (Lombry *et al.*, 2000:36). A molecular weight of < 500 Da is required for penetration through the stratum corneum. Fluvastatin, lovastatin, rosuvastatin and simvastatin have a molecular weight ranging between 404.540 and 418.600 Da.

2.7.2 Fick's law of diffusion

The rate at which drugs permeate the skin can be described using Fick's law of diffusion (Ng & Lau, 2015:5). The use of this application will be where gas-phase diffusion is limited to enhance migration from low-permeable layers to the subsurface (Webb & Preuss, 2003:329). Random motion of molecules leads to migration (diffusion) that occurs at different rates and through adsorption, separation of molecules and catalytic conversion (Kärger & Ruthven, 2016:4027). The permeability through the skin is defined in the equation that Fick's law is based on, the measurement of the partition coefficient (P) that predicts the partition of the permeant into the skin and the diffusion coefficient (D) that predicts how often the permeant diffuses through the skin (Ng & Lau, 2015:5). Fick's law of diffusion is highly dependent on the physicochemical properties of a molecule (molecular mass) and the hydrophilicity (log P). As a rule, the log P value for skin permeation (K_p) must be above 3, which indicates higher lipophilicity, and a log P lower than -1.66, according to Roberts and Aptula, (2008:380), indicates a substance of higher hydrophilic nature. Lovastatin and rosuvastatin indicate a higher lipophilic nature, which will make diffusion easier than the more hydrophilic statin that will have a problem permeating through.

2.7.3 Aqueous solubility

The aqueous solubility is a physical property that affects the adsorption, dissolution, metabolism and elimination of a compound (Delaney, 2005:289). Aqueous solubility can attribute to the physicochemical properties of an API's characterisation, which is a key during discovery (Elder & Holm, 2013:3). A good aqueous solubility can be described as a compound having a 1 mg/kg potency and dissolving 100 µg/ml to have an adequate solubility; a poor solubility can be described as having a solubility of < 10 µg/ml (Elder & Holm, 2013:4). According to Naik *et al.* (2000:321), a higher aqueous solubility insures penetration through the skin (> 1.0 mg/ml). A molecule that is too hydrophilic will not be able to transfer into the stratum corneum, but must

reach the more aqueous epidermis beneath. Molecules with a higher lipophilicity can transfer through the stratum corneum, thus molecules must possess both lipid and aqueous soluble properties. Fluvastatin, lovastatin and simvastatin possess properties of lipophilicity and rosuvastatin is more hydrophilic (Odriozola-Serrano *et al.*, 2014:1; Kela & Kaur, 2013:9203). The solubility of statins in *n*-octanol and phosphate-buffered solution (PBS at pH 7.4) were determined and results are given in Table 2.2 (Marais, 2018:199; Maree, 2018:231).

Table 2.2: Solubility (mg/ml) of selected the statins in *n*-octanol and PBS (pH 7.4) (Marais, 2018:199; Maree, 2018:231).

Statin	<i>n</i> -Octanol (mg/ml)	PBS (mg/ml)
Fluvastatin	11.03 ± 0.60	0.53 ± 0.09
Lovastatin	8.90 ± 0.41	0.01 ± 0.01
Rosuvastatin	0.28 ± 0.05	0.31 ± 0.07
Simvastatin	0.64 ± 0.02	0.24 ± 0.06

The solubility for fluvastatin and lovastatin in *n*-octanol was much higher than for rosuvastatin and simvastatin. Solubility of rosuvastatin and fluvastatin was the highest in PBS (pH 7.4), but all the statins had poor aqueous solubility, lower than 1.0 mg/ml (required for ideal skin permeation). Hence, the statins may struggle to permeate into and through the skin.

2.7.4 Melting point

According to Pathan and Setty. (2009:176), a lower melting point correlates with a good solubility; a substance with a higher melting point can decrease the mobility and have a decrease in the permeation effects (Kanikkannan & Singh, 2002:223). The ideal melting point for transdermal delivery is < 200 °C (Naik *et al.*, 2000:319). A definite correlation can be drawn between the melting point and the amount of API transferred through the stratum corneum (Williams, 2003:37). Each of the selected statins showed a melting point of below 200 °C; fluvastatin was the statin with the closest value to 200 °C with a melting point value of 194 – 197 °C, which might influence the rate of transdermal delivery (Schmidt *et al.*, 2006:729).

2.7.5 Log P/Log D

The log P value can be described as the partition coefficient defined by the unionised species between octanol and water. This can have an effect on the pH when particles are ionised (Hadgraft & Valenta, 2000:244). Molecules with an intermediate partition coefficient will solubilise in the lipophilic stratum corneum and show diffusion (Benson, 2005:25). Movement of molecules across the skin barrier consists of fundamental concepts, of which partition coefficient,

thermodynamic activity and diffusion coefficient are the most important (Narishetty & Panchagnula, 2004:372).

The partition coefficient can be seen as an indication of how good the distribution of molecules is between the lipophilic and hydrophilic layers of the skin (Williams, 2013:676). The brick and mortar structure is a distribution of the lipophilic and hydrophilic properties (Naik *et al.*, 2000:319). For an API to possess an ideal log P, the values must fall within 1 – 3 (Williams, 2003:36). Rosuvastatin can be too hydrophilic and can have difficulty permeating through the barriers.

Table 2.3: Determined log D values of the selected statins (Marais, 2018:199; Maree, 2018:231).

Statin	Log D
Fluvastatin	1.36 ± 0.00
Lovastatin	4.50 ± 0.04
Rosuvastatin	- 0.20 ± 0.00
Simvastatin	1.80 ± 0.00

From the results obtained, it was evident that lovastatin had the highest lipophilicity, while rosuvastatin was the most hydrophilic when compared to the other statins. This data correlates with Wang *et al.* (2008:245), who stated rosuvastatin had a log D value of - 0.3. The log D of fluvastatin correlated well with literature, with a value of 1.3 as per Wang *et al.* (2008:245). Lovastatin presented with a value higher than that of Joshi *et al.* (1999:270), who determined a value of 1.8 for lovastatin. The lipophilicity of APIs to permeate the skin should be between 1 and 3 (Naik *et al.*, 2000:319) to be considered as an acceptable balanced lipophilicity (log D) (Wiedersberg & Guy, 2014:150). It can be stated that the log D values of simvastatin and fluvastatin were considered the most ideal for transdermal delivery.

2.7.6 Ionisation, pH and pKa

The stratum corneum plays an important role in enzymatic activities and skin regeneration, therefore the skin has a pH value ranging between 4.2 and 5.6 (Schmid-Wendtner & Korting, 2006:296). It is known that skin permeation decreases with pH levels of lower than 3 and higher than 9 (Murthy *et al.*, 2003:49). The permeability coefficient of skin permeation is directly equivalent to pH; the pH needs to be above the pKa of a weak acid and below the pKa of a weak base (Chantasart *et al.*, 2015:3459).

It can be stated that anionic and cationic surfactants can interact with intercellular keratin that lies within the stratum corneum. Anionic substances permeate poorly through the skin (Williams & Barry, 2012:133), whereas permeation of non-ionic substances have a better permeation rate, as

they have the characteristics of polar groups that disrupt the skin layer (Hadgraft, 1999:3; Williams & Barry, 2012:133). Non-ionic structures can influence percutaneous absorption by changing the structure of the stratum corneum's intercellular regions; non-ionic surfactants include sucrose fatty acids. These fatty acids alter the barrier function of the membranes (Cázares-Delgadillo *et al.*, 2005:205). The degree of unionisation of APIs can be determined with the Henderson-Hasselbalch equation by using the pH and pKa of each of the APIs (Maitani *et al.*, 1995:167).

$$\% \text{unionised} = 1 / [1 + \text{antilog} (\text{pH} - \text{pKa})] \quad \text{Equation 2.1}$$

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

The pKa values obtained were as follows:

- * Fluvastatin: 4.60 (Schönherr *et al.*, 2015:163).
- * Lovastatin: 13.49 (Schönherr *et al.*, 2015:163).
- * Rosuvastatin: 4.80 (Koçak *et al.*, 2012:138).
- * Simvastatin: 4.30 at a pH of 5.5 (Selvasudha & Koumaravelou, 2017:77).

2.8 Overcoming the skin barrier

The intercellular matrix structure consists of fatty acids, ceramides, cholesterol and cholesterol sulphates. For transdermal delivery, deeper penetration of active ingredients is needed. Transdermal delivery of APIs, must consist of the ideal physicochemical properties, such as the lipophilicity of the drug, particle size and the protein binding capacity of the API (Trommer & Neubert, 2006:107).

2.8.1 Penetration enhancers

The ideal penetration enhancer should increase the permeability of the skin by reversible disruption of the structure of the stratum corneum. It must serve as a force of transport for chemicals into the skin and avoid damaging the tissue that lies deeper within the skin (Prausnitz & Langer, 2008:1265). There are different passive enhancement ways to permeate the skin barrier. The first mechanism of penetration is by increasing the diffusion through the skin and that is done by rearranging the lipids in the stratum corneum. This can be done by using fatty acids that induce phase separation and reduce the barrier function. Oleic acid is an example of the fatty acids used for these purposes. The second mechanism of penetration enhancement is by increasing the drug solubility in the skin and a combined increase in membrane solubility and diffusion coefficient. Increasing the degree of saturation can be done by increasing the solubility of the drug in the vehicle containing it, to increase the skin permeability (Moser *et al.*, 2001:106).

2.8.1.1 Types of penetration enhancers

Types of penetration enhancers can include ceramides, azone analogues, menthol derivatives, essential oils and fatty acids (Chen *et al.*, 2014:51; Williams & Barry, 2012:128). Penetration enhancers must increase the transport of molecules across the skin without disrupting the natural layers of the skin (Karande & Mitragotri, 2009:2365). Ceramides can be found in the stratum corneum and are amphiphilic molecules, as molecules of hydrophobic nature can weaken the ceramide bilayers (Chen *et al.*, 2014:52). Azones enhance the effects of permeation by interacting with the lipid domains of the stratum corneum and disrupting the lipid bilayer (Chen *et al.*, 2014:53). Menthol derivatives also disrupt the lipid layer of the stratum corneum and do not cause skin irritability (Aqil *et al.*, 2007:1062). Essential oils' main mechanism of action is to disrupt the hydrogen bond of the ceramides in the stratum corneum and increase the diffusion into the skin (Aqil *et al.*, 2007:1063).

2.8.1.2 Fatty acids as penetration enhancers

Fatty acids have a *cis-trans* configuration and can be classified as short to long chain unsaturated fatty acids, the most important of which are oleic acid and linoleic acid (Katdare *et al.*, 2019:142). Absorption of drugs can be enhanced by long chain fatty acids such as oleic acid, which increases the flux through the human skin in such a manner that it interacts with the lipids inside the stratum corneum and in turn enhances the drug penetration (Pathan & Setty, 2009:177). Another mode of action for fatty acids is to bind the isopropyl-group with the polar region of the layer (Brinkmann & Müller-Goyman, 2004:219). Fatty acids can be found in natural seeds, in the formation of C₁₆ and C₁₈ saturated fats and can consist of a mixture between oleic and linoleic acids together with palmitic or palmitoleic acids (Ratlledge, 2004:813). The ideal fatty acid chain is the C₁₈ configuration (18:2). This describes the notation of chain length and geometry of double bonds (Gocen *et al.*, 2018:143). The structural properties of these fatty acids play an important role in the biological processes, and the chain length (containing the number of carbons) classifying them as saturated or unsaturated (Czamara *et al.*, 2014). Unsaturated fatty acids were found to have a greater impact on trans-epidermal water loss (TEWL), than the saturated fatty acids.

2.9 Transdermal delivery system selection

Transdermal drug delivery is an important alternative route to oral drug delivery and can enhance the effect of low bioavailable drugs taken orally (Prausnitz & Langer, 2008:1268). Nano-emulsions present higher storage stability, lower preparation costs and they are thermodynamically stable and are dispersions of oil and water stabilised by surfactants (Shakeel & Ramadan, 2010:356).

2.9.1 Nano-emulsions as delivery systems

Nano-emulsions have been developed to protect drugs against environmental factors (Zhang *et al.*, 2014:1000), and to target a specific organ by enhancing permeability (Jiang *et al.*, 2013:3142). Nano-emulsions can be both oil in water (o/w) or water in oil (w/o) (biphasic) nano-emulsions (Singh *et al.*, 2017:29). Nano-emulsions generally consist of 5 – 20% oil/lipids, surfactants and co-surfactants that stabilise the emulsion and can absorb at the oil water interface. Some nano-emulsions can contain stabilisers for storage and heat protection or antibacterial invasion (Singh *et al.*, 2017:31). Particle size ranges can be between 20 and 200 nm and are intermediate between nano-emulsions or micro-emulsions, because of these small droplet sizes nano-emulsions can withstand sedimentation or creaming (Lin Ee *et al.*, 2008:626). To lower these particle sizes for a dispersion with small droplet formation, high energy methods are used (Calderó *et al.*, 2011:409).

Nano-emulsions only need a reasonable surfactant concentration and have a low surface tension of o/w droplets which ensures droplet uniformly (Tadros *et al.*, 2004:304). Emulsions can undergo phase inversion, where water is added to the oil phase to form the emulsion and the surfactant molecules changes from negative to positive, forming an o/w emulsion, or from positive to negative forming a w/o emulsion when chemical energy is released (Zhang *et al.*, 2017:316). The o/w mixture was used during this study, where the droplet size of the emulsions depended strongly on the oil phase composition (Mayer *et al.*, 2013:126). Lipophilic molecules were dispersed within the aqueous medium. The tails of the non-polar surfactant molecules entered the hydrophobic core (oil phase), while the polar heads entered the surrounding hydrophilic environment. It can be said that the surfactant molecules get dispersed within the water. This system of micelle formation helps to provide a thermodynamically stable environment for the nano-emulsion formed (McClements, 2012:1720).

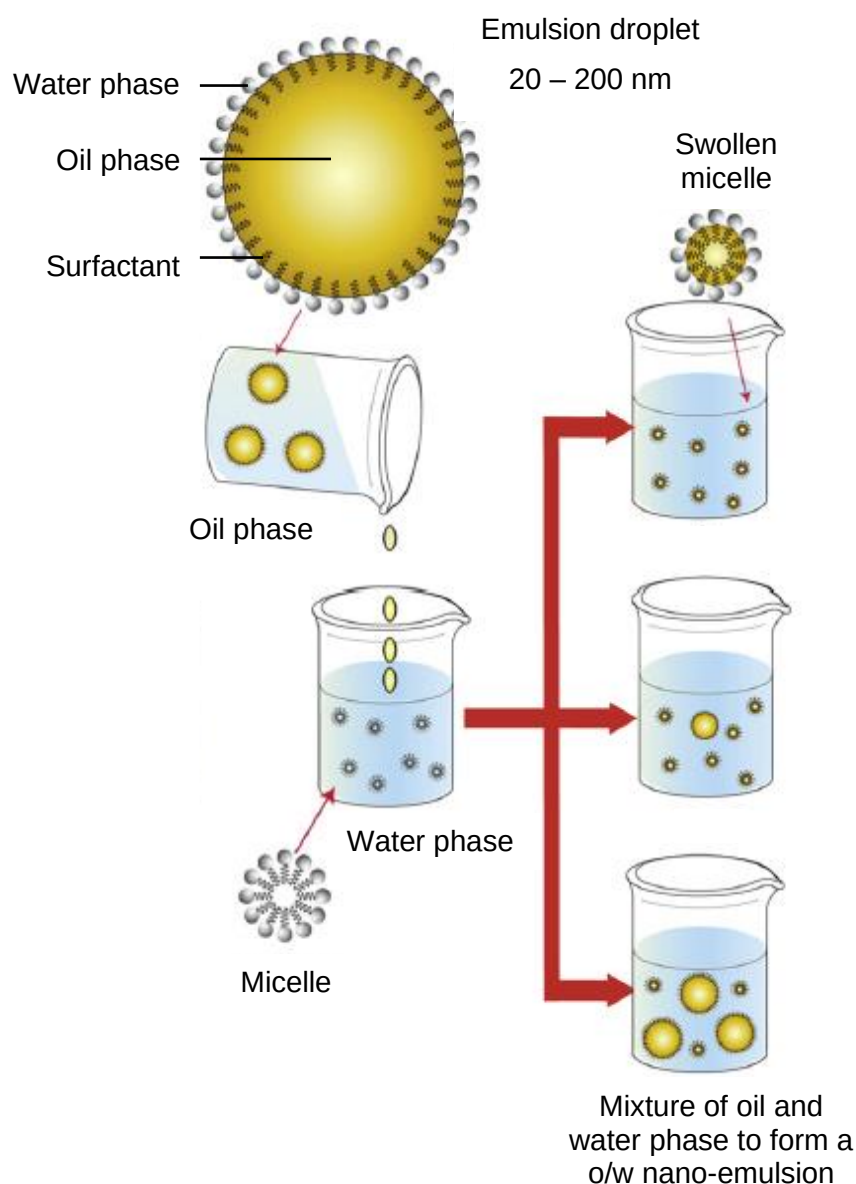


Figure 2.7: Representation of an o/w nano-emulsion formation with the droplet acting as a reservoir for the lipophilic APIs (adapted from Rao & McClements, 2012:327).

2.9.2 Applications of nano-emulsions in transdermal drug delivery

2.9.2.1 Advantages of nano-emulsions

These formulations have been developed to overcome the problems with conventional delivery systems (Jaiswal *et al.*, 2015:123). They have the ability to dissolve large quantities of hydrophobic substances and to protect substances from hydrolysis and degradation (Lovelyn & Attama, 2011:626). The small droplet sizes reduce the gravitational force, prevent flocculation, keep the system dispersed and prevent fluctuations. Nano-emulsions are efficient in their delivery of ingredients through the skin, with the large surface area of the emulsions. This allows for rapid penetration of the active constituents through the skin (Lovelyn & Attama, 2011:629). The nano-

sized particles ranging from 20 – 200 nm were perfect for penetration through the stratum corneum (Jaiswal *et al.*, 2015:123).

Delivery of a nano-emulsion system is based on active compounds encapsulated into the system for delivery (Sessa *et al.*, 2014:42). These systems can increase aqueous solubility of APIs that are poorly water soluble. Factors that determine the transdermal permeation of the system can depend on the mobility of the drug into the nano-emulsions, the release of the API out of the nano-emulsion and the amount of API that permeates through the skin (Thakur *et al.*, 2013:16). Water in combination with surfactant is needed for producing nano-emulsions and can cultivate the inversion of phases and increase droplet size and polydispersity of a formulation (Wang *et al.*, 2007:232). Dilution of surfactants into water increases the stability of a dispersion and in turn can enhance the application factor on the skin and increase the barrier function of the nano-emulsion. This is done by increasing the viscosity and permeability rate of the substance to ensure controlled release (Kaplan *et al.*, 2019:198). The o/w nano-emulsions were formulated to help lipophilic drugs improve their bioavailability (Daull *et al.*, 2013:531). The surfactants and co-surfactants, as well as the oil phase increases drug diffusion by enhancing the partition coefficient through the skin (Abd *et al.*, 2016:213). The interfacial regions used to stabilise the molecules between the oil and the water droplets can lead to an increase in the release of drugs (Klang *et al.*, 2010:159). These formulations and their fluidity, and the lack of gelling or thickening agents give them a pleasant feeling on the skin (Lovelyn & Attama, 2011:629).

2.9.2.2 Disadvantages of nano-emulsions

Preparation of nano-emulsions requires special techniques to obtain the needed particle size for transdermal delivery. To achieve the desired droplet size of the particles in a nanometric range, a method of high-pressure homogenisation needs to be used (Sharma *et al.*, 2015:546). Interfacial tension between particles can be reduced by the addition of emulsifiers, surfactants and amphiphilic compounds, as this prevents the droplets from aggregating and re-coalescence, and the type of surfactant used determines the particle size of the droplet formation (Santana *et al.*, 2013). Large amounts of surfactants or co-surfactants are needed for the formation of nano-emulsions to provide stabilisation for microdroplets, where temperature and pH can also affect the stability (Bhosale *et al.*, 2014:123).

2.10 Semi-solid formulations

Semi-solid dosage forms provide a way of carrying APIs to the skin for topical and transdermal delivery. Semi-solid dosage forms can prolong the period of time substances have contact with the skin and in this way enhance drug delivery through the skin. These formulations enhance the physicochemical properties of the API for application on the skin (Gupta & Garg, 2002:144). Dosage forms include creams, gels and ointments (Olejnik *et al.*, 2012:4032). Semi-solid dosage

forms are non-greasy and cause less irritation to the skin and can be easily removed. This route of administration avoids the first-pass effect and in turn reduces gastro-intestinal irritation, this can slow down the process of drug uptake, but prolong local effects (Bharat *et al.*, 2011:421).

2.10.1 Emulgel

Emulgels are an ideal dosage form to deliver hydrophobic drugs. This formulation is a combination between a gelling agent and an emulsion, where the gelling agent is incorporated into the water phase (Khullar *et al.*, 2011:63). These formulations can decrease the surface tension by increasing the viscosity of the aqueous phase; they exert better application profiles and have a faster release of drugs from vehicles to the skin (Yassin, 2014:1015).

Although emulgels have advantages of formulating drugs in this manner, this formulation method can have disadvantages. These formulations can cause poor permeability of some APIs through the skin because of the large particles present in the formulation and thus cannot be absorbed (Singla *et al.*, 2012:487); this can be a huge limitation for this formulation. According to Jivani *et al.* (2018:1), these formulations can be made into a nano-emulgel to increase drug permeability.

2.10.2 Nano-emulgel

Nano-emulgels can be described as two immiscible liquids (oil and water) that are stabilised by surfactants or co-surfactants and then incorporated into a gel matrix (Dhawan *et al.*, 2014:65). Due to the nano-sized droplets, ranging from 20 – 200 nm (same as nano-emulsions), dispersions can overcome the obstacle of skin penetration as this will improve the permeation through the skin barrier (Chellapa *et al.*, 2015:43); the API is successfully released out of the vehicle.

Formulations in a nano-emulgel-based formula increases patient compliance due to the non-sticky and easily spreadable nature of the formulation. Besides this formulation having an extended release controlling effect on the drugs, it also increases the half-life of APIs (Chellapa *et al.*, 2015:45). A nano-emulgel formulation of each of the selected statins will be formulated by utilising the o/w nano-emulsion method with the addition of Carbopol® Ultrez 20 as the gelling agent, since this formulation is the most advantageous for transdermal delivery.

2.11 Conclusion

Hypercholesterolemia is focused on reducing the levels of LDL cholesterol by the use of statins (HMG-CoA reductase inhibitors). Familial hypercholesterolemia (FH) has high concentrations of LDL-cholesterol and this enhances the deposits of cholesterol into peripheral tissue that gives rise to atherosclerosis and in turn, coronary heart disease (Soutar & Naoumova, 2007:214). FH affects 1 in 500 persons worldwide and secondary causes of hypercholesterolemia can include

hypothyroidism, diabetes and renal or hepatic disease. In a study implemented on a group of 100 German people, mutations in 39 patients were found (Dedoussis *et al.*, 2004:3). Statins are a great advancement in the treatment of FH by decreasing the LDL levels (Raal & Santos, 2012:266), although statins have a tendency to cause side-effects that include myalgia (Ramkumar *et al.*, 2016:632), and rhabdomyolysis (Golomb & Evans, 2008:377).

Due to these side-effects, poor patient compliance with the use of statins are associated (Casula *et al.*, 2012:805), therefore the transdermal route of delivery can be proposed as an alternative route of administration to overcome the limitation set out by the oral route. The transdermal route proposes many advantages such as avoiding the stomach environment where APIs are degraded (Singh *et al.*, 2014:282). It avoids the first-pass metabolism, which in most cases is responsible for the side-effects of certain APIs. The transdermal route ensures a steady plasma level of drugs, is a non-invasive route and improves bioavailability (Marwah *et al.*, 2014:564). Consequently, for transdermal delivery, the barrier of the stratum corneum must be crossed, thus the physicochemical properties of the selected statin must be of the desired measurements in the formulation (nano-emulsions and nano-emulgels). These formulations will be compared in this study for the purpose of membrane release studies and *in vitro* skin diffusion studies.

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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 Johandré van Jaarsveld.

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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 high-performance liquid chromatography (HPLC) method was developed and validated to simultaneously analyse statins (atorvastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin). A Venusil XBP C₁₈ (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 started at 25% acetonitrile (ACN) and increased linearly of 90% after 1.0 min, subsequently it was held at 90% until 6.5 min and then re-equilibrated to 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 were indicative of concentration ranging from 0.2-206.4 µg/ml and correlated with the (R²) 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, and is characterised by high plasma levels of low-density lipoprotein cholesterol (LDL), which in turn can increase coronary heart diseases [Civeira, 2004]. Worldwide, approximately 80% of males and 50% of 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. The preferred cholesterol lowering treatment is 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 [Gazzerro et al. 2012; McFarland et al. 2014; McFarlane et al. 2002].

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 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 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, and 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 $\mu\text{g/ml}$). 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 $\mu\text{g/ml}$), 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 $\mu\text{g/ml}$). 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. The results obtained are 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, it could be concluded there was a strong relationship between the peak area and the statin concentration; the 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 percentage relative standard deviation (%RSD) for each of the statins were as follows: 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 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 was used for inter-day precision, but only one concentration was selected. For this validation, the intermediate amount of 0.8 g of the each of the statin 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 Rafael et al. 2007 (%RSD should be ≤3%), and 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 have 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) water (H₂O), 2) hydrochloric acid (HCl), 3) sodium hydroxide (NaOH) and 4) hydrogen peroxide (H₂O₂), as illustrated in Figure 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 are ≤20% [FDA 2001; González et al. 2014]. The results shown in Table 1 fell within 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, as the results of each of the selected statins analysed by this HPLC method, were satisfactory (see Fig. 2).

3. Experimental

The developed method was done 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 C₁₈ (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 active pharmaceutical ingredients (APIs); the selected run time was 10.5 min at an injection volume of 10 µl. The mobile phases used were 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 increased linearly to 90% after 1.0 min, held at 90% for 6.5 min and then re-equilibrated to the starting conditions. The solvent 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.

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152 Campus, South Africa.

153 **Disclaimer**

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

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 224 [and%20Protocol%20in%20the%20Quality%20Sytem%20Compliance%20Industry.pdf](http://www.ivtnetwork.com/sites/default/files/StepbyStep%20Analytical%20Methods%20Validation%20and%20Protocol%20in%20the%20Quality%20Sytem%20Compliance%20Industry.pdf) Date of access:
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239

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

	Atorvastatin	Fluvastatin	Lovastatin	Mevastatin	Pitavastatin	Pravastatin	Rosuvastatin	Simvastatin
Linearity								
Correlation coefficient R²	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

241

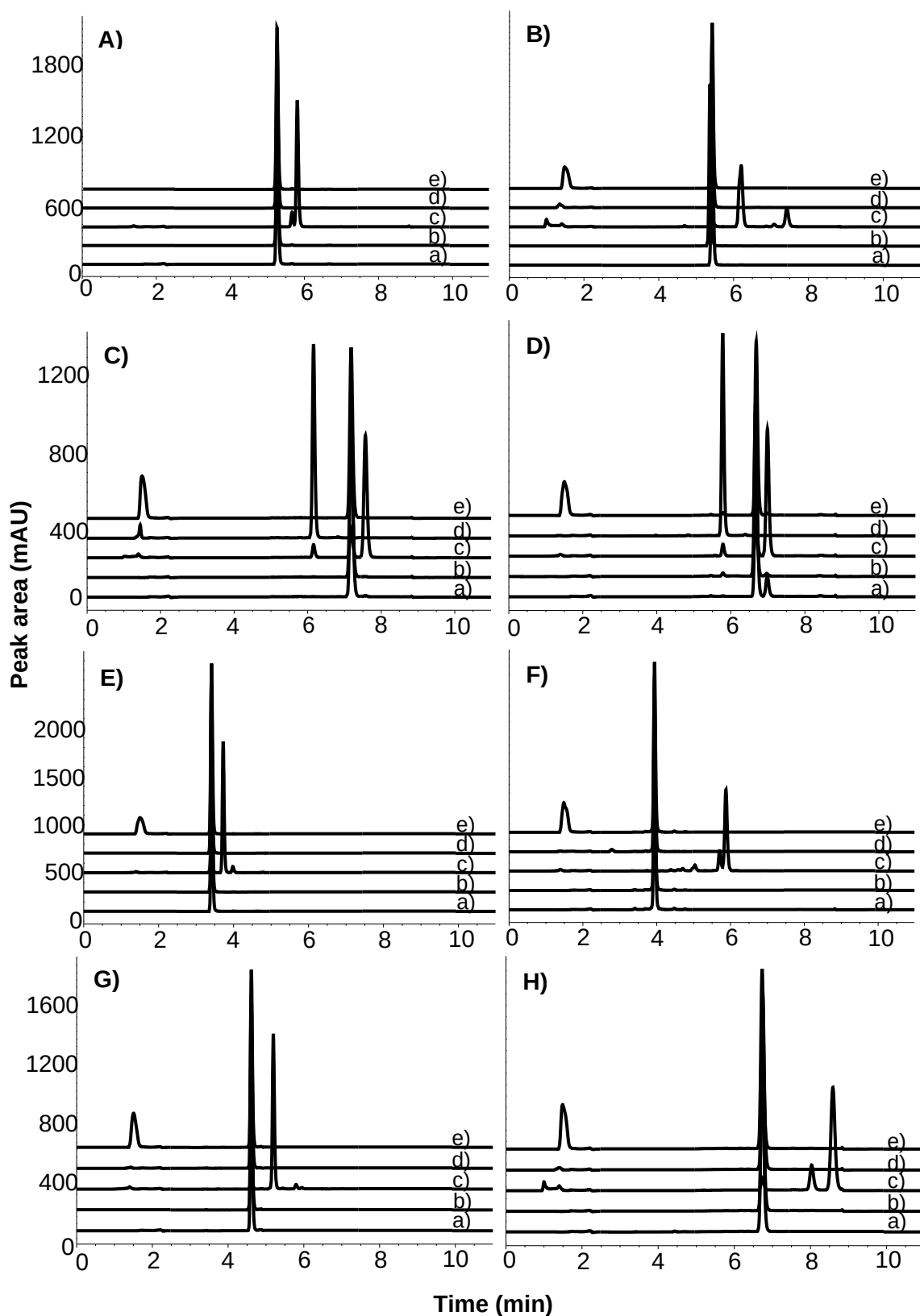


Fig. 1: HPLC chromatogram showing specificity data obtained for: A) atorvastatin, B) fluvastatin, C) lovastatin, D) mevastatin, E) pitavastatin, F) pravastatin, G) rosuvastatin and H) simvastatin; in addition: a) standard solution of each sample and samples stressed with 200 μ l of b) H_2O , c) HCl ,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

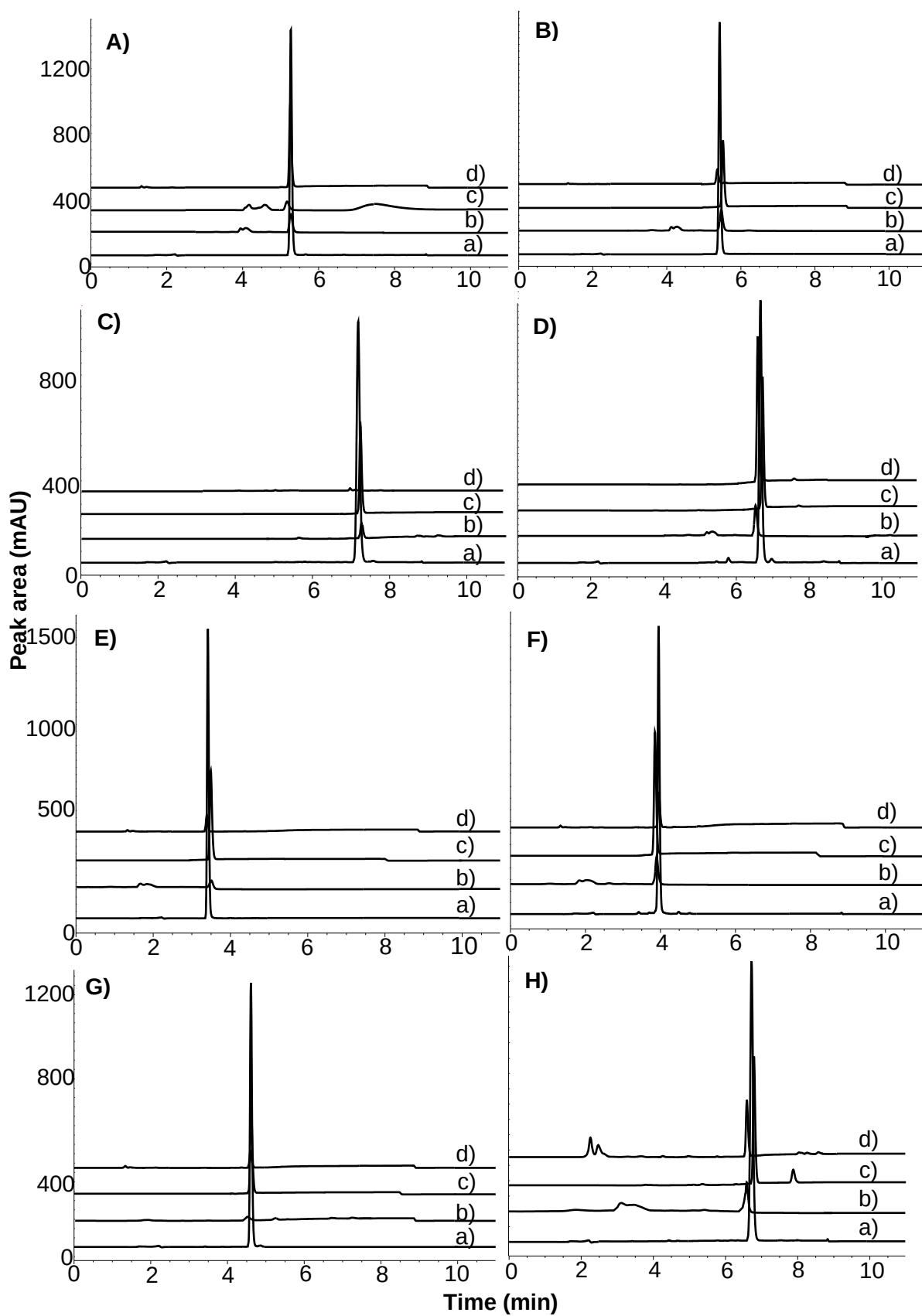


Fig .2: Chromatogram representation of: A) atorvastatin, B) fluvastatin, C) lovastatin, D) mevastatin, E) pitavastatin, F) pravastatin, G) rosuvastatin and H) simvastatin; in addition: a) standard solution, b) receptor phase extraction, c) tape-stripping (SCE) and d) skin samples (ED)

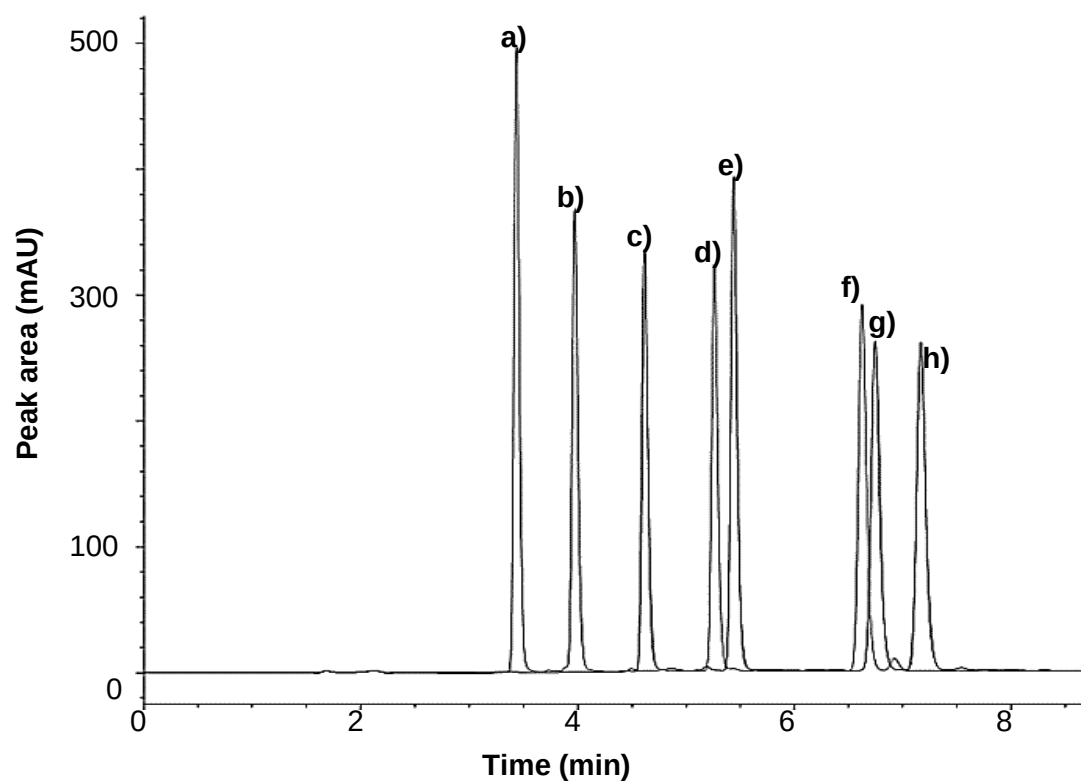


Fig. 3: Chromatogram representation of the simultaneous detection of the different statins: a) pitavastatin, b) pravastatin, c) rosuvastatin, d) atorvastatin, e) fluvastatin, f) mevastatin, g) simvastatin, h) lovastatin

Chapter 4

ARTICLE FOR THE PUBLICATION IN THE JOURNAL OF PHARMACEUTICAL SCIENCES

This chapter is written for publication in the Journal of Pharmaceutical Sciences. The complete author's guide is listed in Appendix G. The author's guide states that the font size should preferably be written in 10 pt Helvetica and that the author has a choice of writing in UK or US English. This chapter is subsequently written in 10 pt Helvetica and UK English. Numbering of text references are not included for this article, as it will be numbered before being submitted for publishing.

Selected statins formulated in nano-emulsions and nano-emulgels for transdermal delivery

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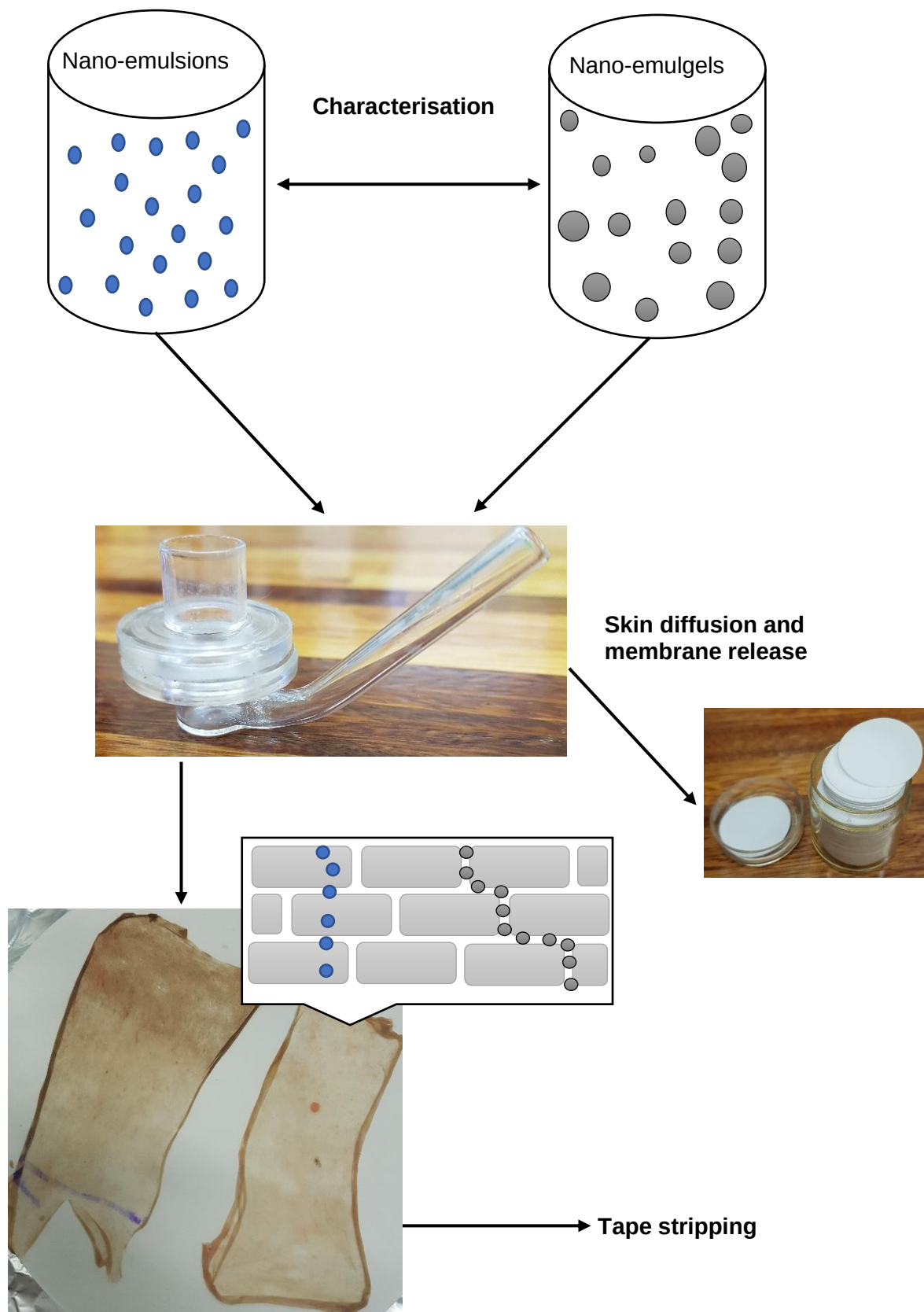
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Abstract

During this study four statins (fluvastatin, lovastatin, rosuvastatin and simvastatin), in a concentration of 2%, were incorporated separately into nano-emulsions and nano-emulgels (both containing grapeseed oil (penetration enhancer) as oil phase), and subsequently compared. The aim of this study was to enhance the transdermal delivery of the selected statins, which have different and less than ideal physicochemical properties, and to determine which delivery vehicle proved most favourable for drug delivery. During the membrane release studies, fluvastatin and rosuvastatin were the most favourable active pharmaceutical ingredients (APIs) released respectively from the nano-emulsion and nano-emulgel. The aforementioned trend was also observed during the 12 h *in vitro* skin diffusion study. Tape stripping showed that the nano-emulsion containing fluvastatin obtained the highest results in both the stratum corneum epidermis (SCE) and epidermis (ED), while the nano-emulgel containing rosuvastatin delivered the highest median concentration in the SCE and ED. Although the nano-emulsions obtained the best results for transdermal delivery in this study, it can be proposed that nano-emulgels are the desired vehicle for transdermal delivery, since they release the API in a more controlled manner, which may lead to more steady plasma levels.

Keywords: Statins, nano-emulsion, nano-emulgel, penetration enhancer, physicochemical properties, membrane release, skin diffusion, tape stripping, transdermal delivery

Graphical abstract



1 Introduction

Cholesterol is found naturally in the body and is present in the cell walls to ensure that the cells in the body function as one unit (Scherr & Zidenberg-Cherr, 2016). Cholesterol has an impact on coronary heart disease, once the serum cholesterol levels are elevated (Lütjohann *et al.*, 2019). Elevated serum cholesterol levels, known as hypercholesterolemia, include the accumulation of cholesterol in the liver and spleen (Klein *et al.*, 2006). Hypercholesterolemia is caused by the shortage of circulating high-density lipoproteins (HDL), which poses a risk to the cardiovascular system (Cuchel *et al.*, 2010), and can be treated by cholesterol lowering agents that include statins (classified as HMG-CoA (as 3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors), niacin and ezetimibe. HMG-CoA reductase inhibitors are classified as the first line of treatment for hypercholesterolemia (Raaijmakers *et al.*, 2018) and are seen as vasculature protectors, since they have the ability to lower low density lipoproteins (LDL) and increase circulating HDL (Stalker *et al.*, 2001). HMG-CoA reductase inhibitors serve as a well-established form of treatment for hypercholesterolemia as an oral dosage form.

Since the statins are administered orally, they possess many disadvantages, which include gastrointestinal disturbances such as bloating, diarrhoea and constipation; these side effects may be caused by the disturbances in the cholesterol-bile excretion (Wierzbicki *et al.*, 2003). Due to the side effects statins exert after being taken orally, other routes of administration have been investigated. The transdermal route of delivery involves delivery of the statins through the layers of the skin and directly into the bloodstream; this route enhances the bioavailability of statins and ensures a more uniform plasma concentration that has a longer duration of action (Ahad *et al.*, 2015).

The skin consists of three layers, namely the epidermis (outer layer contains the stratum corneum), the dermis and the hypodermis (Mikesh *et al.*, 2013). The stratum corneum is the layer that provides protection for the skin against environmental factors and acts as a penetration barrier (Kumar *et al.*, 2015). The stratum corneum consists of a lipid matrix, therefore for active pharmaceutical ingredients (APIs) to penetrate through they must consist of the ideal physicochemical properties (Kumar *et al.*, 2015), which include factors such as diffusion coefficient, pKa and pH and also an octanol-water partition coefficient (log P) between 1 and 3, molecular weight of <500 Da, aqueous solubility of >1 mg/ml and a melting point of <200°C (Naik *et al.*, 2000; Rastogi & Yadav, 2012). The statins (fluvastatin, lovastatin, rosuvastatin and simvastatin) selected during this study exert non-ideal physicochemical properties and should therefore be formulated in a carrier system that enhances the permeation through the skin barrier (Shakeel *et al.*, 2007). Subsequently, nano-emulsions were chosen due to their small droplet size and because the delivery vehicle can either be hydrophilic or lipophilic (Kong *et al.*, 2011). Nano-emulsions were compared to nano-emulgels (nano-emulsion with an added gelling agent), since nano-emulgels are known to act as a controlled release system with the API entrapped (Ajazuddin *et al.*, 2013). Due to the barrier function of the skin, it was decided to include a chemical penetration enhancer during the formulation of the nano-emulsions and the nano-emulgels. Penetration enhancers (like fatty acids) regulate between the lipid junction of the skin to obtain the desired permeation (He *et al.*, 2009), since the epidermal layer consists of ceramides and free fatty acids, i.e. oleic and linoleic acid (Elias & Wakefield, 2015). Oleic and linoleic acids are important unsaturated fatty acids that improve permeation through human skin (Pathan & Setty, 2009). Grapeseed oil consists of phenolic compounds and has a high linoleic acid content, which was incorporated as the oil phase of the nano-emulsions and nano-emulgels during this study (Mbah, 2007).

The nano-emulsions and nano-emulgels were formulated with grapeseed oil, as mentioned above, and containing the statins with the aim of determining if these statins could be delivered transdermally, and if successful, which formulation was optimal. The different formulations tested were nano-emulsions with fluvastatin (**NEF1**), lovastatin (**NEL1**), rosuvastatin (**NER1**) and simvastatin (**NES1**) and nano-emulgels with fluvastatin (**NEGF**), lovastatin (**NEGL**), rosuvastatin (**NEGR**) and simvastatin (**NEGS**).

2 Materials and Methods

2.1 Materials

Fluvastatin, lovastatin, rosuvastatin and simvastatin were obtained from DB Fine Chemicals (Johannesburg, RSA), while grapeseed oil was obtained from CJP Chemicals (Johannesburg, RSA). Tween® 80, Span® 60, Carbopol® Ultrez 20 and methanol (all of analytical grade) were obtained from Sigma-Aldrich (Johannesburg, RSA). To prepare the phosphate buffer solution (PBS) at pH 7.4, dihydrogen orthophosphate (KH₂PO₄) and sodium hydroxide (NaOH) were used, which were obtained from Sigma-Aldrich (Johannesburg, RSA). For the high performance liquid chromatography (HPLC) deionised grade water (Millipore, Milford, USA) was used together with mobile phases consisting of octane sulphonic acid-Na (Sigma-Aldrich, Johannesburg, RSA) in combination with Milli-Q® water (deionised) attained from a Milli-Q® Academic water purification system (Merck-Millipore, Midrand, RSA).

2.2 Methods

2.2.1 Formulation of nano-emulsions and nano-emulgels

Eight formulas were formulated, each containing the four statins respectively, in o/w nano-emulsions (**NE1**) and nano-emulgels (**NEG**). The (**NE1**) were formulated firstly by weighing all the excipients and mixing the Tween® 80 in the water phase with a magnetic stirrer on a hot plate (water phase). Secondly, the oil was heated to ±70°C on a heat plate, where after the Span® 60 was dissolved in the grapeseed oil (oil phase), after which the API was added. When all the excipients were dissolved in the respective phases, the oil phase was added to the water phase in a drop wise manner, resulting in the formation of the emulsion. After stirring to ensure proper mixing, the emulsion was ultrasonicated to obtain nano-sized particles; this resulted in a nano-emulsion. To formulate the (**NEG**), Tween® 80 was dissolved in water followed by the addition of Carbopol® Ultrez 20, the gelling agent, then stirred with a magnetic stirrer for 10 min to ensure proper dissolution. The oil phase for the (**NEG**) was prepared as for the (**NE1**). After stirring of the water phase, it was ultrasonicated for 3 min to free any entrapped air, after which it was transferred to an overhead mechanical stirrer, the oil phase and the dissolved excipients were then transferred in a drop-wise manner to the water phase. This formulation was then stirred for 15 min at 850 rpm whereafter the final (**NEG**) was ultrasonicated for 3 min to ensure droplet sizes were in the nano-metric range. The (**NEG**)'s pH was then adjusted according to that of the (**NE1**); due to the addition of Carbopol® to the gel formulations, the pH is lower than that of the nano-emulsions. The pH was adjusted with the use of sodium hydroxide (NaOH) to ensure it was close to that of the skin to avoid skin irritation (Barry 2002:512). The formulas used to prepare the (**NE1**) and (**NEG**) are presented in Table 1.

Table 1:

Excipients used in the formulation of the nano-emulsions and the nano-emulgels (50 ml)

2.2.2 HPLC analysis of statins

An HPLC method was developed and validated for the determination of the selected statins. An HP1100 series equipped with a gradient pump, UV detector and autosampler injector mechanisms was fitted to the system. A Venusil XBP C18 (2) reverse phase column (150 x 4.6 mm) with a particle size of 5 µm (Agela Technologies, Newark, DE) was utilised. The UV detector was set at 240 nm, flow rate of 1.0 ml/min was set and the injection volume was 10 µl. Mobile phases were made with 0.005 M (0.2%) octane sulphonic acid-Na (pH 3.5) and acetonitrile (ACN). The gradient was started at 25% acetonitrile and increased with linearity to 90% after 1.0 min, then held at 90% until 6.5 min and then re-equilibrated to start conditions. A total run time of 10.5 min was set for the validation of statins. Retention times for selected statins were $\pm$ 3.65, 4.09, 4.50 and 7.12 min for fluvastatin, lovastatin, rosuvastatin and simvastatin, respectively. For these statins, methanol was used as the solvent and all experiments were performed under a controlled temperature of 25°C. Limit of detection (LOD) and limit of quantification (LOQ) were determined for the use in skin diffusion studies (see Table 2).

Table 2:

LOD (µg/ml) and LOQ (µg/ml) of the respective statins

2.2.3 Standard preparation

Standard preparation of statins was utilised by weighing $\pm$ 20 mg of each of the selected statins into a 100 ml volumetric flask, whereafter they were made up to volume with methanol. Two dilutions were made of the stock solution: for the first dilution, 5 ml of the stock solution was made up to volume (100 ml) with methanol, and for the second dilution, 5 ml of the first dilution was made up to volume (100 ml) with methanol to obtain concentrations ranging from 0.5 to 200.0 µg/ml. The solution along with the two dilutions were injected in the HPLC at different volumes (2.5, 5.0, 7.5, 10.0 µl) to obtain a standard curve.

2.3 Characterisation of formulations

Each of the selected statins (fluvastatin, lovastatin, rosuvastatin and simvastatin) were characterised in terms of transmission electron microscopy (TEM), pH, droplet size and zeta-potential, and viscosity for the nano-emulsions and nano-emulgels.

2.3.1 TEM

An FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope (HRTEM) (Czech Republic, EU) with an Oxford INCA X-Sight EDS System was used to determine the morphology of the **(NE1)**. TEM was only performed on the selected optimised **(NE1)** due to the cost of the experiment. Dilutions were made of the **(NE1)** with Milli-Q® water, then a small amount of the diluted nano-emulsion was transferred to a microscopic carbon-coated 300 mesh copper grid, left to dry for 10 min, whereafter it was stained with osmium tetroxide to provide high contrast images (Vatsraj *et al.*, 2012).

2.3.2 pH

For determination of the pH, a Mettler Toledo® pH meter (Mettler Toledo, CU), equipped with a Mettler Toledo® InLab® 410 electrode, was used to obtain values. Calibration of the apparatus was performed before the measurements took place; these measurements were done at 32°C in triplicate.

2.3.3 Viscosity

Viscosity readings were done with a Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA). The formulas were acclimatised in a water bath at 25°C and placed into the water bath half an hour prior to the test. Viscosity was determined by measuring the resistance of the spindle in the formulation; for nano-emulsions a SC4-18 cylindrical spindle was used, while a T-bar was used to measure viscosity of the nano-emulgels (they have much higher viscosity). Measurements were taken every 10 sec for 2 min on Rheocalc T 1.2.19 software, which allowed an average viscosity value to be determined.

2.3.4 Droplet size

Droplet size of all eight formulas was measured with a Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom). These readings were made in triplicate by placing 1 ml of the selected formulation in 20 ml Milli-Q® water and transferring it by means of a syringe into a clear disposable zeta-cell (DTS1070 folded capillary cell).

2.3.5 Zeta-potential

This method was identical to the method used in Section 2.3.4, and formulations were placed in a clear disposable zeta-cell (DTS1070 folded capillary cell). Triplicate measurements were taken for each of the formulations over a 300 sec period. Average zeta-potential was determined from results obtained.

2.4 Diffusion studies

2.4.1 Membrane release studies

Membrane release studies were performed by preheating the eight formulas to 32°C in a water bath. The PBS mixture with 10% denatured ethanol, prepared at a pH of 7.4, was pre-heated in a second water bath to 37°C prior to the membrane release studies. A Franz cell was used during membrane release studies and prepared by using Dow Corning® high vacuum grease to apply on both the donor and receptor compartments of the Franz cell. A magnetic stirrer was placed on the inside of the receptor compartment, whereafter a synthetic membrane (polyvinylidene fluoride (PVDF)), Pall® Life Sciences, Michigan, United States) was placed on the receptor compartment with a pore size of 0.45 µm and 25 mm in diameter. After the membrane was placed on the receptor compartment, the donor compartment was placed on top. The two compartments were sealed thoroughly with vacuum grease on each side to prevent the Franz cell from leaking, and horseshoe clamp used to fasten the two compartments tightly. The receptor compartments contained 2 ml PBS/ethanol (9:1), which was inspected to ensure no air bubbles formed. The donor compartments were filled with 1 ml of the formulas and a piece of Parafilm® used to cover the donor compartment with a plastic cap. The Franz cells were then transferred into a water bath (Grant Instruments, United Kingdom), pre-heated to 37°C, with a magnetic stirring plate (Variomag, United States). The receptor phase was extracted every hour for six consecutive hours and refilled with PBS/ethanol (9:1) kept at 37°C. The extracted receptor phases were then placed into vials for HPLC analysis to determine the concentration of API that permeated through the membrane into the receptor fluid.

2.4.2 Skin preparation

Skin that was obtained after abdominoplasty of Caucasian females (Ethics approval number: NWU-00111-17-A1-04) and a Dermatome™ (Zimmer TDS, United Kingdom) were used to obtain a thickness of ±400 µm. The dermatomed skin samples were placed on Whatman® filter paper and enfolded in aluminium foil then stored in the fridge at a -20°C until needed for skin diffusion studies. Before commencing with an *in vitro* skin diffusion study, the skin was cut into circles and placed between the donor and receptor phase of the Franz diffusion cell. Skin samples were inspected for any defects before the study began.

2.4.3 Skin diffusion

For the skin diffusion studies, the same technique was used as in Section 2.4.1. During the membrane release studies, a PVDF synthetic membrane was used, and for the *in vitro* skin diffusion studies the dermatomed skin was used. The skin was placed between the donor and receptor compartments, where after the compartments were sealed with vacuum grease and a horseshoe clamp. The receptor compartment was filled with 2 ml PBS/ethanol (9:1) and the donor compartment with 1 ml of formulation. Extractions were made only once after

12 h and the receptor phase was placed into vials for HPLC analysis to determine the amount of formulation that diffused through the skin (Ubaidulla *et al.*, 2007).

2.4.4 Tape stripping

Tape stripping was done after the 12 h *in vitro* skin diffusion studies to evaluate the effectiveness of the formulations and the absorbance through the skin (Demierre *et al.*, 2012). Tape stripping was performed by separating the horseshoe clamp and the Franz cells from each other. The skin samples were carefully removed and placed on a piece of Parafilm®, then pinned to a hard surface. The skin was dabbed with a piece of hand towel to remove any excess formulation left on the skin. 3M Scotch® Magic™ tape was cut into ± 16 pieces, in sufficient sizes that covered the diffusion area. The first tape strip was discarded, as it contained formulation left on the skin and could cause contamination. Each of the strips used was placed into a polytop with 5 ml PBS/ethanol (9:1) and served as the stratum corneum epidermis (SCE) results. Another polytop with 5 ml PBS/ethanol (9:1) was used for the remaining skin, also known as the epidermis-dermis (ED). These polytops for each study were left in a fridge ($\pm 4^{\circ}\text{C}$) for approximately 8 h. After this period, a volume of 1 ml was extracted by means of a syringe fitted with a $0.45\ \mu\text{m}$ polytetrafluoroethylene (PTFE) filter and injected into HPLC vials for analysis.

2.5 Data analysis

Each of the samples for the membrane release and *in vitro* skin diffusion studies were analysed by means of HPLC. The raw data obtained was then analysed according to a linear graph that was obtained after the standard solutions were injected (Section 2.2.3). For the membrane release studies, the average cumulative amount of each statin per area diffused was plotted against time (h) (Shakeel *et al.*, 2007) to obtain the flux of each Franz cell (Ng *et al.*, 2010). For the 12 h skin diffusion studies, the median amount per area ($\mu\text{g}/\text{cm}^2$) that diffused through the skin was used.

2.6 Statistical analysis

Statistical analysis was performed on each of the selected statins in their formulations for membrane release and skin diffusion studies, as well as the tape stripping for SCE and ED. Statistical data analysis was performed by using the analysis of variance (ANOVA) statistical model testing between the mean differences in more than two conditions at the same time (Boisgontier & Cheval, 2016). The summarised data were given by means of box plots to illustrate the obtained data in terms of a lower quartile, media, upper quartile, extreme value and mean (Krzywinski & Altman, 2014). After the ANOVA test were performed, Levene's test for homogeneity of variances was performed to test the equal variances of the data from different groups. The mean differences were determined after the Levene's test by utilising the Tukey Honest Significant Difference (HSD) multiple comparison test; this test ranks the groups according to their homogeneity (Hamada, 2018). The p-values are used to summarise the data and draw a conclusion. The p-value normally draws a conclusion of statistical significance ($p < 0.05$) and statistical insignificance ($p > 0.05$) (Consonni & Bertazzi, 2017).

3 Results and Discussion

3.1 Formulation of nano-emulsions and nano-emulgels

Eight formulations were prepared using 2% of API (statins), four nano-emulsions and four nano-emulgel formulations. Nano-emulsions exerted lower viscosity, while nano-emulgels consisted of a higher viscosity. Nano-emulgels were formulated to overcome the limitations set out by the nano-emulsions.

3.2 Characteristics of semi-solid formulations

The data obtained from TEM, pH, droplet size, zeta-potential and viscosity are summarised in Table 3.

Table 3:

Characterisation results for both the **(NE1)** and the **(NEG)**

pH measurement is a ration between hydroxide ions and their concentrations (Hach Company, 2010). A pH value lower than 3 or higher than 9 can influence the permeability through the skin, as it compromises the integrity of the skin (Barry, 2002). It can be seen from Table 3 that the pH of **(NE1)** was between 4 and 8, which was lower than 9. The pH of **(NEG)** was lower than that of **(NE1)**, because Carbopol® Ultrez 20 has an acidic value (Gupta & Vyas, 2010), but still fell within the acceptable pH range for transdermal delivery. A zeta-potential value of +30 mV and -30 mV, or more, is considered stable over time (Eid *et al.*, 2014; Marsalek, 2014; Silva *et al.*, 2012). It can be stated that the charge of the **(NEG)** was much higher than the charge of the **(NE1)** (except for **(NEL1)** and **(NEGL)**), thus it can be stated that the semi-solid dosage forms will be more stable than the nano-emulsion. The droplet size should range preferably between 50 and 200 nm for successful transdermal delivery (Tadros *et al.*, 2004); all the **(NE1)** and the **(NEG)** fell within the droplet size range, except for **(NEGS)**, which had a slightly higher value of 200.83 nm. The droplet size readings for the **(NE1)** correlated well with the results obtained from the TEM micrographs (Table 3). The determination of viscosity was compared to literature, which states that the addition of surfactants and oil components increases the viscosity by means of increasing interfacial tension between oil and water (Chime *et al.*, 2014); concerning **(NEG)**, the addition of the gelling agent (Carbopol® Ultrez 20) increased the viscosity (Bowen-Leaver *et al.*, 2006). After the **(NEG)** formulations were neutralised, a higher viscosity reading was obtained, as literature suggested (Lubrizol, 2009).

3.3 Membrane diffusion experiments

In Fig. 1, the median flux values for the **(NE1)** and the **(NEG)** are indicated; it can be seen that **(NEF1)** showed the highest median flux value, followed by **(NER1)**, **(NEL1)** and lastly **(NES1)** when the **(NE1)** were compared. For the **(NEG)**, **(NEGR)** revealed the highest median flux, followed by **(NEGF)**, **(NEGS)** and lastly, **(NEGL)**. The **(NE1)** presented with an overall higher median flux than **(NEG)**. Hence, the statins were released from all the different formulations.

Fig. 1: Box-plots indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of the **(NE1)** and the **(NEG)** of all four statins during the membrane release studies over 6 h

For the membrane release studies, statistical analyses were performed by means of an ANOVA test, t-test for independent samples and Levene's test for homogeneity. A two-way ANOVA was performed on the **(NE1)** and the **(NEG)**, and for both the p-value was <0.001 indicating the results were statistically significant. After the two-way ANOVA, a one-way ANOVA was separately performed on the **(NE1)** and the **(NEG)** to observe if there were any interactions between the statins in different formulations. Both the **(NE1)** and the **(NEG)** had a p-value of <0.001 , which indicated statistical significance. A Tukey's HSD was performed for the statins, and it was suggested that simvastatin and lovastatin were homogenously grouped together, followed by rosuvastatin and fluvastatin, which had no similarities for **(NE1)**. On inspection of **(NEG)**, it was observed that no statin shared similar properties and it was grouped individually.

3.4 Diffusion experiment

3.4.1 Diffusion study

It was found that the **(NE1)** with the highest median amount per area diffused through the skin ($\mu\text{g}/\text{cm}^2$) was **(NEF1)**, followed by **(NES1)**, **(NER1)** and lastly, **(NEL1)**. The **(NEG)** with the highest median amount per area diffused ($\mu\text{g}/\text{cm}^2$) was **(NEGR)**, followed by **(NEGF)**, **(NEGS)** and lastly, **(NEGL)**. The **(NE1)** (**NEF1**, **NEL1**, **NER1**, **NES1**) presented with higher median amounts per area diffused ($\mu\text{g}/\text{cm}^2$) than their **(NEG)** counterparts (**NEGF**, **NEGL**, **NEGR**, **NEGS**). The lower median amount per area diffused for the **(NEG)** can be ascribed to the fact that Carbopol® Ultrez® 20 was added to these formulations, which in turn increased viscosity that delivered the formulation in a more controlled manner (Shah *et al.*, 2012). In both **(NE1)** and **(NEG)**, lovastatin presented with the lowest median amount per area diffused. These results can be attributed to the poor physicochemical properties the statins exert for transdermal delivery (Naik *et al.*, 2000).

Fig. 2: Box plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) for each of the selected statins in a) **(NE1)** after 12 h, and b) **(NEG)** after 12 h

Skin diffusion studies were statistically analysed by means of a two-way ANOVA followed by the Kruskal-Wallis ANOVA (one-way ANOVA). For the two-way ANOVA, the statins had a p-value of <0.001 in both the **(NE1)** and the **(NEG)**, implying statistical significance; thereafter statins were ranked according to their cumulative concentrations performed by the Kruskal-Wallis test. For the **(NE1)**, statins were placed in order from the highest to the lowest cumulative concentration as follows: **(NEL1)** > **(NER1)** < **(NEGR)** > **(NEF1)**; the same was done for the **(NEG)**: **(NEGL)** > **(NEGS)** > **(NEGR)** > **(NEGF)**.

3.5 Tape stripping

Fig. 3: Box plots indicating the mean and median concentration ($\mu\text{g/ml}$) of a) **(NE1)** and **(NEG)** in the SCE (excluding fluvastatin), and b) **(NE1)** and **(NEG)** for each of the selected statins (excluding fluvastatin) present in the ED, where c) indicated the mean and median concentration ($\mu\text{g/ml}$) of **(NE1)** and **(NEG)** in the SCE (including fluvastatin), and d) **(NE1)** and **(NEG)** in (including fluvastatin) in the ED

3.5.1 Stratum corneum-epidermis

The median concentrations for the **(NE1)** and the **(NEG)** in the SCE were inspected and it was found that **(NEF1)** had the highest median concentration, followed by **(NER1)**, **(NES1)** and lastly, **(NEL1)**. When results of the **(NEG)** in the SCE were compared **(NEGR)** obtained the highest median concentration, followed by **(NEGL)**, **(NEGS)** and lastly, **(NEGF)**. When the results of the **(NE1)** and the **(NEG)** were compared, it was evident that the results of the **(NEG)** were much higher than **(NE1)**, except for **(NEF1)**, which indicated a much higher median concentration than **(NEGF)**.

3.5.2 Epidermis-dermis

The median concentrations for the **(NE1)** and the **(NEG)** in the ED were inspected. For the **(NE1)**, **(NEF1)** prevailed with the highest median concentration, followed by **(NES1)**, **(NER1)** and lastly, **(NEL1)**. Results compared for the **(NEG)** showed that **(NEGR)** obtained the highest median concentration, followed by **(NEGS)**, **(NEGF)** and lastly, **(NEGL)**. The **(NE1)** were compared to **(NEG)** in the ED and results showed that **(NE1)** had the overall highest median concentration. **(NE1)** presented with higher median concentrations than **(NEG)**.

A one-way ANOVA was performed for each of the four statins from the formulations in the different layers of the skin. The p-values for the **(NE1)** in the SCE and the ED were <0.001 and 0.005 , respectively, while the p-values for the **(NEG)** in the SCE and the ED were <0.000 and <0.001 , respectively. The Unequal HSD-test was performed for the **(NE1)** in SCE, and the results showed that **(NEF1)** had the highest mean log flux and **(NEL1)** had the lowest value of mean flux out of the four statins. The Levene's test was performed for **(NE1)** in the ED, where a p-value of 0.000167 was obtained. An Unequal HSD-test was also performed on **(NE1)** in the ED, and no results were obtained for fluvastatin. The Unequal HSD-test was performed for **(NEG)** in SCE, and the results showed that **(NEGR)** had the highest mean log flux and **(NEGF)** had the lowest value of mean flux out of the four statins. The Levene's test was performed for **(NEG)** in the ED, where a p-value of 0.000025 was obtained. An Unequal HSD-test was also performed on **(NEG)** in the ED, and no results were obtained for fluvastatin, as none were obtainable.

4 Conclusion

Characterisation was done on the formula of nano-emulsions, whereafter it was developed into a nano-emulgel by adding a gelling agent. The optimised formulas were utilised for membrane release studies, skin diffusion studies and tape stripping. With the membrane release studies, it was evident that **(NE1)** showed a better median flux than **(NEG)**. This correlates with literature stating that a **(NEG)** will be release in a more controlled manner, whereas release of a **(NE1)** is much faster (Gopalasatheeskumar *et al.*, 2017). All the formulations showed a release, although a significant lower release was obtained from lovastatin in both formulations, which

could be due to o/w nano-emulsions that favour lipophilic substances, and lovastatin can be seen as too lipophilic for penetration (Yoshida *et al.*, 2011). The highest median flux values were obtained from **(NEF1)** and **(NEGR)**.

The aim of the study was to determine if the selected statins from their different formulations could successfully be delivered transdermally. It can be proposed that the highest median amount diffused was obtained by **(NE1)**, when **(NE1)** and **(NEG)** are compared. The statins that had the highest median amount per area diffused were obtained by **(NEF1)** and **(NEGR)** from the **(NE1)** and the **(NEG)** respectively, while lovastatin obtained the lowest median amount per area diffused for both the **(NE1)** and the **(NEG)**. When a comparison was drawn between the different statins, it was observed that fluvastatin exerted the ideal physicochemical properties (Williams, 2003:36). The low amount diffused by **(NEL1)** and **(NEGL)** could be due to poor physicochemical properties of the statin.

After skin diffusion studies, tape stripping was performed on the skin to determine the amount of statins in the SCE and ED. The highest median concentration was obtained by **(NEF1)** in the SCE, and can be ascribed to the ideal physicochemical properties, such as the ideal log D, ideal melting point and the ideal molecular weight (Chapman & McTaggart, 2002; Greupink *et al.*, 2011; Ruan *et al.*, 2012). Results of the SCE for **(NEL1)** and **(NEGL)** showed a higher median concentration than for membrane release and skin diffusion studies, therefore it can be proposed that lovastatin, which has a high lipophilicity, is more likely to accumulate in the SCE, as the SCE is more lipophilic (Naik *et al.*, 2000). The highest median concentrations for the ED was obtained by **(NEF1)** and **(NEGR)**, which can be the result of ideal physicochemical properties of the statins (Chang *et al.*, 2013; Naik *et al.*, 2000). The higher concentration of fluvastatin and rosuvastatin in the SCE and ED can be attributed to the salt form of the statin (Chaudhary *et al.*, 2012). It can be stated that the salt form of lovastatin and rosuvastatin can be altered for enhanced solubility and better permeation. Finally, it can be said that fluvastatin exerted ideal physicochemical properties for better permeation and can be proposed to be the reason that higher amounts were obtained in the SCE and ED and in turn, the systemic circulation.

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Declaration of interest

None

Disclaimer

Any finding, opinions, conclusions and recommendations are those of the authors and therefore the NRF does not accept any liability in regards thereto.

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Tables:

Table 1:

Excipients used in the formulation of the nano-emulsions and the nano-emulgels (50 ml)

	Nano-emulsion	%m/m	Nano-emulgel	%m/m
Phase A	Statin	2.00%	Statin	2.00%
	Grapeseed oil	8.00%	Grapeseed oil	8.00%
	Span® 60	6.00%	Span® 60	6.00%
Phase B	Milli-Q® water	78.00%	Milli-Q® water	77.54%
	Tween® 80	6.00%	Tween® 80	6.00%
			Carbopol® Ultrez 20 polymer	0.46%

Table 2:

LOD ($\mu\text{g/ml}$) and LOQ ($\mu\text{g/ml}$) of the respective statins

Statin	LOD	LOQ
Fluvastatin	0.14	0.55
Lovastatin	0.15	0.62
Rosuvastatin	0.13	0.53
Simvastatin	0.13	0.52

Table 3:

Characterisation results for both the **(NE1)** and the **(NEG)**

	TEM (nm)	pH	Zeta-potential (mV)	Droplet size (nm)	Viscosity (cP)
(NEF1)	60.80-164.55	7.35	-66.53	162.60	5.78
(NEGF)		6.44	-88.53	146.10	1301.83
(NEL1)	58.80-389.46	6.17	-57.17	154.23	3.43
(NEGL)		5.98	-45.83	117.96	2463.08
(NER1)	50.14-575.09	6.11	-31.90	151.20	3.86
(NEGR)		5.96	-42.67	183.16	1141.92
(NES1)	42.82-171.19	7.13	-42.73	151.73	5.66
(NEGS)		6.06	-70.53	200.83	56189.14

Figures:

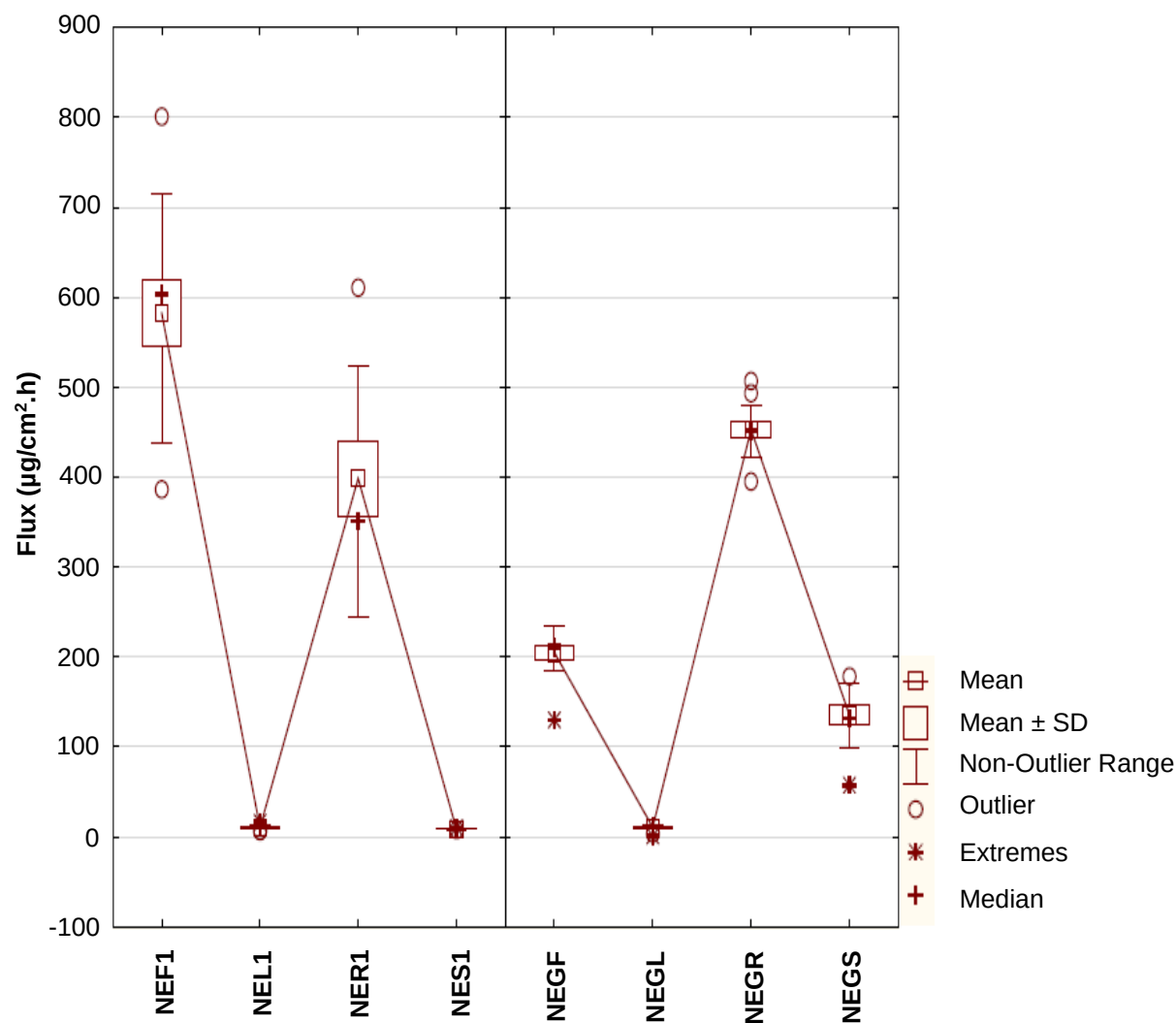


Fig. 1: Box-plots indicating the flux (µg/cm².h) of the (NE1) and the (NEG) of all four statins during the membrane release studies over 6 h

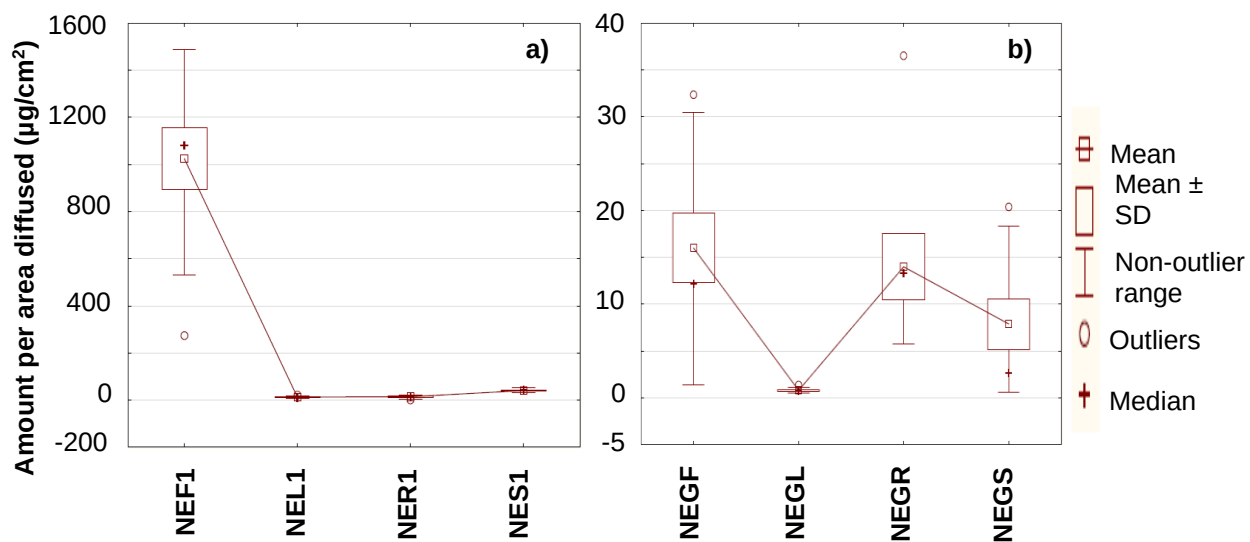


Fig. 2: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) for each of the selected statins in the a) **(NE1)** after 12 h and b) the **(NEG)** after 12 h

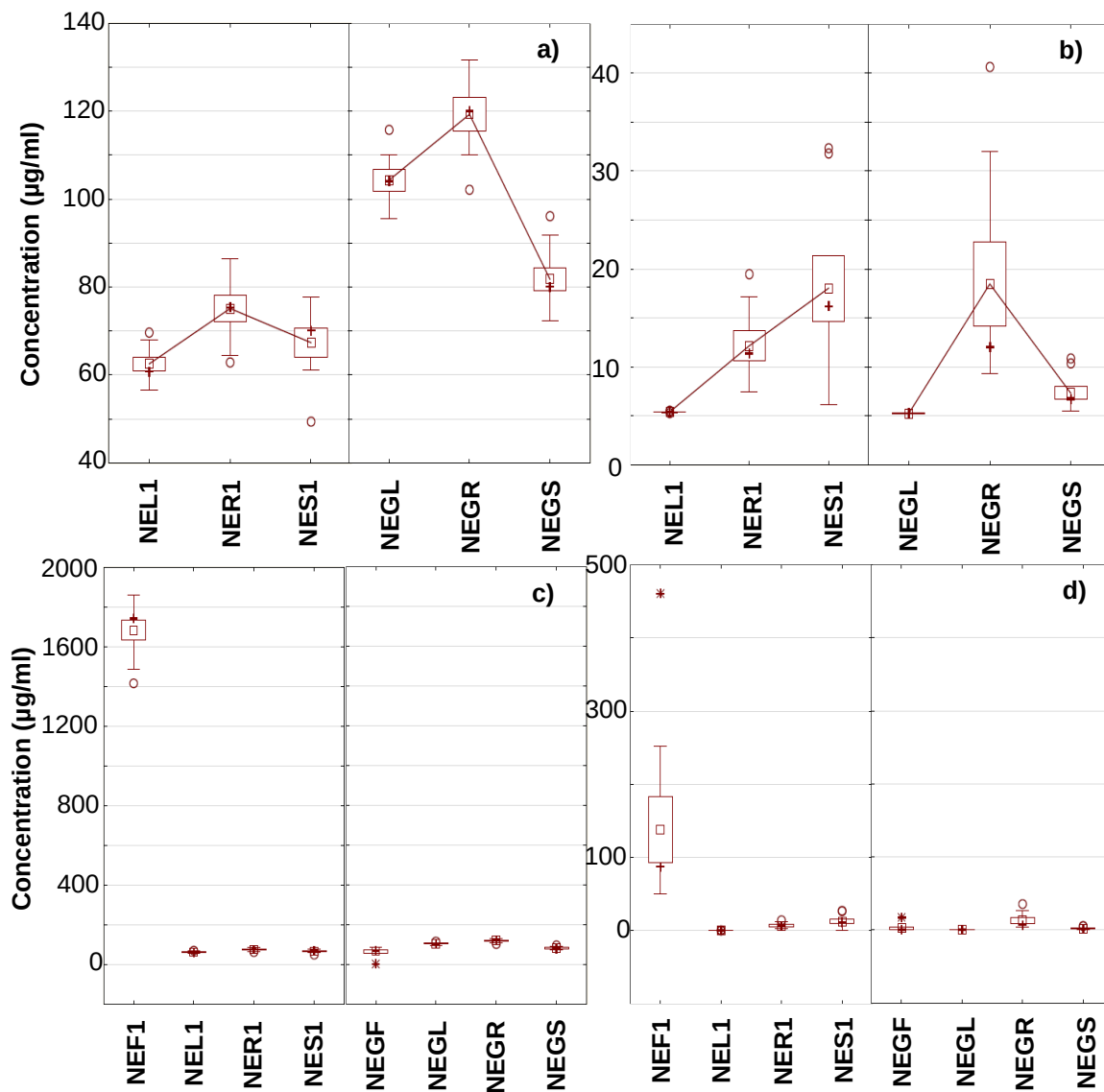


Fig. 3: Box-plots indicating the mean and median concentration (µg/ml) of: a) **(NE1)** and **(NEG)** in the SCE (excluding fluvastatin) and b) **(NE1)** and **(NEG)** for each of the selected statins (excluding fluvastatin) present in the ED, where c) indicates the mean and median concentration (µg/ml) of **(NE1)** and **(NEG)** in the SCE (including fluvastatin) and d) **(NE1)** and **(NEG)** in (including fluvastatin) in the ED

Chapter 5

CONCLUSION AND FUTURE PROSPECTS

Familial hypercholesterolemia can be seen as inherited cardiomyopathies that can cause arrhythmias, which may lead to death if undiagnosed and untreated (Sturm *et al.*, 2018:665). Familial hypercholesterolemia can be caused by a genetic mutation in lipid and lipoprotein metabolisms. Cholesterol in general can be caused by a high carbohydrate diet and by the lack of exercise (Vinson *et al.*, 2019:50). LDL is the factor of cholesterol that causes cardiovascular disease. High cholesterol levels can be treated with statins (HMG-CoA reductase inhibitors) (Breuker *et al.*, 2018:195), which are beneficial for preventing recurrent stroke, however they can produce side-effects with oral use (Wu *et al.*, 2019:1), including muscular side-effects caused by muscle organic anion transporters (Takeda *et al.*, 2004:138).

For the reduction of side-effects caused by statin therapy, the transdermal route was investigated. The transdermal route of delivery possesses many advantages over oral delivery, including better bioavailability (since the API is directly available in the bloodstream) and it does not undergo first-pass metabolism (Ahad *et al.*, 2015:588). For the transdermal delivery of statins, the statins must pass through the stratum corneum into the deeper layers of the skin into systemic circulation, thus statins must exert ideal physicochemical properties (Kumar *et al.*, 2015:169). To help APIs pass through the layers of the skin, they must be formulated in a carrier system of ideal properties. Nano-sized formulations were looked into as carrier systems to enhance penetration (Shakeel *et al.*, 2007:E1).

The aim of this study was to incorporate 2% of the selected statins (lovastatin, fluvastatin, rosuvastatin and simvastatin separately) into four nano-emulsions and four nano-emulgel formulations: all contained grapeseed oil as the oil phase. After formulation, the permeation through the skin was investigated together with the cytotoxic effects.

The objectives of this study were to:

- * develop and validate the analytical method used to determine the concentrations of the selected APIs, i.e. HPLC;
- * formulate four nano-emulsions and four nano-emulgels containing the APIs separately (the APIs will be encapsulated in the oil phase (grape seed oil));
- * characterise the emulsions (both nano-emulsions and nano-emulgels) with regard to EE%, droplet size, zeta-potential, pH, viscosity, morphology and visual examination;
- * use membrane release studies to determine the release of the selected APIs from the four nano-emulsions and the four nano-emulgels;

- * perform Franz cell diffusion studies on the skin and tape stripping techniques to determine the extent of transdermal and topical delivery of the APIs from the nano-emulsions and nano-emulgels;
- * evaluate the cytotoxic effects of the APIs (alone) and the combined formulation on HaCaT cells by means of the IC₅₀ values for both Methylthiazol tetrazolium (MTT) and Neutral red (NR).

The HPLC method was successfully developed and validated, as observed from the results obtained (Appendix A), the method was reliable, accurate and reproducible.

The nano-emulsions were formulated with the appropriate surfactant ratios; these ratios were tested via characterisation and the best dispersion, which was the formula with equal surfactant rations Tween® 80:Span® 60 (1:1) (**NE1**), was selected. The APIs in the nano-emulsions were subsequently named fluvastatin (**NEF1**), lovastatin (**NEL1**), rosuvastatin (**NER1**) and simvastatin (**NES1**). After the nano-emulsions were formulated, nano-emulgels were developed by adding a gelling agent (Carbopol® Ultrez 20) to the water phase. These nano-emulgels (**NEG**) with the selected APIs were named fluvastatin (**NEGF**), lovastatin (**NEGL**), rosuvastatin (**NEGR**) and simvastatin (**NEGS**), and subsequently characterised.

Characterisation was done by means of pH, droplet size, viscosity, zeta-potential and morphology. Measurement of pH was done on both the (**NE1**) and (**NEG**), and both obtained pH values between 5 and 7. This falls within the acceptable range, as literature stated the pH levels for formulations applied to the skin must range between 3 and 9 to avoid skin irritation, since the skin has a pH of 5 (Barry, 2002:512). Droplet size, according to Naik *et al.* (2000:1453), should be < 200 nm and have a Pdl value of 0 to 1 to obtain successful transdermal delivery (Elmataeeshy *et al.*, 2018:24); both the (**NE1**) and (**NEG**) had droplet sizes below 200 nm and Pdl values below 1. Zeta-potential of the (**NE1**) and (**NEG**) both showed values of less than - 30 mV, which makes these formulations ideal for transdermal delivery (Eid *et al.*, 2014:2; Marsalek, 2014:13; Silva *et al.*, 2012:860). Viscosity values between the (**NE1**) and the (**NEG**) differed significantly, since the (**NEG**) had a gelling agent added, which led to an increased viscosity.

Membrane release studies were performed with the (**NE1**) and the (**NEG**) containing the different statins, where the highest median flux value was obtained by (**NEF1**) among the (**NE1**) (followed by **NEGL**, **NEGR** and **NEGS**), while (**NEGR**) obtained the highest median flux value for the (**NEG**) (followed by **NEGF**, **NEGL** and **NEGS**). For the membrane release studies, the (**NEG**) obtained higher median flux values than their (**NE1**) counterpart, except for (**NEF1**) which obtained the highest median flux value.

During the skin diffusion studies, it was evident that the **(NE1)** obtained overall higher median amounts per area diffused than their **(NEG)** counterpart. For the **(NE1)**, **(NEF1)** had the highest median concentration in the stratum corneum epidermis (SCE), while **(NEGR)** obtained the highest median concentration in the SCE for the **(NEG)**. In the SCE, it was observed that the **(NEG)** obtained higher median concentrations than their **(NE1)** counterpart, except for **(NEF1)** which presented with the highest median concentration for all the formulas. For results in the epidermis dermis (ED), the **(NE1)** obtained higher median concentrations than their **(NEG)** counterparts, where **(NEF1)** obtained the highest results for **(NE1)** and **(NEGR)** for the **(NEG)**. All four statins in their different formulas showed promising results for transdermal delivery, thus transdermal delivery could be looked into as an alternative route of administration.

A cytotoxic assay on HaCaT cells was performed with different concentrations of the statins alone and different concentrations of the **(NE1)** with the APIs (fluvastatin, lovastatin, rosuvastatin and simvastatin); a placebo nano-emulsion (without any APIs) was also tested. For the nano-emulsions tested, **(NES1)** showed the highest cytotoxicity value for MTT-assay with the IC₅₀ determination. For the APIs alone, lovastatin was the most cytotoxic for cells tested with MTT-assay for IC₅₀ determination. For the NR-assay, lovastatin had the lowest IC₅₀-value and was the statin with the highest cytotoxicity value of all the nano-emulsions tested and for the APIs alone. When the different concentrations of each tested formulation or API alone were compared during the MTT-assay, it was evident the **(NEF1)** had the most cytotoxic value. For the APIs alone, lovastatin presented as the most cytotoxic on the HaCaT cells in different concentrations; only fluvastatin and simvastatin became more cytotoxic at higher concentrations. For the NR-assay it was evident that **(NEF1)**, **(NEL1)**, **(NER1)** and **(NES1)**, at higher concentrations, became cytotoxic to the cells; **(NEL1)** was the most cytotoxic statin in formulation. With the APIs alone, only the higher concentrations became cytotoxic to the cells; lovastatin was also the most cytotoxic to the cells. Concentrations used for transdermal studies were much lower than those tested for cytotoxic effects, thus in conclusion, it can be stated that the statins would not be cytotoxic to the skin when applied.

Prospects for the future:

- * Different formulations for the statins can be developed for testing.
- * Stability tests could be performed to investigate the stability of statins in their formulations over a long-term period.
- * Different gelling agents can be used for formulations to investigate results and compare to standing observations.
- * Other salt forms of statins could be used to see if they exert ideal properties for transdermal penetration.

- * Penetration enhancers can be changed to possibly enhance penetration through the stratum corneum and possibly dissolve statins better for enhanced delivery.
- * Cytotoxic experiments could be performed on other cell lines to see if formulations were cytotoxic on other cell lines in different concentrations.

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Appendix A

VALIDATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR SELECTED STATINS

A.1 Objective of validation

The objective was to verify an HPLC assay to determine an analytical method that was suitable and sensitive enough with regard to the quantification of selected statins in the nano-emulsions and nano-formulations. Validation criteria needed to be investigated to validate this analytical method, which included linearity, accuracy, precision, ruggedness, limit of detection (LOD) and lower limit of quantification (LLOQ) (ICH, 2005:2). Determination and quantification of the concentration of the APIs in the receptor phase, collected during the *in vitro* diffusion studies, was also analysed with this HPLC assay.

A.2 Chromatographic conditions

This HPLC method for fluvastatin, lovastatin, rosuvastatin and simvastatin was verified under the supervision and guidance of Prof. JL du Preez at the Analytical Technology Laboratory (ATL), North-West University (NWU). Chromatographic conditions, with regard to the analytical instrument used, the column, mobile phase, detection wavelength, injection volume and the flow rate, have to be controlled during the verification process of the analytic method. The following chromatographic conditions were used during the verification of the HPLC method:

Analytical instrument:	HP1100 series HPLC, equipped with a gradient pump, UV detector and autosampler injector mechanism, was fitted to the system. ChemStation Rev. A.10.03 acquisition and analysis software were used to analyse chromatograms. Laboratory environment with a controlled temperature of 25°C was maintained.
Column:	A Venusil XBP C18(2) reverse phase column (150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, Newark, DE).
Mobile phase:	The mobile phase consisted of acetonitrile/water with 0.005 M (0.2%) octane sulphonic acid-Na (pH 3.5).
Gradient conditions	Start at 25% acetonitrile and increase linearity to 90% after 1.0 min. Hold at 90% until 6.5 min and then re-equilibrate at start conditions.
Solvent:	Methanol
Injection volume:	An injection volume of 10 µl was chosen.
Detection:	The UV detector was set at 240 nm for the detection of APIs.

Flow rate: A flow rate of 1.0 ml/min was set.

Retention time: Retention time for fluvastatin, lovastatin, rosuvastatin and simvastatin was $\pm$ 3.65, 4.09, 4.50 and 7.12 min, respectively.

Run time: Selected statins run time was 10.5 min.

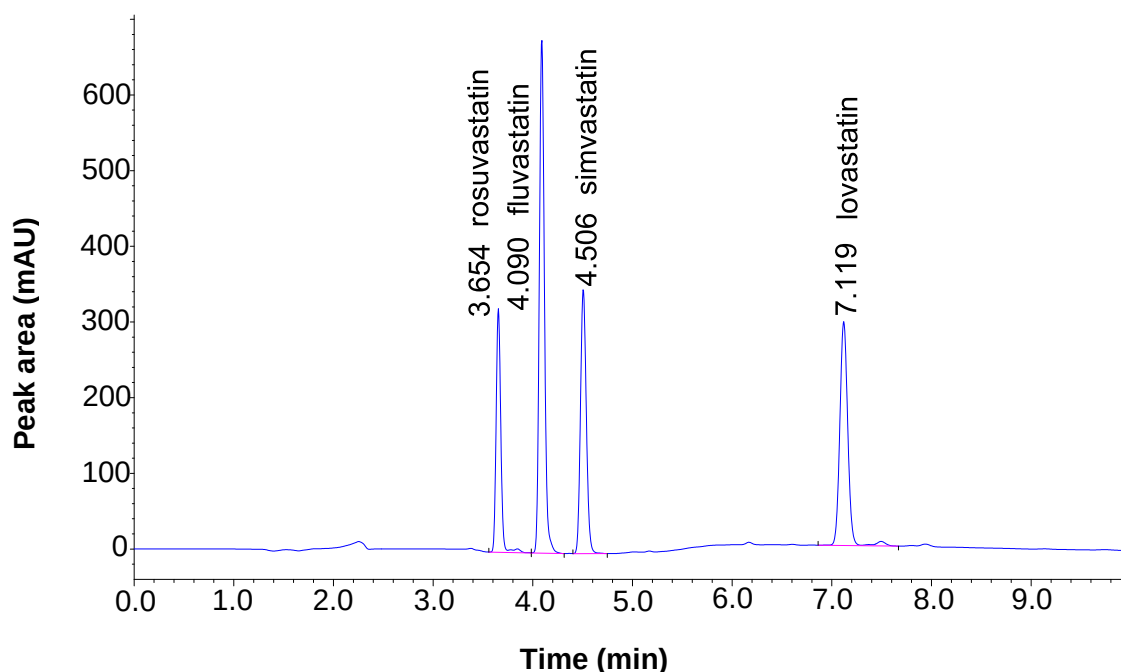


Figure A.1: Chromatographic representation of the HPLC values for fluvastatin, lovastatin, rosuvastatin and simvastatin

A.3 Validation criteria

A.3.1 Linearity

Linearity is obtained when the test results are directly equivalent to the analyte concentration of the sample (APVMA, 2004:5; ICH, 2005:5). Linearity is determined with a linear regression curve of the data plots formed by the peak areas (y-axis) against the analyte concentration ($\mu\text{g/ml}$; x-axis). The linear regression equation (Equation A.1) can best describe linearity, where y represents the peak area of the analyte (fluvastatin, lovastatin, rosuvastatin and simvastatin), m represents the slope, x represents the concentration of the analyte (fluvastatin, lovastatin, rosuvastatin and simvastatin) and c is the y-intercept (Snyder *et al.*, 1997:644). A straight line should form, according to Snyder *et al.* (1997:691), with a regression value of $R^2 \geq 0.98$ (Johnson & Van Buskirk, 1998:96). The slope measured should show a correlation between response and analyte concentrations to be indicative of a high degree of linearity (APVMA, 2004:4).

$$y = mx + c$$

Equation A.1

Linearity of the HPLC method was determined by preparing a stock solution and making two dilutions of each statin. Each statin (20 mg) was weighed and dissolved in a 100 ml volumetric flask, and made up to volume with methanol. Of the stock solution, 5 ml was transferred into a 50 ml volumetric flask and made up to volume with methanol. Another dilution was made by transferring 5 ml of the solution into a 50 ml volumetric flask and made up to volume with methanol. These dilutions, together with the stock solutions, were injected into the HPLC at a default injection volume of 10 µl.

Tables A.1 to A.4 and Figures A.2 to A.5 demonstrate the linearity data of the selected statins (fluvastatin, lovastatin, rosuvastatin and simvastatin). All the selected statins demonstrated an R^2 value of 0.9998, obtained from the linear regression curve (see Tables A.1 – A.4 and Figures A.2 – A.5). All figures indicate perfect linear relationship.

Table A.1: Linearity results of fluvastatin standard solution

Standard (µg/ml)	Peak area (mAU)	
0.2	8.2	
0.5	16.6	
1.0	31.0	
1.5	45.8	
2.0	53.8	
5.0	135.1	
10.0	266.4	
15.0	397.9	
20.1	530.7	
50.2	1190.2	
100.3	2380.6	
150.5	3555.6	
200.6	4726.5	
	R^2	0.9999
	y-intercept	19.43
	Slope	23.49

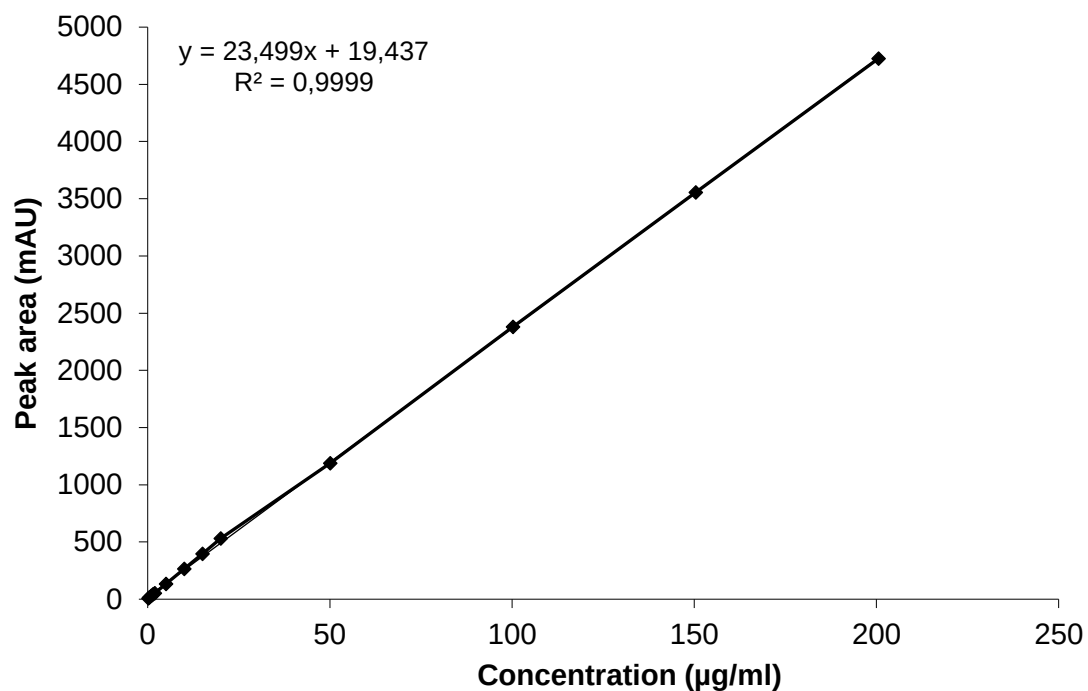


Figure A.2: Linear regression curve of fluvastatin standard solution

Table A.2: Linearity results of lovastatin standard solution

Standard (µg/ml)	Peak area (mAU)	
0.2	8.2	
0.5	16.6	
1.0	31.0	
1.5	45.8	
2.0	53.8	
5.0	135.1	
10.0	266.4	
15.0	397.9	
20.1	530.7	
50.2	1190.2	
100.3	2380.6	
150.5	3555.6	
200.6	4726.5	
	R²	0.9999
	y-intercept	19.44
	Slope	22.84

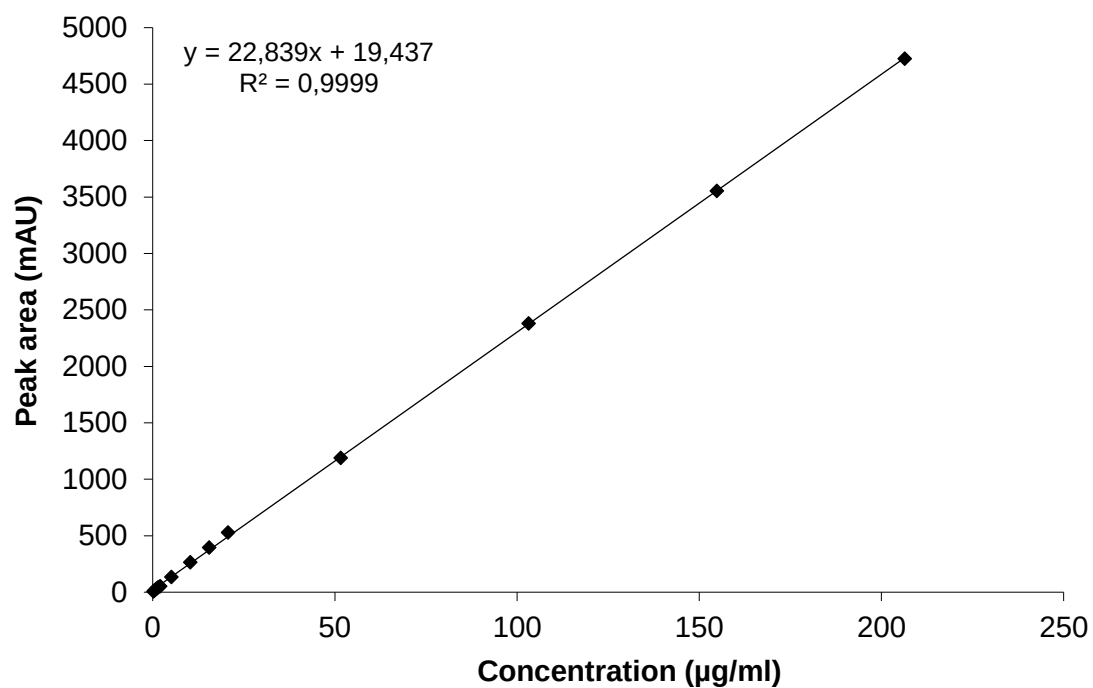


Figure A.3: Linear regression curve of lovastatin standard solution

Table A.3: Linearity results of rosuvastatin standard solution

Standard (µg/ml)	Peak area (mAU)	
0.2	8.2	
0.5	16.6	
1.0	31.0	
1.5	45.8	
2.1	53.8	
5.2	135.1	
10.3	266.4	
15.5	397.9	
20.6	530.7	
51.6	1190.2	
103.2	2380.6	
154.7	3555.6	
206.3	4726.5	
	R²	1
	y-intercept	19.44
	Slope	22.85

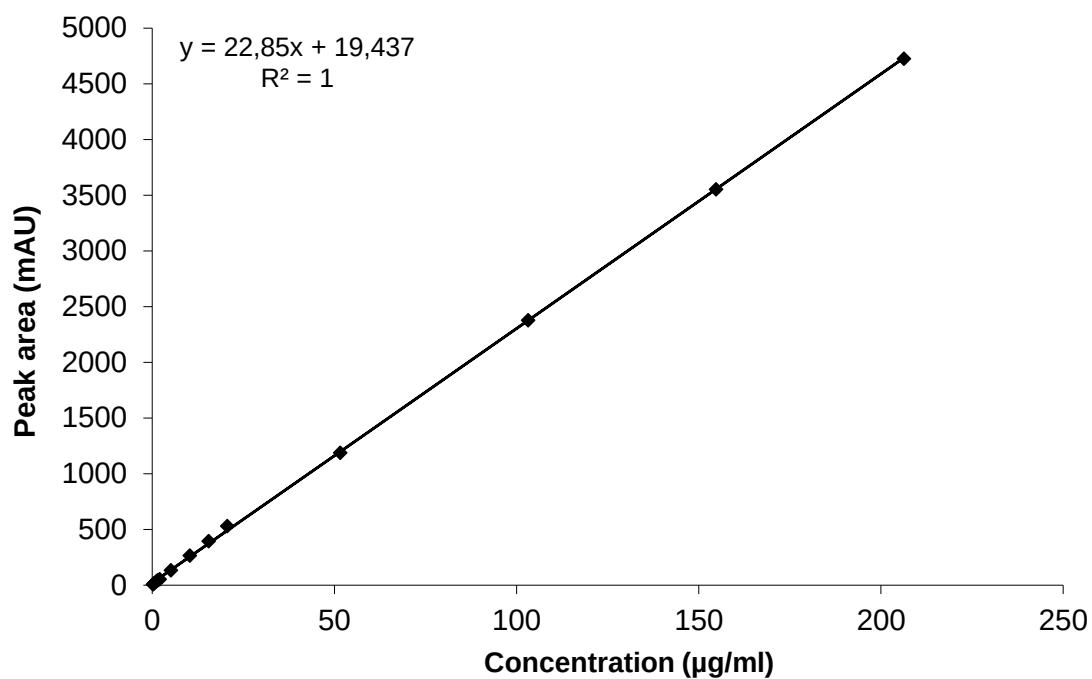


Figure A.4: Linear regression curve of rosuvastatin standard solution

Table A.4: Linearity results of simvastatin standard solution

Standard (µg/ml)	Peak area (mAU)	
0.2	8.2	
0.5	16.6	
1.0	31.0	
1.5	45.8	
2.0	53.8	
5.0	135.1	
10.0	266.4	
14.9	397.9	
19.9	530.7	
49.8	1190.2	
99.7	2380.6	
149.5	3555.6	
199.3	4726.5	
R²		0.9999
y-intercept		19.44
Slope		23.65

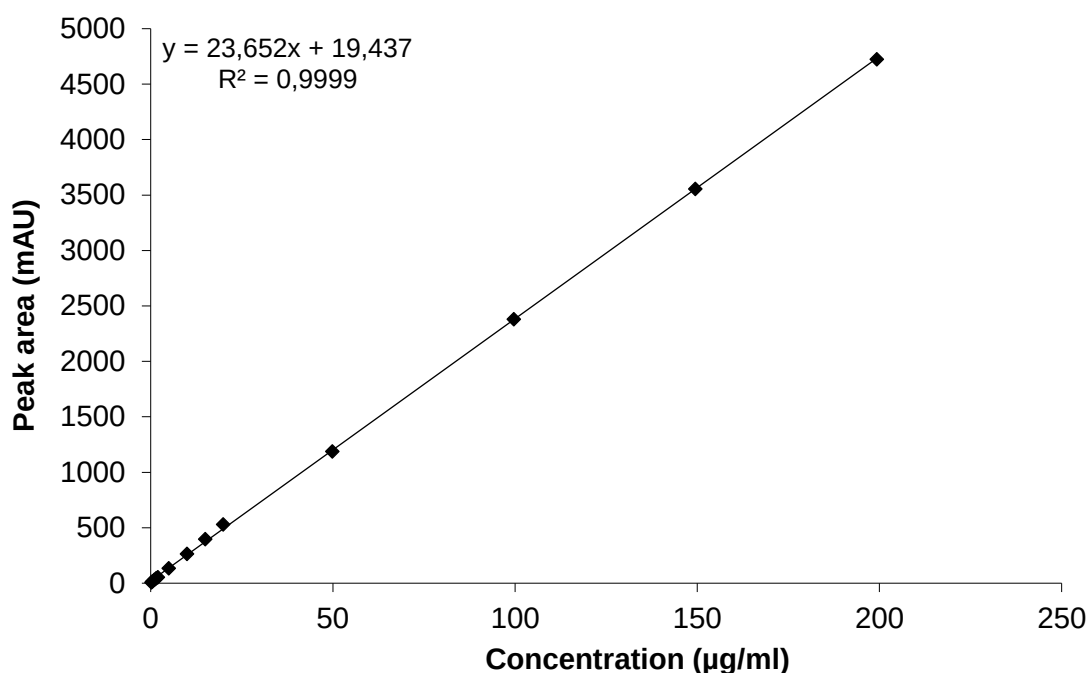


Figure A.5: Linear regression curve of simvastatin standard solution

A.3.2 Accuracy

The test results can be viewed by performing accuracy on the selected statins. Accuracy should be determined across a specific range of the analytical procedure (ICH, 2005:9). Accuracy was determined by analysing a minimum of nine determinations over a range of three different concentrations (Snyder *et al.*, 1997:691). The accuracy measured must represent the full calibration curve range.

Accuracy was determined by preparing three spiked solutions in triplicate. An amount of ± 200 mg of each statin was added to a 100 ml volumetric flask and made up to volume (100 ml) with methanol; this process was prepared for the four statins.

Nine volumetric flasks were used for each statin:

- * Three flasks (100 ml) were prepared with 3 ml spiked solution and 0.6 ml placebo nano-emulsion; made up to volume (100 ml) with methanol.
- * Three flasks (100 ml) were prepared with 4 ml spiked solution and 0.8 ml placebo nano-emulsion; made up to volume (100 ml) with methanol.
- * Three flasks (100 ml) were prepared with 5 ml spiked solution and 1.0 ml placebo nano-emulsion; made up to volume (100 ml) with methanol.

These flasks were mixed thoroughly in an ultrasonic bath. Small quantities (1.5 ml) were transferred to vials to be injected in the HPLC.

Standard solutions were prepared by weighing ± 20 mg of each statin (active constituent) in separate volumetric flasks (100 ml); each statin was made up to volume (100 ml) by adding methanol. Small quantities of 1.5 ml were added to the HPLC vial for injection. The standard solution was then injected, in duplicate, in five different concentrations into the HPLC. The vials were then assayed, and the results compared against expected results; the accepted percentage recovery should range between 90 and 110% (APVMA, 2004:5; Shabir, 2005:9). The resulting peak areas are given in Tables A.5, A.7, A.9 and A.11, and the accuracy results are given in Tables A.6, A.8, A.10 and A.12.

Table A.5: Peak areas for the fluvastatin standard solution

Sample (Standard)	Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)		
		1	2	Average
Sample 1	80.4	2534.1	2526.3	2530.2
Sample 2	120.6	3779.3	3778.5	3778.9
Sample 3	160.8	5009.0	5014.2	5011.6
Sample 4	201.0	6220.6	6234.9	6227.7
Sample 5	241.2	7416.4	7422.5	7419.4

Table A.6: Fluvastatin accuracy parameters

Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)			Recovery	
	1	2	Average	($\mu\text{g/ml}$)	%
61.4	1892.3	1890.8	1891.5	58.8	95.7
61.4	1931.4	1934.7	1933.1	60.2	97.9
61.4	1940.5	1945.0	1942.8	60.5	98.5
81.9	2556.9	2556.1	2556.5	80.7	98.5
81.9	2569.7	2580.8	2575.3	81.3	99.2
81.9	2579.2	2583.5	2581.3	81.5	99.5
102.4	3203.6	3208.0	3205.8	102.0	99.6
102.4	3234.6	3227.3	3230.9	102.8	100.4
102.4	3217.0	3218.8	3217.9	102.4	100.0
				Mean	98.8
				SD	1.3
				%RSD	1.3

Fluvastatin had an average percentage recovery of 98.8%, which falls within the acceptable criteria range for recovery (90 – 110%) (APVMA, 2004:5; Shabir, 2005:9).

Table A.7: Peak areas for the lovastatin standard solution

Sample (Standard)	Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)		
		1	2	Average
Sample 1	82.0	2250.0	2239.0	2244.5
Sample 2	123.0	3339.9	3331.0	3335.1
Sample 3	164.0	4399.9	4403.6	4401.7
Sample 4	205.0	5463.6	5507.8	5485.7
Sample 5	246.0	6535.7	6584.2	6560.0

Table A.8: Lovastatin accuracy parameters

Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)			Recovery	
	1	2	Average	($\mu\text{g/ml}$)	%
69.6	1946.1	1940.7	1943.4	70.4	101.1
69.6	1950.1	1951.8	1950.9	70.7	101.5
69.6	1941.5	1954.9	1948.2	70.6	101.4
81.4	2558.3	2552.2	2555.3	93.6	115.1
81.4	2572.5	2569.4	2570.9	94.2	115.8
81.4	2581.3	2585.6	2583.5	94.7	116.4
116.0	3179.4	3196.6	3188.0	117.7	101.5
116.0	3215.3	3203.1	3209.2	118.5	102.2
116.0	3211.5	3220.1	3215.8	118.8	102.4
				Mean	106.4
				SD	6.7
				%RSD	6.3

In Table A.8, it can be seen that lovastatin demonstrated a percentage recovery of 106.4%, which falls within the acceptable criteria range for recovery (90 – 110%) (AVPMA, 2004:5; Shabir, 2005:9).

Table A.9: Peak areas for the rosuvastatin standard solution

Sample (Standard)	Concentration spiked ($\mu\text{g/ml}$)	Peak area (mAU)		
		1	2	Average
Sample 1	90.0	2122.2	2155.7	2138.9
Sample 2	135.0	3167.1	3165.2	3166.1
Sample 3	180.0	1203.2	4206.6	4204.9
Sample 4	225.0	5232.0	5242.0	5237.0
Sample 5	270.0	6268.1	6273.6	6270.8

Table A.10: Rosuvastatin accuracy parameters

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	1	2	Average	(µg/ml)	%
62.6	1452.4	1452.4	1452.4	60.2	96.2
62.6	1474.7	1477.4	1476.0	61.2	97.9
62.6	1479.9	1482.6	1481.3	61.5	98.2
83.4	1952.3	1951.4	1951.9	82.0	98.2
83.4	1959.2	1962.1	1960.6	82.3	98.7
83.4	1967.7	1971.5	1969.6	82.7	99.1
104.3	2444.9	2447.8	2446.3	103.5	99.2
104.3	2459.4	2455.0	2457.2	104.0	99.7
104.3	2455.7	2459.0	2457.3	104.0	99.7
				Mean	98.5
				SD	1.0
				%RSD	1.0

Rosuvastatin presented with an average percentage recovery of 98.5%, which falls within the acceptable criteria range for recovery (90 – 110%) (AVPMA, 2004:5; Shabir, 2005:9).

Table A.11: Peak areas for the simvastatin standard solution

Sample (Standard)	Concentration spiked (µg/ml)	Peak area (mAU)		
		1	2	Average
Sample 1	88.8	2299.8	2294.1	2297.0
Sample 2	133.2	3414.2	3414.7	3414.4
Sample 3	177.6	4512.3	4521.0	4516.6
Sample 4	222.0	5603.9	5625.2	5614.5
Sample 5	266.4	6682.7	6687.9	6685.3

Table A.12: Simvastatin accuracy parameters

Concentration spiked (µg/ml)	Peak area (mAU)			Recovery	
	1	2	Average	(µg/ml)	%
61.0	1568.9	1557.9	1563.4	58.6	96.0
61.0	1582.7	1588.2	1585.4	59.5	97.5
61.0	1580.9	1587.0	1584.0	59.4	97.4
81.4	2088.4	2091.1	2089.8	79.9	98.2
81.4	2099.7	2100.9	2100.3	80.3	98.7
81.4	2104.5	2108.5	2106.5	80.6	99.0
101.7	2612.2	2616.0	2614.1	101.1	99.4
101.7	2628.4	2621.1	2624.7	101.5	99.8
101.7	2624.2	2628.5	2626.4	101.6	99.9
				Mean	98.4
				SD	1.2
				%RSD	1.2

Simvastatin had an average percentage recovery of 98.4%, which falls within the acceptable criteria range for recovery (90 – 110%) (AVPMA, 2004:5; Shabir, 2005:9).

A.3.3 Precision

Precision describes the closeness of individual measures. The procedure is applied for repeated measurements of multiple aliquots of a single homogenous sample (FDA, 2001:5; Shah *et al.*, 1992:1589). A minimum of three concentrations must be tested and fall within the expected concentration range (FDA, 2001:5). Precision can be expressed as the variance of the standard deviation of a series of measurements (ICH, 2005:4). Precision was examined in terms of intra-day precision (repeatability) and inter-day precision (reproducibility).

A.3.3.1 Repeatability (intra-day precision)

Precision can be considered as a level of repeatability (intra-day) where samples were tested within a short period under the same operating conditions (ICH, 2005:5). This assay was prepared using nine samples consisting of three concentrations.

Three solutions were made with each statin emulsion:

- * 0.6 g were weighed and diluted with methanol and made up to 100 ml;
- * 0.8 g were weighed and diluted with methanol and made up to 100 ml;
- * 1.0 g were weighed and diluted with methanol and made up to 100 ml.

Each dilution was made in triplicate and placed in HPLC vials. Each vial was injected in duplicate on the same day (Day 1). The acceptance criteria for intra-day precision is a percentage relative standard deviation (%RSD) equal or less than 2.00% (APVMA, 2004:5; Snyder *et al.*, 1997:691). Tables A.13 to A.16 represent the repeatability data obtained.

Table A.13: Repeatability (intra-day-precision) results of fluvastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
120.0	4077.1	4075.2	4076.1	135.9	113.2
120.0	4313.7	4307.2	4310.5	135.9	113.3
120.0	4274.4	4276.5	4275.5	135.2	112.6
160.0	5593.7	5603.6	5598.6	184.0	115.0
160.0	5558.0	5567.4	5562.7	184.6	115.4
160.0	5455.7	5454.3	5455.0	184.1	115.1
200.0	6976.5	6989.6	6983.0	227.3	113.6
200.0	6900.7	7006.6	6953.7	228.7	114.4
200.0	6908.5	6855.3	6881.9	227.3	113.7
				Mean	114.1
				SD	0.9
				%RSD	0.8

Table A.14: Repeatability (intra-day-precision) results of lovastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
120.0	3595.3	3647.9	3621.6	121.9	101.5
120.0	3666.2	3695.2	3680.7	122.5	102.1
120.0	3784.3	3816.0	3800.1	123.2	102.7
160.0	4822.9	4792.3	4807.6	165.7	103.5
160.0	4878.0	4870.5	4874.3	165.8	103.6
160.0	4806.3	4760.4	4783.4	162.3	101.4
200.0	5956.1	6020.1	5988.1	204.4	102.2
200.0	5834.8	5741.2	5788.0	202.4	101.2
200.0	5938.1	5874.0	5906.0	203.2	101.6
				Mean	102.3
				SD	0.9
				%RSD	0.8

Table A.15: Repeatability (intra-day-precision) results of rosuvastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
120.0	3589.9	3588.5	3589.2	156.6	130.5
120.0	3478.7	3485.2	3482.0	156.5	130.4
120.0	3588.8	3594.0	3591.4	158.0	131.7
160.0	4718.3	4721.9	4720.1	209.5	131.0
160.0	4684.5	4685.3	4684.9	208.8	130.5
160.0	4738.6	4747.7	4743.1	209.2	130.7
200.0	5905.5	5827.9	5866.7	265.3	132.7
200.0	5813.6	5822.3	5817.9	260.3	130.2
200.0	5892.7	5902.4	5897.6	260.9	130.5
				Mean	131.0
				SD	0.8
				%RSD	0.6

Table A.16: Repeatability (intra-day-precision) results of simvastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
120.0	3451.3	3457.9	3454.6	126.4	105.3
120.0	3305.9	3295.5	3300.7	121.6	101.3
120.0	3269.6	3267.8	3268.7	120.8	100.7
160.0	4293.4	4300.2	4296.8	161.9	101.2
160.0	4333.9	4268.9	4301.4	159.7	99.8
160.0	4326.6	4323.3	4325.0	159.2	99.5
200.0	5223.7	5240.7	5232.2	193.4	96.7
200.0	5407.6	5413.8	5410.7	201.2	100.6
200.0	5379.6	5377.8	5378.7	203.4	101.7
				Mean	100.6
				SD	2.2
				%RSD	2.2

From Tables A.13 to A.15, it is observed that the repeatability results obtained from this procedure was satisfactory; each statin (fluvastatin: 0.8%; lovastatin: 0.8% and rosuvastatin: 0.6%) represented with a %RSD $\leq$ 2.00%. Simvastatin (2.2% in Table A.16) was still within the acceptable range according to Okafo and Roberts (2003:54), where the %RSD values must be less than 10%.

A.3.3.2 Reproducibility (inter-day precision)

Reproducibility can be defined as an agreement between numerous measurements that have been collected using the same method (ICH, 2005:5). Reproducibility was performed using three homogenous samples of approximately equivalent concentrations; a concentration of 0.8 g was used, in triplicate, and made up to 100 ml with methanol. Acceptable criteria for inter-day precision is a %RSD of $\leq 3.00\%$, according to Rafael *et al.* (2007:100).

Table A.17: Inter-day precision results of fluvastatin (Day 1)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	($\mu\text{g/ml}$)	%
822.30	5593.7	5603.6	5598.6	184.0	115.0
814.40	5558.0	5567.4	5562.7	184.6	115.4
800.10	5455.7	5454.3	5455.0	184.1	115.1
				Mean	115.2
				SD	0.18
				%RSD	0.16

Table A.18: Inter-day precision results of fluvastatin (Day 2)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	($\mu\text{g/ml}$)	%
804.100	5388.2	5417.1	5403	179.3	112.1
805.300	5422.1	5444.9	5434	180.1	112.5
806.100	5444.9	5450.4	5448	180.4	112.7
				Mean	112.44
				SD	0.28
				%RSD	0.25

Table A.19: Inter-day precision results of fluvastatin (Day 3)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	($\mu\text{g/ml}$)	%
803.700	5431.9	5444.2	5438	177.9	111.2
812.800	5512.0	5527.5	5520	178.6	111.6
828.000	5659.7	5643.4	5652	179.6	112.2
				Mean	111.67
				SD	0.44
				%RSD	0.39

Table A.20: Reproducibility results of fluvastatin

	Day 1	Day 2	Day 3	
	115.0	112.1	111.2	
	115.4	112.5	111.6	
	115.1	112.7	112.2	
				Between days
Mean	115.2	112.4	111.7	113.1
SD	0.2	0.3	0.4	0.3
%RSD	0.2	0.3	0.4	0.3

Table A.21: Inter-day precision results of lovastatin (Day 1)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
812.50	4822.9	4792.3	4807.6	165.7	103.5
823.50	4878.0	4870.5	4874.3	165.8	103.6
825.10	4806.3	4760.4	4783.4	162.3	101.4
				Mean	102.8
				SD	1.2
				%RSD	1.2

Table A.22: Inter-day precision results of lovastatin (Day 2)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
805.00	4729.7	4748.4	4739	159.9	99.9
821.700	4854.4	4902.8	4879	161.4	100.9
810.900	4850.1	4871.6	4861	162.9	101.8
				Mean	100.88
				SD	0.78
				%RSD	0.78

Table A.23: Inter-day precision results of lovastatin (Day 3)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
829.600	5255.7	5189.4	5223	171.6	107.2
818.200	5249.7	5176.4	5213	173.6	108.5
820.600	5105.3	5096.8	5101	169.3	105.8
				Mean	107.18
				SD	1.11
				%RSD	1.04

Table A.24: Reproducibility results of lovastatin

	Day 1	Day 2	Day 3	
	103.5	99.9	107.2	
	103.6	100.9	108.5	
	101.4	101.8	105.8	
				Between days
Mean	102.80	100.88	107.18	103.62
SD	1.20	0.78	1.11	1.03
%RSD	1.20	0.78	1.04	1.01

Table A.25: Inter-day precision results of rosuvastatin (Day 1)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
824.10	4718.3	4721.9	4720.1	209.5	131.0
820.80	4684.5	4685.3	4684.9	208.8	130.5
829.60	4738.6	4747.7	4743.1	209.2	130.7
				Mean	130.7
				SD	0.23
				%RSD	0.17

Table A.26: Inter-day precision results of rosuvastatin (Day 2)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
823.00	5159.9	5167.3	5164.0	215.7	134.8
820.700	5018.3	5453.3	5236.0	219.4	137.1
811.300	5113.1	5165.4	5165.4	217.8	136.1
				Mean	136.00
				SD	0.94
				%RSD	0.69

Table A.27: Inter-day precision results of rosuvastatin (Day 3)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
806.700	5222.1	5223.9	5223	222.6	139.1
813.600	5235.1	5233.3	5234	221.2	138.3
812.300	5263.6	5281.4	5272	223.2	139.5
				Mean	138.96
				SD	0.52
				%RSD	0.38

Table A.28: Reproducibility results for rosuvastatin

	Day 1	Day 2	Day 3	
	131.0	134.8	139.1	
	130.5	137.1	138.3	
	130.7	136.1	139.5	
				Between days
Mean	130.70	136.00	138.96	135.22
SD	0.23	0.94	0.52	0.56
%RSD	0.17	0.69	0.38	0.41

Table A.29: Inter-day precision results of simvastatin (Day 1)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
805.8	4293.4	4300.2	4296.8	161.9	101.2
814.8	4333.9	4268.9	4301.4	159.7	99.8
825.0	4326.6	4323.3	4325.0	159.2	99.5
				Mean	100.17
				SD	0.9
				%RSD	0.9

Table A.30: Inter-day precision results of simvastatin (Day 2)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
811	4487.8	4377.0	4432.4	167.2	104.5
836.8	4510.4	4506.0	4508.2	164.9	103.0
812.9	4384.5	4401.4	4393.0	165.3	103.3
				Mean	103.6
				SD	0.6
				%RSD	0.6

Table A.31: Inter-day precision results of simvastatin (Day 3)

Mass weighed (mg)	Peak area			Recovery	
	1	2	Average	(µg/ml)	%
825.400	4458.9	4445.6	4452	175.3	109.5
812.000	4358.9	4355.7	4357	174.2	108.9
819.500	4413.9	4429.3	4422	175.3	109.5
				Mean	109.31
				SD	0.31
				%RSD	0.28

Table A.32: Reproducibility results of simvastatin

	Day 1	Day 2	Day 3	
	101.2	104.5	109.5	
	99.8	103.0	108.9	
	99.5	103.3	109.5	
				Between days
Mean	100.17	103.60	109.31	104.36
SD	0.89	0.61	0.31	0.6
%RSD	0.89	0.61	0.28	0.6

From the results observed in Tables A.17 to A.32 for the selected statins, it is apparent the reproducibility results fell within the accepted criteria of $\%RSD \leq 3.00\%$, as seen as follows:

- * Fluvastatin: 0.30%
- * Lovastatin: 1.01%
- * Rosuvastatin: 0.41%
- * Simvastatin: 0.60%

A.3.4 Robustness

Robustness shows the reliability of the analysis by deliberately changing the variations of the method parameters. The change should be sensitive and robust in such a way that the results obtained via the analytical method can accurately reflect on relationship between the active concentration and the peak area (APVMA, 2017). Sensitivity and robustness can be analysed between the response in the peak area and the mobile phase. For this test, 20 mg of each statin was weighed, added to a 100 ml volumetric flask and made up to volume with methanol. It was placed in an ultrasonic bath for proper dissolution then transferred into HPLC vials for analysis.

The standard solution of the statins was injected three times at different parameters. The first injection was done at the normal parameters, according to the method (flow rate: 1.0 ml; wavelength: 240 nm, and 25% acetonitrile), and used as a reference. The second injection (flow rate: 1.2 ml; wavelength: 236 nm, and 20% acetonitrile) and third injection (flow rate: 0.8 ml; wavelength: 244 nm, and 30% acetonitrile) were the varied parameters. Tables A.33 to A.36 represent the robustness data obtained for each of the statins, respectively. Figure A.6 shows the chromatogram for the three injections at different parameters for the statins.

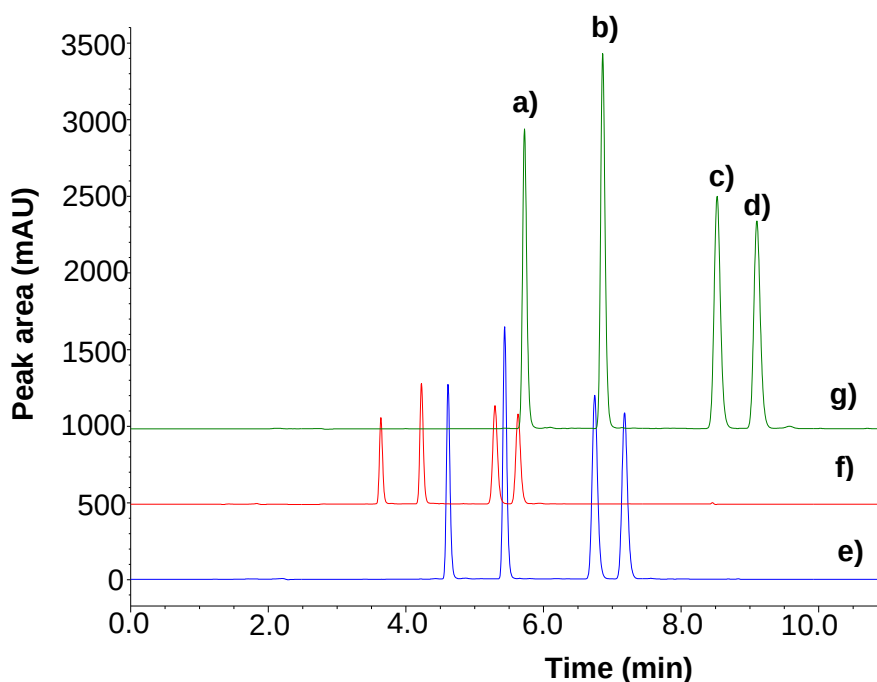


Figure A.6: HPLC chromatogram representing robustness data of a standard solution injected for a) rosuvastatin, b) fluvastatin, c) simvastatin, d) lovastatin at different test parameters: e) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 25% acetonitrile, f) 1.2 ml/min flow rate, 236 nm wavelength and 20% acetonitrile, and g) 0.8 ml/min flow rate, 244 nm wavelength and 30% acetonitrile

Table A.33: Robustness data for fluvastatin

	HPLC parameters				
	%ACN mobile phase (%)	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	25	1.0	240	4.1	6458.10
Method 2	20	1.2	236	4.2	2788.59
Method 3	30	0.8	244	6.9	8954.67

Table A.34: Robustness data for lovastatin

	HPLC parameters				
	%ACN mobile phase (%)	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	25	1.0	240	7.1	6216.30
Method 2	20	1.2	236	5.6	2903.89
Method 3	30	0.8	244	9.1	9041.81

Table A.35: Robustness data for rosuvastatin

	HPLC parameters				
	%ACN mobile phase (%)	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	25	1.0	240	3.7	4689.50
Method 2	20	1.2	236	3.6	1827.82
Method 3	30	0.8	244	5.7	8452.14

Table A.36: Robustness data for simvastatin

	HPLC parameters				
	%ACN mobile phase (%)	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	25	1.0	240	4.5	6702.60
Method 2	20	1.2	236	5.3	3138.64
Method 3	30	0.8	244	8.5	9813.43

The reason for testing robustness is to show the change in method, changes the chromatographic conditions, but deviation in peak areas are still seen and can be analysed. This is an indication that the variation of the peak areas was minimal and that the method used can withstand changes in parameters. The shifts in peak retention times for each of the selected statins were as follows:

- * Fluvastatin: 4.1 – 6.9 min
- * Lovastatin: 7.1 – 9.1 min
- * Rosuvastatin: 3.6 – 3.9 min
- * Simvastatin: 4.5 – 8.5 min

A.3.5 Ruggedness

The ruggedness of an analytical method is the ability of reproducibility of test results under different conditions and variations, such as different analysts, instruments and reagents. Ruggedness can be tested by changing the conditions and not the method parameters (González *et al.*, 2014:14; Snyder *et al.*, 1997:701), and can be viewed in terms of system stability and system repeatability.

A.3.5.1 Sample stability

Sample stability is defined as the ability of the sample to resist any chemical changes and physical degradation or decomposition (UNODC, 2009:63). The amount of degradation that occurred is measured by the %RSD. Each of the statins were prepared by measuring 10 mg and subsequently adding methanol up to volume (100 ml). Thereafter 1 000 µl was transferred with a micropipette into

an HPLC vial to be injected and analysed by the HPLC. Each sample was injected in 1 h intervals over a 24 h period to determine the stability and method. Acceptance criteria for sample stability is a %RSD of around 2% or less (Cowley, 2012:113). According to González *et al.* (2014:22), the deviation of the values should be within $\pm 15\%$. Sample stability parameters can be observed in Tables A.37 to A.40.

Table A.37: Sample stability parameters for fluvastatin

Time (h)	Peak area	%Remaining
0	3267.7	100.0
1	3267.4	100.0
2	3268.0	100.0
3	3272.4	100.1
4	3270.4	100.1
5	3270.4	100.1
6	3268.6	100.0
7	3263.8	99.9
8	3262.8	99.9
9	3263.2	99.9
10	3263.2	99.9
11	3263.5	99.9
12	3266.9	100.0
13	3264.3	99.9
14	3267.6	100.0
15	3268.1	100.0
16	3267.2	100.0
17	3265.1	99.9
18	3265.6	99.9
19	3267.1	100.0
20	3270.1	100.1
21	3268.4	100.0
22	3266.6	100.0
23	3269.0	100.0
24	3271.6	100.1
Mean	3267.2	100.0
SD	2.66	0.08
%RSD	0.08	0.08

Table A.38: Sample stability parameters for lovastatin

Time (h)	Peak area	%Remaining
0	3002.5	100.0
1	2975.8	99.1
2	3009.0	100.2
3	2952.2	98.3
4	2958.3	98.5
5	2961.1	98.6
6	2960.9	98.6
7	2966.6	98.8
8	2938.5	97.9
9	2936.6	97.8
10	2921.6	97.3
11	2911.2	97.0
12	2873.8	95.7
13	2906.0	96.8
14	2860.6	95.3
15	2847.1	94.8
16	2856.0	95.1
17	2814.5	93.7
18	2847.9	94.9
19	2787.1	92.8
20	2767.9	92.2
21	2760.0	91.9
22	2743.3	91.4
23	2751.3	91.6
24	2738.2	91.2
Mean	2881.9	96.0
SD	85.10	2.83
%RSD	2.95	2.95

Table A.39: Sample stability parameters for rosuvastatin

Time (h)	Peak area	%Remaining
0	2409.6	100.0
1	2408.0	99.9
2	2405.6	99.8
3	2412.2	100.1
4	2410.8	100.0
5	2409.4	100.0
6	2405.4	99.8
7	2403.7	99.8
8	2403.9	99.8
9	2406.1	99.9
10	2405.6	99.8
11	2408.1	99.9
12	2404.9	99.8
13	2407.7	99.9
14	2406.9	99.9
15	2406.9	99.9
16	2405.6	99.8
17	2406.6	99.9
18	2405.6	99.8
19	2409.0	100.0
20	2409.5	100.0
21	2407.3	99.9
22	2410.6	100.0
23	2410.4	100.0
24	2409.1	100.0
Mean	2407.5	99.9
SD	2.23	0.09
%RSD	0.09	0.09

Table A.40: Sample stability parameters for simvastatin

Time (h)	Peak area	%Remaining
0	2852.9	100.0
1	2846.3	99.8
2	2827.6	99.1
3	2829.4	99.2
4	2813.2	98.6
5	2806.0	98.4
6	2796.4	98.0
7	2779.5	97.4
8	2771.7	97.2
9	2760.2	96.7
10	2753.1	96.5
11	2750.4	96.4
12	2736.6	95.9
13	2733.9	95.8
14	2720.0	95.3
15	2717.8	95.3
16	2700.7	94.7
17	2693.2	94.4
18	2688.0	94.2
19	2684.2	94.1
20	2679.4	93.9
21	2669.2	93.6
22	2666.2	93.5
23	2656.6	93.1
24	2656.4	93.1
Mean	2743.6	96.2
SD	61.36	2.15
%RSD	2.24	2.24

From the sample stability results found in Tables A.37 to A.40, it was observed the %RSD for the different statins were within the acceptable range of around 2% or less (Cowley, 2012:113), or $\pm 15\%$ according to González *et al.* (2014:22).

- * Fluvastatin: 0.08%
- * Lovastatin: 2.95%
- * Rosuvastatin: 0.09%
- * Simvastatin: 2.24%

A.3.5.2 System repeatability

Repeatability was measured in terms of accuracy and precision by preparing samples of each statin at a concentration of 10 mg and made up to volume (100 ml) with methanol; 1 000 µl was transferred with a micropipette to HPLC vials. The samples were then injected into the HPLC seven consecutive times, to evaluate repeatability of peak areas and retention times on the same day and under the same conditions. A %RSD of $\leq 2\%$ is viewed as acceptable criteria (Cowley, 2012:114). Tables A.41 to A.44 review the system repeatability of the selected statins.

Table A.41: System repeatability of fluvastatin

Injection	Peak area	Retention time (min)
1	3283.3	5.453
2	3270.6	5.452
3	3258.9	5.453
4	3263.2	5.449
5	3261.2	5.456
6	3263.2	5.456
7	3260.6	5.459
Mean	3265.9	5.454
SD	7.93	0.003
%RSD	0.24	0.055

Table A.42: System repeatability of lovastatin

Injection	Peak area	Retention time (min)
1	3025.4	7.897
2	3016.4	7.898
3	2993.1	7.913
4	3025.3	7.885
5	3006.8	7.901
6	3002.4	7.908
7	3005.9	7.907
Mean	3010.7	7.901
SD	11.20	0.009
%RSD	0.37	0.108

Table A.43: System repeatability of rosuvastatin

Injection	Peak area	Retention time (min)
1	2414.9	4.606
2	2413.2	4.604
3	2407.6	4.604
4	2409.9	4.601
5	2408.9	4.608
6	2410.4	4.607
7	2407.4	4.611
Mean	2410.3	4.606
SD	2.60	0.003
%RSD	0.11	0.065

Table A.44: System repeatability of simvastatin

Injection	Peak area	Retention time (min)
1	2871.6	7.067
2	2865.3	7.066
3	2867.9	7.079
4	2859.3	7.057
5	2866.1	7.070
6	2856.0	7.072
7	2854.7	7.075
Mean	2863.0	7.069
SD	5.92	0.007
%RSD	0.21	0.093

In Tables A.41 to A.44 it was noticed the %RSD for the peak areas and retention times of the different statins were below 2% and therefore fall within the accepted criteria (Cowley, 2012:114).

A.3.6 Specificity

Specificity can be defined as a measurement to investigate accurately the concentration of the analyte in the presence of other compounds to determine the effect impurities has on the analyte (ICH, 2005:4). For an analytical procedure to be acceptable, specificity needs to be tested (Snyder *et al.*, 1997:96). This method was tested with different components at the same retention times where the statins were detected.

For this assay a standard solution was prepared by weighing 20 mg of each statin and adding it to a 100 ml volumetric flask, the flask was then made up to volume with methanol. The standard solution

(1 ml) was then transferred into four different test tubes. To the first test tube, 200 µl of water (H₂O) was added, to the second test tube 200 µl of 2 M hydrochloric acid (HCl) was added, to the third test tube 200 µl of 2 M sodium hydroxide (NaOH) was added, and the fourth test tube contained 200 µl of 2 M hydrogen peroxide (H₂O₂); additional tubes were used for the placebo diluted with methanol and a standard solution. The test tubes were left to stand for 10 min, where after 1000 µl were transferred into HPLC vials by means of a micropipette. The samples were injected in duplicate into the HPLC.

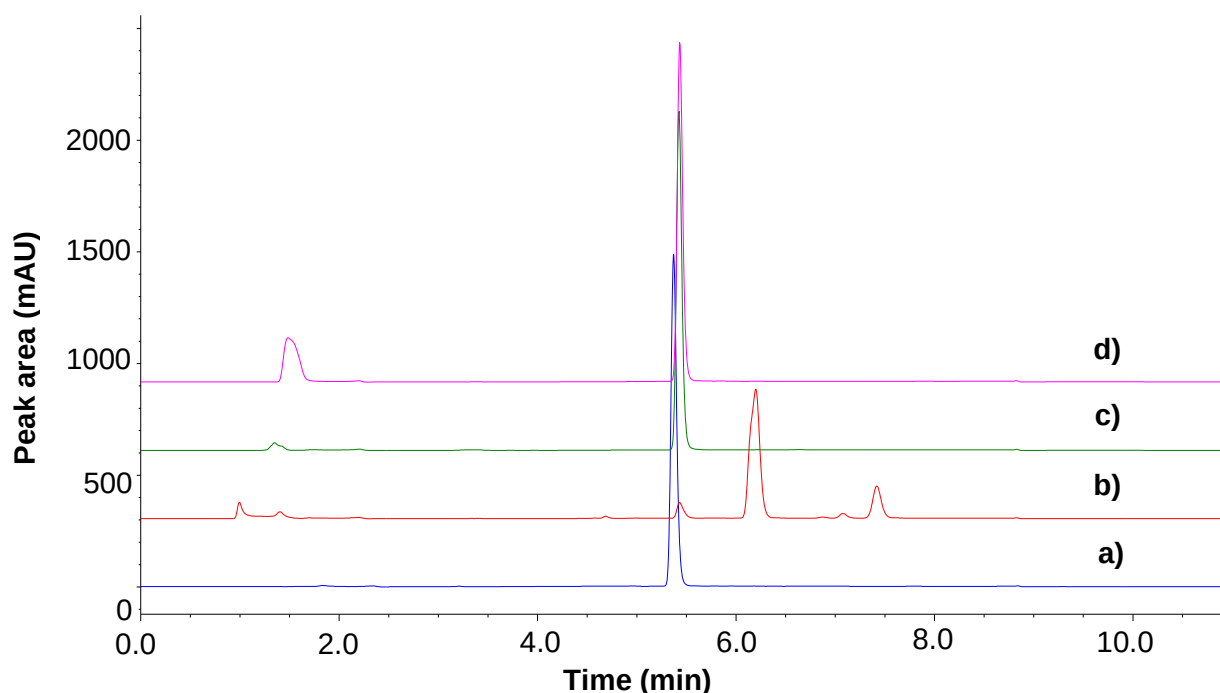


Figure A.7: HPLC chromatogram showing specificity data obtained for fluvastatin with 200 µl of a) H₂O, b) HCl, c) NaOH and d) H₂O₂

Table A.45: Specificity data for fluvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
H ₂ O (a)	5955.1	5901.9	5928.5	5.37
HCl (b)	4052.6	3959.3	4005.9	6.20
NaOH (c)	5968.2	5950.1	5959.2	5.43
H ₂ O ₂ (d)	5995.5	5989.9	5972.7	5.43

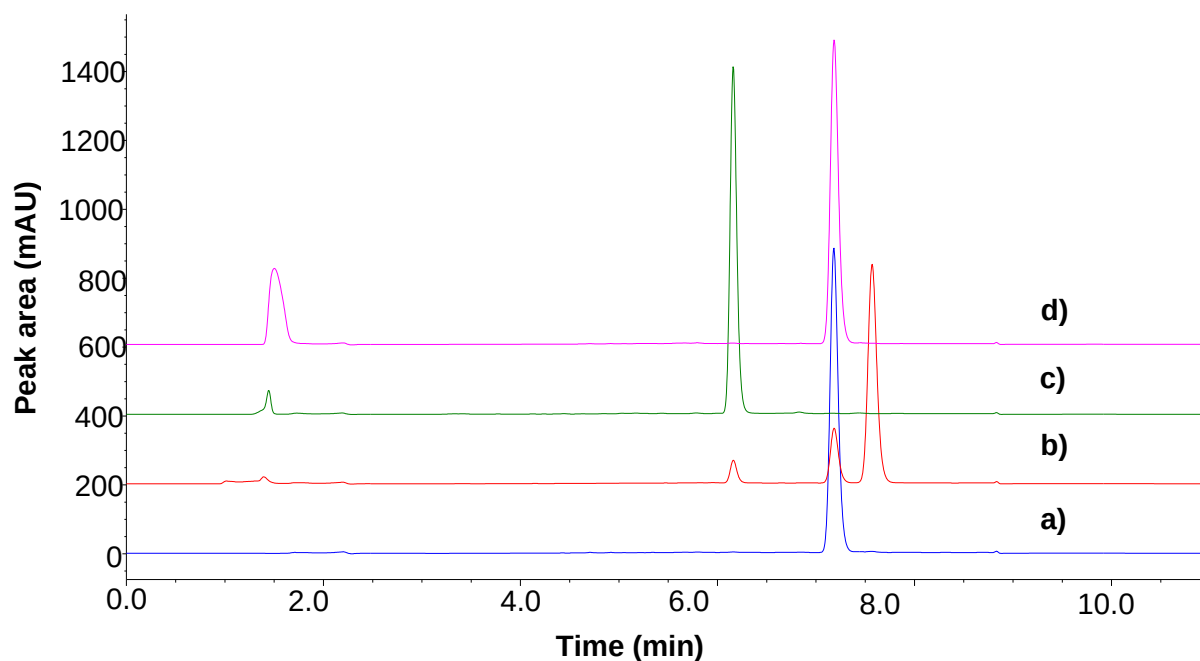


Figure A.8: HPLC chromatogram showing specificity data obtained for lovastatin with 200 μ l of a) H_2O , b) HCl , c) NaOH and d) H_2O_2

Table A.46: Specificity data for lovastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
H_2O (a)	5079.4	5084.3	5081.9	7.19
HCl (b)	4051.9	4054.3	4053.1	7.56
NaOH (c)	4848.1	4854.1	4851.1	6.15
H_2O_2 (d)	5119.8	5090.9	5105.4	7.18

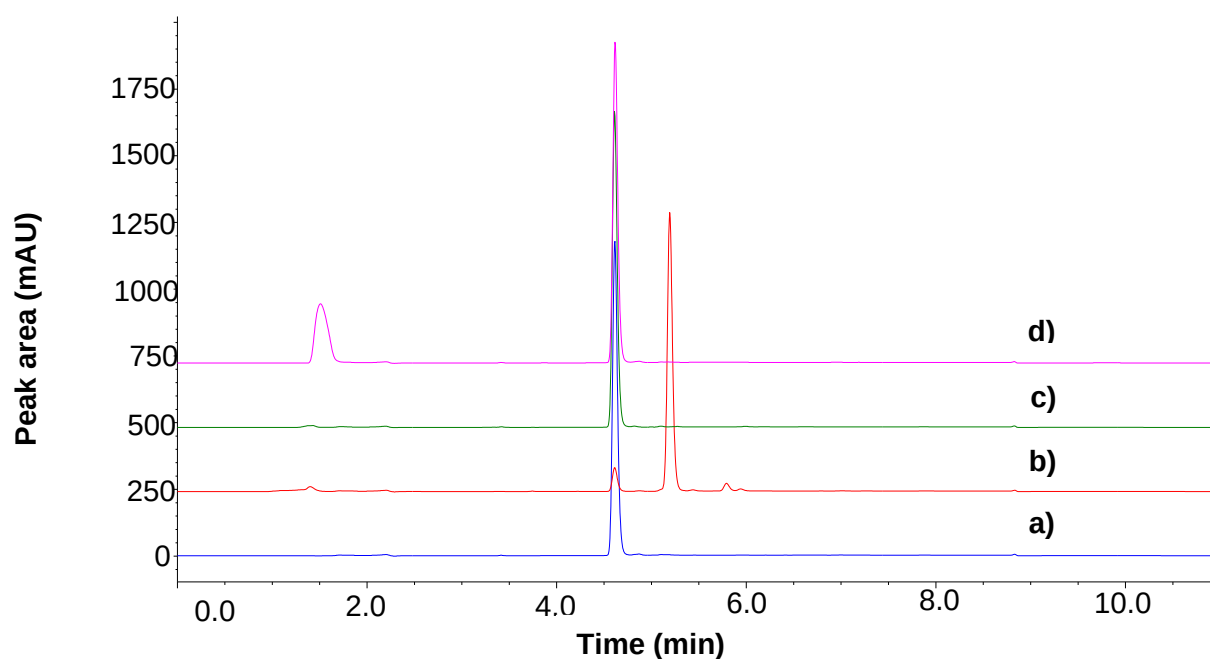


Figure A.9: HPLC chromatogram showing specificity data obtained for rosuvastatin with 200 μ l of a) H_2O , b) HCl , c) NaOH and d) H_2O_2

Table A.47: Specificity data for rosuvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
H_2O (a)	4308.0	4313.4	4310.7	4.61
HCl (b)	3990.6	3982.7	3986.7	5.19
NaOH (c)	4292.7	4294.5	4293.6	4.61
H_2O_2 (d)	4385.8	4381.6	4383.7	4.61

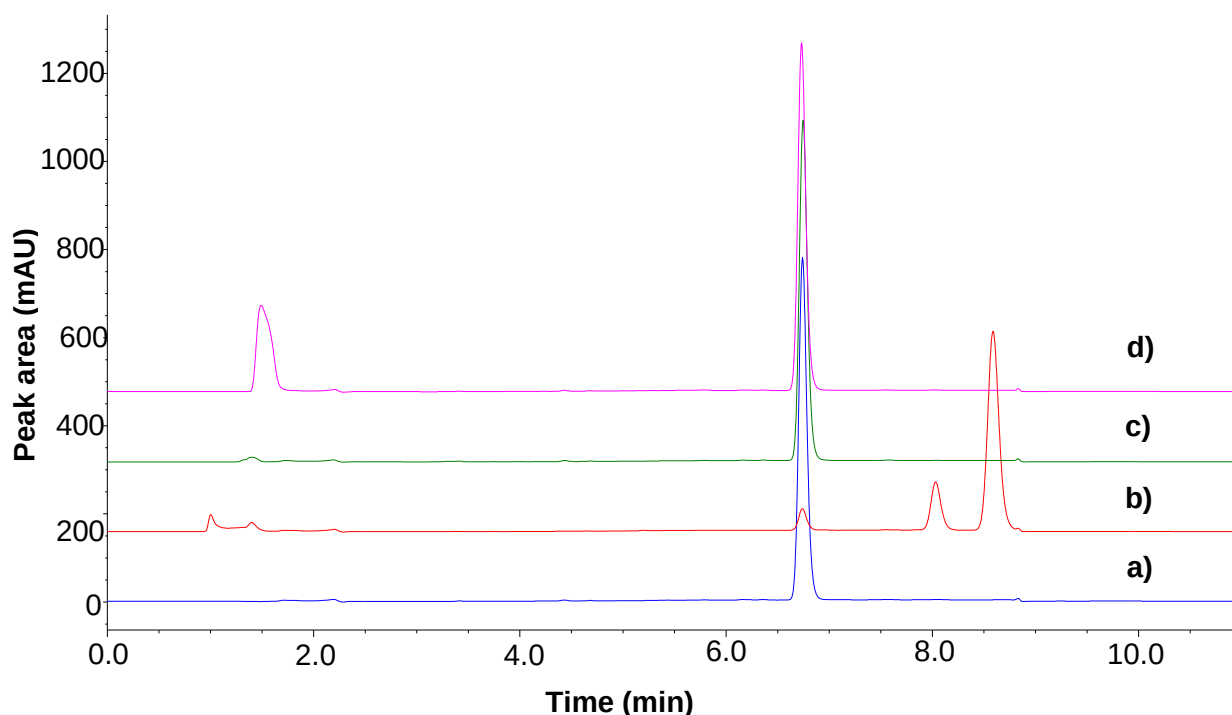


Figure A.10: HPLC chromatogram showing specificity data obtained for simvastatin with 200 μ l of a) H_2O , b) HCl , c) NaOH and d) H_2O_2

Table A.48: Specificity data for simvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
H_2O (a)	4350.8	4347.5	4349.2	6.74
HCl (b)	3559.8	3551.8	3555.8	8.58
NaOH (c)	4287.2	4293.3	4290.3	6.73
H_2O_2 (d)	4374.8	4374.3	4374.5	6.73

A.3.7 Limit of detection and lower limit of quantification

LOD can be described as the lowest amount of analyte that can be detected in a sample (USP, 2011). This level of substance detected merely tests the amount of analyte and if it is above or below a certain level.

The LLOQ is the lowest amount of analyte in a standard curve that can be quantitatively determined in a sample with acceptable precision and accuracy under experimental conditions (Rozet *et al.*, 2011:854; Wolfinger *et al.*, 2018:2). The LLOQ expresses as a concentration of analyte in the sample (FDA, 2001:10).

A.3.7.1 Limit of detection

To determine the LOD of fluvastatin, lovastatin, rosuvastatin and simvastatin, a stock solution was prepared by weighing 5 mg of each statin, respectively and placing it in a 100 ml volumetric flask. This was made up to 100 ml by adding methanol to the statins whereafter 1 ml of the stock solution was placed into another 100 ml volumetric flask by means of a micropipette and made up to volume with methanol. A volume of 10 μ l of the diluted solution was placed into HPLC vials for analysis. The solution was injected six times at four different injection volumes:

- * 2.5 μ l
- * 5.0 μ l
- * 7.5 μ l
- * 10 μ l

A %RSD of $\leq 15\%$ is seen as acceptable for LOD determination (FDA, 2001:10; Rathmann *et al.*, 2015:54), hence, from Tables A.49 to A.52 it was observed that all the statins were within the acceptable range:

- * Fluvastatin: 9.74%
- * Lovastatin: 6.92%
- * Rosuvastatin: 12.74%
- * Simvastatin: 7.39%

A.3.7.2 Lower limit of quantification

To determine the LLOQ of an analyte, a sample was prepared as described in Section A.3.7.1. A %RSD of 20% is seen as acceptable for determining LLOQ (Bansal & DeStefano, 2007:3; Meyer *et al.*, 2014:23). Tables A.49 to A.52 display the data for the selected statins, which were within the acceptable range:

- * Fluvastatin: 0.27%
- * Lovastatin: 0.97%
- * Rosuvastatin: 4.28%
- * Simvastatin: 1.51%

Table A.49: LOD and LLOQ results for fluvastatin

	LOD	LLOQ
Injection volume (µl)	2.50	10.00
Concentration (µg/ml)	0.14	0.55
Peak area	3.81	19.25
	3.87	19.27
	4.01	19.34
	4.47	19.32
	3.76	19.38
	3.19	19.38
Mean	3.85	19.32
SD	0.38	0.05
%RSD	9.74	0.27

Table A.50: LOD and LLOQ results for lovastatin

	LOD	LLOQ
Injection volume (µl)	2.50	10.00
Concentration (µg/ml)	0.15	0.62
Peak area	4.72	21.82
	4.86	21.68
	4.25	21.52
	4.51	21.24
	4.55	21.77
	5.29	21.84
Mean	4.69	21.65
SD	0.33	0.21
%RSD	6.92	0.97

Table A.51: LOD and LLOQ results for rosuvastatin

	LOD	LLOQ
Injection volume (μl)	2.50	10.00
Concentration (μg/ml)	0.13	0.53
Peak area	3.42	14.24
	3.28	14.25
	3.18	14.29
	3.00	14.28
	3.39	14.24
	4.37	12.65
Mean	3.44	13.99
SD	0.44	0.60
%RSD	12.75	4.28

Table A.52: LOD and LLOQ results for simvastatin

	LOD	LLOQ
Injection volume (μl)	2.50	10.00
Concentration (μg/ml)	0.13	0.52
Peak area	2.71	14.00
	3.29	14.33
	2.81	14.01
	2.99	14.28
	3.27	14.29
	3.19	14.63
Mean	3.05	14.26
SD	0.23	0.22
%RSD	7.39	1.51

A.4 Conclusion

The analysis method for fluvastatin, lovastatin, rosuvastatin and simvastatin was verified according to set standards and the results were satisfactory for linearity, accuracy, precision, repeatability, stability and specificity. The method was found to be valid and could be used for determining the concentration of the statins during the *in vitro* skin studies of both the nano-emulsions and the nano-emulgels.

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Appendix B

FORMULATION OF A NANO-EMULSION CONTAINING SELECTED STATINS AND GRAPSEED OIL

B.1 Introduction

The skin is the largest organ of the human body and can be seen as an important pathway for the absorption of drugs (Zhou *et al.*, 2018:1713). The epidermis contains the stratum corneum that prevents the penetration of particles through the skin, since the stratum corneum is hydrophobic in nature (Carter & Wang, 2019:49; Palmer & De Louise, 2016:1719). For transdermal delivery, the API should have ideal physicochemical properties and if not, the use of chemical penetration enhancers in a formulation is of great importance (Shafiq *et al.*, 2007:277; Vogt *et al.*, 2016:4). To overcome the barrier of the stratum corneum an o/w nano-emulsion was made.

Nano-emulsions are ideal formulations for transdermal delivery, as they enhance dermal penetration into the depth of the skin via intercellular and intracellular routes. The intercellular route is responsible for the movement of hydrophilic and lipophilic particles of an API through the lipid bilayers of the stratum corneum (Carter & Wang, 2019:49). Poor solubility of an API can lead to a decrease in permeation through the stratum corneum, which in turn may lead to poor bioavailability (Shafiq *et al.*, 2007:227). For nano-emulsions to cross the barrier of the skin, the droplet size must be < 200 nm (Tan *et al.*, 2019:264).

Before 1980, transdermal delivery was limited to only a few compounds passing through for absorption (Wiedersberg & Guy, 2014:150). Small particles show a closer contact to the stratum corneum and therefore, it is a challenge for molecules with a larger molecular weight to permeate the skin (Bos & Meinardi, 2000:165; Naik *et al.*, 2000:319). Successful formulation of APIs into a system that is non-irritant to the skin, is dependent on the ideal formulation (Williams, 2013:685). Characterisation was done on these nano-emulsions to determine if they exerted the ideal properties for transdermal delivery.

B.2 Intended purpose of the formulation

The aim was to formulate a nano-emulsion and nano-emulgel containing selected statins (fluvastatin, lovastatin, rosuvastatin and simvastatin, separately) for transdermal delivery. Although the selected statins do have the ideal molecular weight or aqueous solubility, a nano-emulsion and nano-emulgel would enhance the drug delivery system by improving unfavourable properties (Najafi-Taher *et al.*, 2018:142; Silva *et al.*, 2012:864). Grapeseed was incorporated into the formulations to act as a penetration enhancer, due to the fatty acid component and linoleic acid that contains unsaturated C₁₈ carbon fatty acids similar to those found in the skin (Cizinauskas *et al.*, 2017:1).

B.3 Delivery system selection

The skin acts as a barrier to protect the internal organs from the environment (Saghazadeh *et al.*, 2018:1). A variety of properties can influence transdermal delivery; the selected statin must be delivered by what is referred to as a vehicle that carries the selected statins through the skin (Weiss, 2011:472). The API must move through the skin to the targeted area where the delivery process will proceed into the systemic circulation, thus the vehicle used for transport is important. The chosen vehicle must bind to the excipients in the formulation to deliver the API to the desired place; the API must be stable in the selected formulation, and must be released when needed from the formulation (Weiss, 2011:472). For the purposes of this study, a nano-emulsion (with grapeseed oil) was used as the delivery vehicle for the transdermal delivery of the selected statins. Nano-emulsions as a delivery system have numerous advantages, and they could be used to encapsulate lipophilic drugs to disperse them into aqueous media (Khatri & Shao, 2017:171; Ostertag *et al.*, 2012:95).

B.4 Excipients used to formulate a nano-emulsion

Nano-emulsions have much better stability to gravitational forces and consist of water, oil, surfactant(s), co-surfactant(s) and the API (Chime *et al.*, 2014:90; Reddy *et al.*, 2013:87). Due to nano-emulsions' small particle size, they have better stability against flocculation and coalescence (Qian & McClements, 2011:1000; Tadros *et al.*, 2004:303). The use of surfactants and co-surfactants assists with formulating a nano-emulsion to maintain the solubilisation of the emulsion. Although grapeseed oil was originally incorporated as a penetration enhancer, it should also be noted that the solubility of the API in the nano-emulsion is greatly influenced by the oil phase used (Shafiq *et al.*, 2007:228).

Table B.1: Excipients, functions, suppliers and batch numbers as used during the formulation of the o/w nano-emulsions containing fluvastatin, lovastatin, rosuvastatin and simvastatin in grapeseed oil

Excipient	Function	Supplier	Batch number
Fluvastatin	API	DB Fine Chemicals	1701108447
Lovastatin	API	DB Fine Chemicals	20170423
Rosuvastatin	API	DB Fine Chemicals	ROC/1512B/0008J1
Simvastatin	API	DB Fine Chemicals	IF-SI-170913
Grapeseed oil	Natural oil and penetration enhancer	CJP Chemicals	0712E040876
Span® 60	Lipophilic surfactant	Sigma-Aldrich	BCBT9142
Tween® 80	Hydrophilic surfactant	Sigma-Aldrich	SLBN0127V
Milli-Q® water	Solvent	In house	Direct Pure UP

B.4.1 Statins

Statins have a relatively low aqueous solubility, thus the bioavailability is limited (Korani *et al.*, 2019:568). Lovastatin and simvastatin are natural statins and their chemical structure is closely related, whereas artificial statins have more interactions with HMG-CoA and have a higher lipophilicity than natural statins (Korani *et al.*, 2019:568). Fluvastatin, lovastatin, simvastatin are lipophilic, whereas rosuvastatin is more hydrophilic, hence, the statins were incorporated in the oil phase of the nano-emulsion, which contained 2% of the selected API.

B.4.2 Grapeseed oil

For o/w nano-emulsions to penetrate the skin, oil is needed with high content fatty acids, such as linoleic and oleic acids (Fiori, 2007:43). Grapeseed oil is rich in unsaturated fatty acids, according to Davidov-Pardo and McClements (2015:205), which acts as a penetration enhancer. Grapeseed contains 72.2% linoleic fatty acid and 21.63% oleic acid, which are essential for the human body, since it cannot be synthesised naturally in the body. Oleic acid is used to activate the diffusion of permeants through the skin, working on the skin lipids (Lane, 2013:18), and plays a role in the chemical structure of intercellular lipid layers in the stratum corneum (Engelbrecht *et al.*, 2011:2799). Grapeseed oil is also rich in phenolic and tocopherol components, which helps in crossing the skin barrier (Almeida *et al.*, 2009:166; Göktürk Baydar *et al.*, 2007:29; Kamel *et al.*, 1985:882). Tocopherols can act as an antioxidant by breaking chains in electron donors and inhibits oxidation. The degree of unsaturated fatty acids plays an important role in lowering cholesterol levels, and is ideal for the purpose of the study on the effect of cholesterol.

B.4.3 Emulsifiers

Emulsifiers (better known as surfactants) are needed to form stable oil droplets (Almeida *et al.*, 2009:165). The behaviour of the phase is governed by the size of the droplets, which in turn is defined by the surfactant phase structure (Solans *et al.*, 2005:102). Surfactants are amphiphilic molecules consisting of both hydrophilic and hydrophobic groups on the same molecule (Singh *et al.*, 2017:31). The critical problem with nano-emulsions and large amounts of surfactants used is that it can cause gastrointestinal and skin irritations when administered orally and topically (Azeem *et al.*, 2009:71). Surfactants cause a lowering in interfacial tension, which in turn leads to a smaller droplet size (Tadros *et al.*, 2004:306). Nano-emulsions formed by low-energy methods have shown that the size of the droplet is dictated by the surfactant phase structure (Solans *et al.*, 2005:107). The ideal surfactant should balance in the hydrophilic and lipophilic groups (Jadhav *et al.*, 2015:110). When formulating with surfactants, it must be noted that the higher concentration of surfactant used, the higher the interfacial tension (lowers the droplet size and polydispersity index) (Solans *et al.*, 2005:103).

Emulsions formulated with surfactants must have a hydrophilic-lipophilic balance (HLB) value close to that of the oil used in the formulation to ensure a stable emulsion (Orafidiya & Oladimeji, 2002:241). Emulsifying agents are used in combination with substances to balance both lipophilic and hydrophilic properties in the formulation of a nano-emulsion (Magdassi & Garti, 1999:156; Reddy *et al.*, 2013:87). It is suggested by Eid *et al.* (2014:4) that the HLB values of a nano-emulsion must fall within the range of 9 – 18 (Setya *et al.*, 2014:2218). During this study, Tween® 80 and Span® 60 were used as surfactants in combination with the grapeseed oil.

B.4.4 Span® 60

Span® 60 (sorbitan monostearate), in a formulation, has shown increased permeation across the skin (Fang *et al.*, 2001:91) and has an HLB value of 4.7. For a lipophilic surfactant to be incorporated into the oil-phase of an o/w nano-emulsion, the HLB value must be less than 10 (Reddy *et al.*, 2013:87; Roessler *et al.*, 2002:17; Zhang, 2009a:676). When a surfactant molecule carries a positive charge and dispersed in water, the surfactant is then cationic, which subsequently gives stability during the time of manufacturing and provides a longer shelf life for the emulsion (Phad *et al.*, 2018: S386). Span® 60 is classified as a non-ionic, low HLB surfactant used as a wetting, emulsifying, dispersing and suspending agent (Roessler *et al.*, 2002:9; Zhang, 2009a:675). Span® 60 was utilised during this study in the oil phase with other lipophilic excipients of the emulsion.

B.4.5 Tween® 80

Tween® 80 (polysorbate 80) is best suited for use in nano-emulsions, since it needs a low energy emulsification method to form an emulsion and assists with stabilisation (Ostertag *et al.*, 2012:99; Zhang; 2009b:550). Polysorbate is used in nano-emulsions because of their low toxicity and effectiveness at low concentrations (Ha *et al.*, 2002:2253). Tween® 80 has an HLB value of 15 and is hydrophilic nature, thus it can be incorporated into a water phase (Reddy *et al.*, 2013:87). According to Chime *et al.* (2014:91), hydrophilic and lipophilic surfactants, incorporated into a nano-emulsion will decrease the amount of energy needed for the formulation of the emulsion.

B.4.6 Water

Emulsification takes place when an organic phase and an aqueous phase are mixed. The aqueous phase consists out of a hydrophilic surfactant and water (Bouchemal *et al.*, 2004:241; Hyma *et al.*, 2014:4). Solvents are fundamental for nano-emulsions production, according to Silva *et al.* (2012:857). The water phase contributes to a large part of the nano-emulsion. Milli-Q® water was used as a solvent and constituted the water phase of the nano-emulsion.

B.5 Solubility of selected statins in grapeseed oil

When selecting an oil for the o/w nano-emulsion, the compatibility of the API with the oil must be brought into account, as it determines the amount of API dissolved into the nano-emulsion (Chime *et al.*, 2014:91). Determination of solubility of the APIs in the oil is a critical step in development and evaluation (Duba & Fiori, 2016:44), which was done by preheating the water-shaker bath to 32°C. Grapeseed oil (5 ml) was transferred to the test tubes and thereafter an excess amount of statins were added to the test tubes (in triplicate) and clearly marked. The test tubes were placed into the water-shaker bath for a period of 24 h. Mixtures were constantly checked to see if an excess of API was still present. After 24 h, the test tubes were removed and placed into a centrifuge for 15 min at 4 500 rpm. Supernatant (1 ml) from each test tube was transferred by means of a micropipette into 25 ml volumetric flasks and made up to volume with tetrahydrofuran (THF). The dilution was then transferred into HPLC flasks to determine the concentration of each statin in the grapeseed oil, which were as follows:

- * Fluvastatin: 0.050 mg/ml
- * Lovastatin: 0.225 mg/ml
- * Rosuvastatin: 0.061 mg/ml
- * Simvastatin: 0.007 mg/ml

Literature suggests that oil solubility of grapeseed oil and the API increases with an increase in pressure and decreases with an increase in temperature (Duba & Fiori, 2016:47). According to McClements and Li (2010:217), the solubility of fatty acids and their lipase activation is dependent on the pH of the surrounding elements.

B.6 Formulation of nano-emulsions

Nano-emulsions can absorb active constituents rapidly to penetrate through the skin surface and in this manner enhance penetration. Nano-emulsions are effective transport systems because of their higher surface area and free energy (Bhatt & Madhav, 2011:2483). Nano-emulsions can be prepared using surfactant concentrations in reasonable amounts (Tadros *et al.*, 2004:304). Nano-emulsions in different ratios of Tween® 80 and Span® 60 were used to find the correct formulation of emulsions to pass through the skin, the phase of the emulsion is dependent on the surfactant used (Lovelyn & Attama, 2011:626). Nano-emulsions are thermodynamically unstable and cannot be formed spontaneously, they require either a high-pressure homogeniser, ultrasonic generator or microfluidisers (Bhatt & Madhav, 2011:2294), which will define the particle size of the emulsion (Kela & Kaur, 2013:9203; Tadros *et al.*, 2004:308). Other methods can be used, such as phase inversion temperature emulsification where the formulation of a nano-emulsion is only stable at an optimum storage temperature, if there are any deviation from these temperatures, it will lead to an increase

in droplet size (Ee *et al.*, 2008:626). High-pressure homogenisers were used in this study to reduce the particle size of the nano-emulsion and to reduce the chances of coalescence and flocculation (Kela & Kaur, 2013:9203; Solans *et al.*, 2005:103). Desired results were obtained after sonicating the nano-emulsions for 3 min intervals.

An o/w nano-emulsion was formulated through a high-energy emulsification method (ultrasonication). Nano-sized droplets were obtained with the ultrasonicator with a probe (Model UP200St, Hielscher Ultrasonics, Germany) (Figure B.1), since smaller droplet sizes cross the skin barrier with less effort and are therefore more convenient to reach target areas (Rai *et al.*, 2018:204). The formulated nano-emulsions contained 2% (w/v) of selected statins and 8% or 10% (w/v) grapeseed oil. Three different surfactant ratios were investigated, namely Tween® 80:Span® 60 in the ratio of 3:3; 2:3 and 3:2. Placebo formulations (**(NEP)**) were also formulated and contained no API. The nano-emulsion selected was also used during the formulation of the semi-solid dosage form (nano-emulgel), which will be discussed in Appendix C.



Figure B.1: High-energy emulsification method ultrasonicator (Model UP200St)

B.6.1 Formulation of pre-formulated o/w nano-emulsions

The first group of nano-emulsions contained Tween® 80:Span® 60 (3:3) with 8.31% (w/w) grapeseed oil, and will be referred to as **(NE1)**, which included:

- * **(NEF1)** (2% fluvastatin in nano-emulsion formula 1)
- * **(NEL1)** (2% lovastatin in nano-emulsion formula 1)
- * **(NER1)** (2% rosuvastatin in nano-emulsion formula 1)
- * **(NES1)** (2% simvastatin in nano-emulsion formula 1)
- * **(NEP1)** (placebo of nano-emulsion formula 1)

The second group of nano-emulsions contained Tween[®] 80:Span[®] 60 (3:2) with 10.00% (w/w) grapeseed oil, and will be referred to as **(NE2)**, which included:

- * **(NEF2)** (2% fluvastatin in nano-emulsion formula 2)
- * **(NEL2)** (2% lovastatin in nano-emulsion formula 2)
- * **(NER2)** (2% rosuvastatin in nano-emulsion formula 2)
- * **(NES2)** (2% simvastatin in nano-emulsion formula 2)
- * **(NEP2)** (placebo of nano-emulsion formula 2)

The third group of nano-emulsions contained Tween[®] 80:Span[®] 60 (2:3) with 8.00% (w/w) grapeseed oil, and will be referred to as **(NE3)**, which included:

- * **(NEF3)** (2% fluvastatin in nano-emulsion formula 3)
- * **(NEL3)** (2% lovastatin in nano-emulsion formula 3)
- * **(NER3)** (2% rosuvastatin in nano-emulsion formula 3)
- * **(NES3)** (2% simvastatin in nano-emulsion formula 3)
- * **(NEP3)** (placebo of nano-emulsion formula 3)

B.6.1.1 Formulation of o/w nano-emulsions

The different formulas for the o/w nano-emulsions are outlined in Tables B.2, B.3 and B.4, respectively. All the nano-emulsions were formulated with APIs and then characterised.

Table B.2: Formula of **(NE1)** (50 ml)

Phase	Ingredient	Function	Amount
Oil phase (A)	Selected Statins	API	1 g
	Grapeseed oil	Natural oil and penetration enhancer	4.35 ml
	Span [®] 60	Lipophilic surfactant	3 g
Water phase (B)	Tween [®] 80	Hydrophilic surfactant	2.80 ml
	Milli-Q [®] water	Solvent	40.00 ml

Table B.3: Formula of **(NE2)** (50 ml)

Phase	Ingredient	Function	Amount
Oil phase (A)	Selected Statins	API	1 g
	Grapeseed oil	Natural oil and penetration enhancer	4.37 ml
	Span® 60	Lipophilic surfactant	3 g
Water phase (B)	Tween® 80	Hydrophilic surfactant	1.86 ml
	Milli-Q® water	Solvent	40.00 ml

Table B.4: Formula of **(NE3)** (50 ml)

Phase	Ingredient	Function	Amount
Oil phase (A)	Selected Statins	API	1 g
	Grapeseed oil	Natural oil and penetration enhancer	5.46 ml
	Span® 60	Lipophilic surfactant	2 g
Water phase (B)	Tween® 80	Hydrophilic surfactant	2.79 ml
	Milli-Q® water	Solvent	40.00 ml

B.6.1.2 Formulation method of a nano-emulsion

Formulation of the o/w nano-emulsion contained 2% of the selected statins and 8% grapeseed oil for **(NE1)** and **(NE3)** and 10% grapeseed oil for **(NE2)**. Firstly, a coarse emulsion through self-emulsification was prepared, and then ultrasonication (high-energy emulsification) was used to create the nano-sized droplets. The method used to formulate the different nano-emulsions is illustrated in Figure B.2.



Figure B.2: Formulation method of the **(NE1)**: a) Tween® 20 and water measured separately; b) Tween® 20 and water mixed together; c) Tween® 20 and water pre-heated (phase B); d) grapeseed oil preheated; e) Span® 60 was added to the preheated grapeseed oil (phase A); f) heated and dissolved grapeseed oil and Span® 60; g) API is added to phase A and dissolved; h) phases A and B were mixed together; i) sonication of emulsion for 3 min in 1 min intervals

The following steps were followed to formulate the o/w nano-emulsions:

- * Measure Milli-Q® water (40 ml) in a measuring cylinder and place in a beaker.

- * Measure the hydrophilic surfactant (Tween® 80) using a micropipette.
- * Add the hydrophilic surfactant (Tween® 80) to the water in the beaker.
- * Place a magnetic stirring rod in the water phase and place on a hot plate while ensuring continuous stirring and mix until the Tween® 80 has dissolved completely.
- * Weigh the lipophilic surfactant (Span® 60) in a weighing boat.
- * Measure the grapeseed oil (natural oil and penetration enhancer) in a beaker using a micropipette.
- * Add a magnetic stirring rod to the grapeseed oil and heat on a hot plate whilst continuously stirring.
- * When the grapeseed oil has reached its preferred temperature (70 – 75 °C), add the lipophilic surfactant (Span® 60).
- * When the Span® 60 has completely dissolved, add the selected statins to the oil mixture (phase A) and mix until dissolved.
- * Mix the two phases (phase A into phase B), drop wise until an emulsion is formed, remove the magnetic stirring rods and place back on the hot plate (turn the heat off, the plate remains warm). This is referred to as the coarse emulsion.
- * Remove the beakers from the hot plate and place into a beaker filled with Milli-Q® water. The nano-emulsion must be placed under the ultrasonic probe for 3 min (three 1 min intervals with a few seconds rest in between each probe session).
- * After sonication with the ultrasonic probe, the dispersion must be placed in the ultrasonic bath for 15 min.
- * Nano-sized droplets are created due to energy forces.

All **(NE1)**, **(NE2)** and **(NE3)** formulations were white translucent liquids that showed no aggregation or sedimentation. All three formulations had no oil droplets visible and thus it can be concluded that the dispersion of the oil droplets in water was successful. **(NE1)** had a milky white colour, whereas **(NE2)** and **(NE3)** had a translucent colour. It was observed that **(NE3)** already presented with phase separation directly after formulation. The different nano-emulsions are shown in Figure B.3.

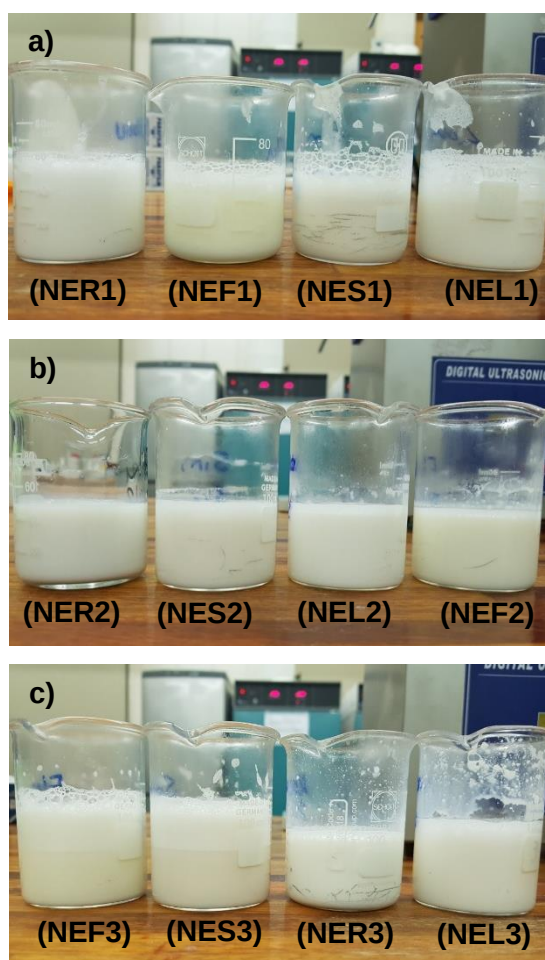


Figure B.3: The formulated dispersions: a) (NE1), b) (NE2) and c) (NE3)

B.7 Characterisation of the pre-formulated nano-emulsions

Effectiveness of the nano-emulsion, reaching its aims for stability and penetration, is set on the characteristics of a dispersion. Evaluating and comparing the properties helps determine which ratio of surfactant would be best to use as an optimal o/w nano-emulsion. The final nano-emulsion was chosen after the three nano-emulsions with different ratios of surfactants were compared and subsequently used during further investigations.

The following characteristics of the three dispersions with different surfactant ratios were determined:

- * pH;
- * droplet size and distribution;
- * zeta-potential;
- * viscosity;
- * drug entrapment efficiency;
- * morphology (this was done only on the selected nano-emulsion, since it is very costly).

B.7.1 pH

The pH of the skin ranges between 4 and 6; these pH values plays a vital role in the functionality of the barrier and integrity of the skin, as normal conditions of the skin is acidic (Ali & Yosipovitch, 2013:261). Measurement of the pH levels must be done to ensure they are within normal range (4 – 6), to determine if the dispersion is basic or acidic. The pH was measured using a Mettler Toledo® pH meter (Mettler Toledo, United States) equipped with a Mettler Toledo® InLab® 410 electrode (Figure B.6). Measurement of pH takes place by measuring the concentration of hydrogen ions $[H^+]$ and is expressed in the ratio between $[H^+]$ and hydroxide ion concentration $[OH^-]$ (Hach Company, 2010:6). A formulation is seen as equal when the ratio of $[H^+]$ and $[OH^-]$ gives a pH of 7 (Hach Company, 2010:7). According to Hempoonsert *et al.* (2010:41), oil droplet size can be influenced by pH. At a lower pH, a less dense packing of droplets occurs, while at a higher pH denser droplet formation occurs. The pH was measured for **(NE1)**, **(NE2)** and **(NE3)** of all four statins in the three different ratios of Tween® 80:Span® 60. Measurements were taken in triplicate by placing the electrode into the freshly prepared dispersions and thereafter the average pH was determined.



Figure B.4: A Mettler Toledo® pH meter with a Mettler Toledo® InLab® 410 electrode

Table B.5: The measured average pH for **(NE1)** containing selected statins

	pH (reading 1)	pH (reading 2)	pH (reading 3)	Average pH
(NEF1)	7.37	7.34	7.34	7.35 ± 0.02
(NEL1)	6.17	6.17	6.18	6.17 ± 0.01
(NER1)	6.08	6.12	6.12	6.11 ± 0.02
(NES1)	7.14	7.12	7.12	7.13 ± 0.01
(NEP1)	6.25	6.27	6.26	6.26 ± 0.01

Table B.6: Average pH for **(NE2)** containing selected statins

	pH (reading 1)	pH (reading 2)	pH (reading 3)	Average pH
(NEF2)	7.55	7.55	7.55	7.55 ± 0.01
(NEL2)	6.36	6.35	6.35	6.35 ± 0.01
(NER2)	6.38	6.37	6.37	6.37 ± 0.02
(NES2)	7.31	7.31	7.37	7.33 ± 0.02
(NEP2)	6.56	6.56	6.56	6.56 ± 0.00

Table B.7: Average pH for **(NE3)** containing selected statins

	pH (reading 1)	pH (reading 2)	pH (reading 3)	Average pH
(NEF3)	7.65	7.65	7.65	7.65 ± 0.01
(NEL3)	6.34	6.27	6.27	6.29 ± 0.01
(NER3)	6.35	6.35	6.35	6.35 ± 0.00
(NES3)	7.50	7.50	7.50	7.50 ± 0.00
(NEP3)	6.40	6.40	6.40	6.40 ± 0.00

From Tables B.5 to B.7, it was observed that all the dispersions of **(NE1)**, **(NE2)** and **(NE3)** had pH values within the acceptable range according to Barry (2002:512), and ranged within 3 – 9, pH higher or lower than these values can influence the permeability through the skin. It was detected that both Span® 60 and Tween® 80 have an acidic value between 2 - 10 and the amount used can influence the pH level of the nano-emulsion (Zhang, 2009b:551). When the average pH values of **(NE1)** are compared to those of **(NE2)** and **(NE3)**, with regard to the respective statins, it is observed that **(NE1)** had a relatively lower pH than **(NE2)** and **(NE3)**, which may be due to the different surfactant ratios used in the different formulations (Zhang, 2009b:551).

B.7.2 Droplet size and distribution

Droplet size and distribution, or light scatter, is measured by the use of instruments that are based on photon correlation spectroscopy (PCS), such as the Zetasizer (Malvern Instruments Limited, 2015:15). PCS measures the light fluctuations between dispersed droplets induced by Brownian motion (Gaur *et al.*, 2014:40; Malvern Instruments Limited, 2015:15). With the interaction of light, the droplet size is determined, which subsequently indicates the average diameter (d.nm) of the droplet on a 90 °angle (Gaumet *et al.*, 2008:2). Each particle is viewed as a sphere where the PCS result is given as the polydispersity index (Pdl) and takes the particle mean size, refraction index, measurement angle and variance of distribution into account (Gaumet *et al.*, 2008:2). In order to choose the ideal formulation, these characteristics are essential.

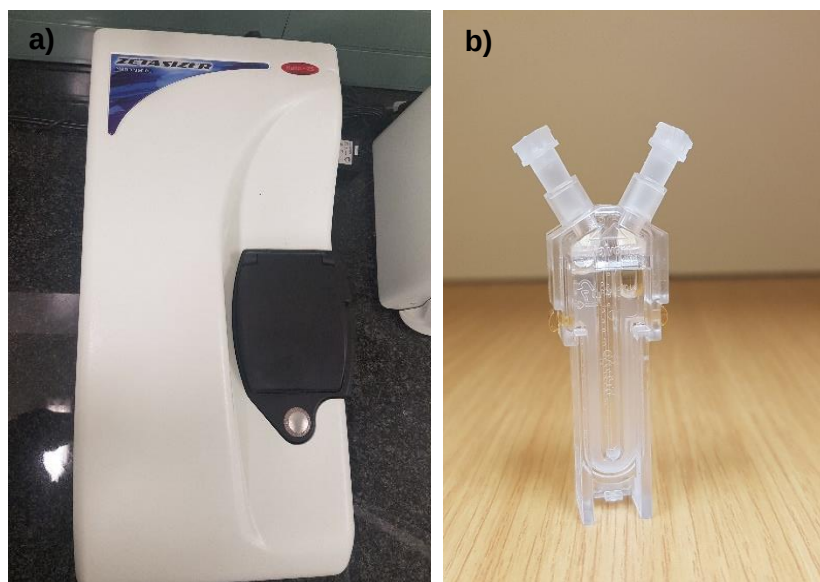


Figure B.5: a) Malvern Zetasizer Nano ZS and b) a clear disposable DTS1070 folded capillary zeta-cell

For the measurement of droplet size and distribution a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) (Figure B.7) was utilised for this study. The **(NE1)**, **(NE2)** and **(NE3)** were diluted using 10 ml of Milli-Q® water and one drop of the dispersion was placed in a polytop. The diluted solution was then transferred to the clear disposable zeta-cell by means of a clear disposable syringe after thorough mixing of the samples. Each zeta-cell of **(NE1)**, **(NE2)** and **(NE3)** were analysed in triplicate and the average of each sample was determined.

Droplet size of 100 nm or less would facilitate inclusion of oil soluble agents. Small droplet sizes lead to transparent emulsions. Ideal droplet size ranges between 50 - 200 nm for delivery of nano-emulsions through the transdermal route (Tadros *et al.*, 2004:303). Small droplet sizes imply that a nano-emulsion can flow easily (Abolmaali *et al.*, 2011:139; Fernandez *et al.*, 2004:53; Leong *et al.*, 2009:721). The average droplet size of selected statins in the following dispersions **(NE1)**, **(NE2)** and **(NE3)** were compared. The average droplet size of the selected statins in **(NE1)**, **(NE2)** and **(NE3)** is given in Figures B.6 to B.8. Comparisons were drawn between the formulations.

- * **(NEF1)**, **(NEF2)** and **(NEF3)**
- * **(NEL1)**, **(NEL2)** and **(NEL3)**
- * **(NER1)**, **(NER2)** and **(NER3)**
- * **(NES1)**, **(NES2)** and **(NES3)**

Table B.8: Average droplet size (nm), as well as polydispersity index (Pdl) of the formulated dispersions of **(NE1)**

(NE1)	Average droplet size (nm)	Average Pdl
(NEF1)	162.60 ± 1.47	0.31 ± 0.02
(NEL1)	154.23 ± 0.68	0.39 ± 0.01
(NER1)	151.20 ± 1.91	0.30 ± 0.04
(NES1)	151.73 ± 2.28	0.30 ± 0.04
(NEP1)		0.39 ± 0.01

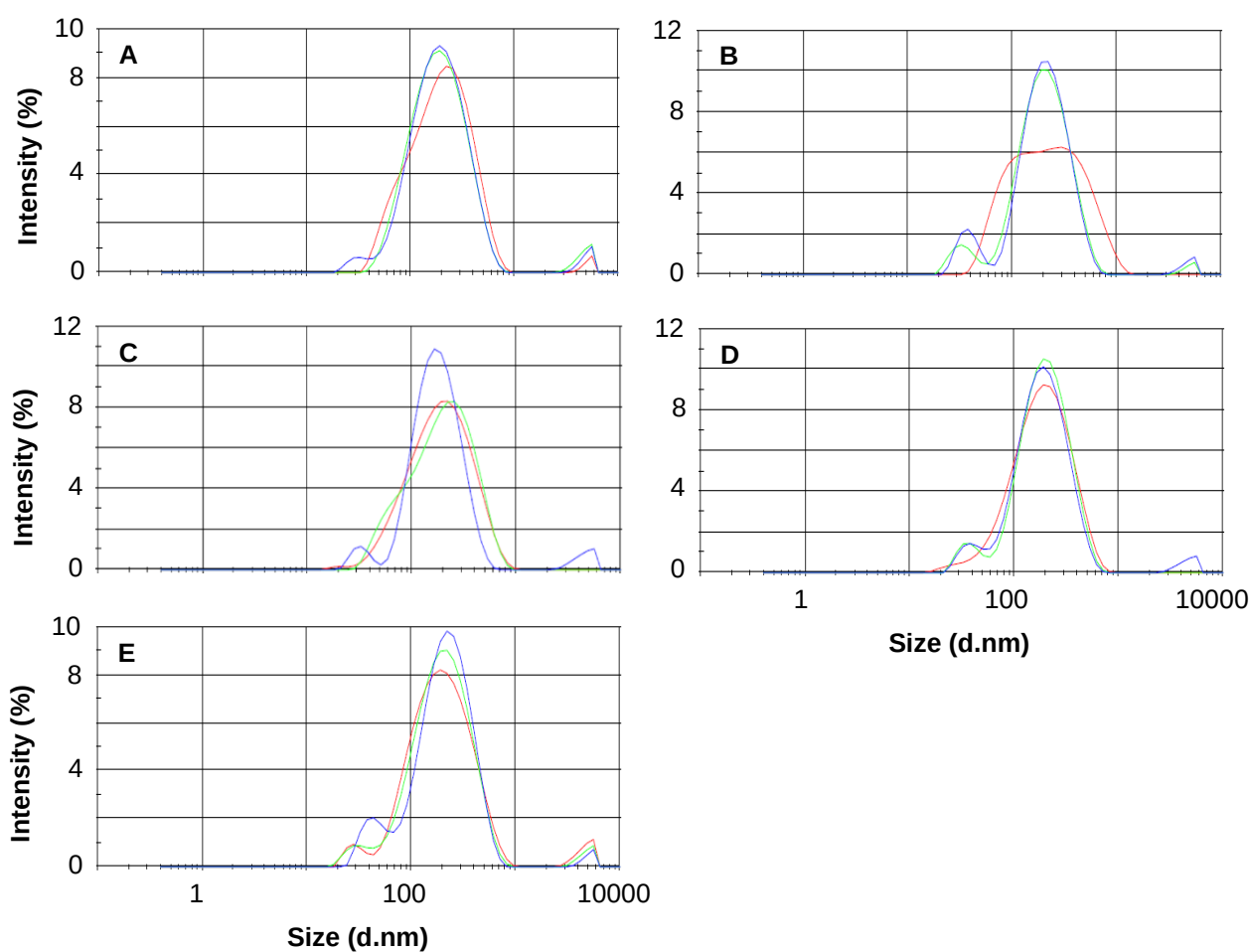


Figure B.6: Average droplet size (nm) of: a) **(NEF1)**, b) **(NEL1)**, c) **(NER1)**, d) **(NES1)**, and e) **(NEP1)**

Table B.9: Average droplet size (nm), as well as polydispersity index (Pdl) of the formulated dispersions of **(NE2)**

(NE2)	Average droplet size (nm)	Average Pdl
(NEF2)	155.20 ± 0.69	0.13 ± 0.01
(NEL2)	160.30 ± 1.04	0.21 ± 0.02
(NER2)	153.53 ± 1.65	0.20 ± 0.02
(NES2)	160.33 ± 0.85	0.25 ± 0.02
(NEP2)	163.73 ± 1.36	0.21 ± 0.01

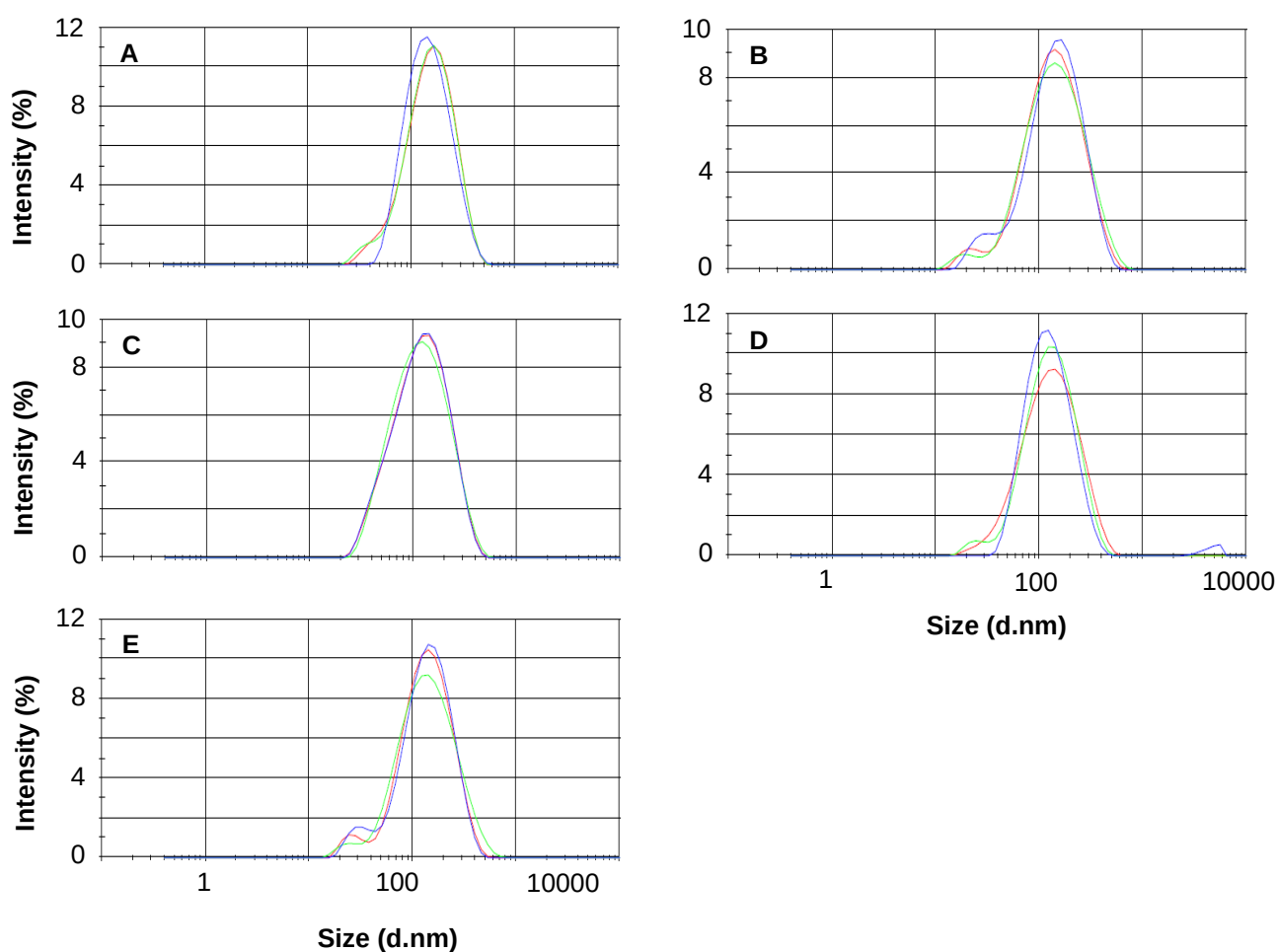


Figure B.7: Average droplet size (nm) of a) **(NEF2)**, b) **(NEL2)**, c) **(NER2)**, d) **(NES2)**, and e) **(NEP2)**

Table B.10: Average droplet size (nm), as well as polydispersity index (Pdl) of the formulated dispersions of **(NE3)**

(NE3)	Average droplet size (nm)	Average Pdl
(NEF3)	126.97 ± 1.64	0.221 ± 0.00
(NEL3)	111.90 ± 0.96	0.291 ± 0.01
(NER3)	99.45 ± 0.43	0.236 ± 0.00
(NES3)	106.63 ± 0.40	0.247 ± 0.01
(NEP3)	110.93 ± 0.85	0.267 ± 0.01

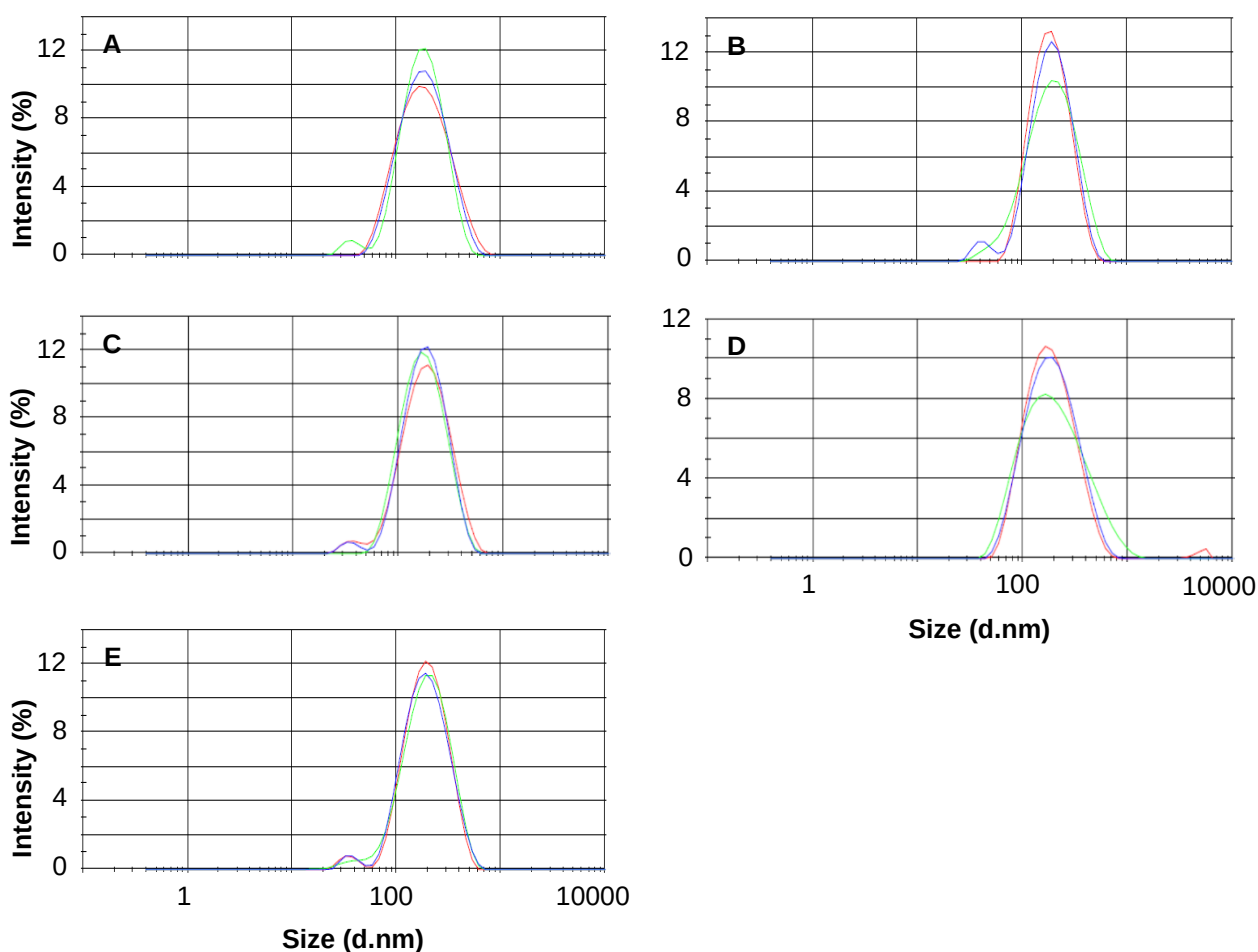


Figure B.8: Average droplet size (nm) of a) **(NEF3)**, b) **(NEL3)**, c) **(NER3)**, d) **(NES3)**, and e) **(NEP3)**

Figures B.6 to B.8 illustrate the average droplets size of each dispersion in triplicate. When fluvastatin is examined, it is evident that **(NEF1)**, **(NEF2)** and **(NEF3)** have relatively symmetrical curves. For lovastatin, the curves of **(NEL2)** and **(NEL3)** are symmetrical, while **(NEL1)** is not; this may be due to the variation of droplet sizes between readings. **(NER1)** seems less symmetrical than **(NER2)** and **(NER3)** when the average droplet size curves of rosuvastatin are compared. All the average droplet size curves of rosuvastatin (**(NES1)**, **(NES2)** and **(NES3)**) show relatively good symmetry, although **(NES3)** shows the best symmetry, which can be because of the small droplet

size of **(NE3)**. The average droplet size range for **(NE1)** was $151.20 \pm 1.91 - 162.60 \pm 1.47$ nm, for **(NE2)** was $153.53 \pm 1.65 - 163.73 \pm 1.36$ nm, and for **(NE3)** was $99.45 \pm 0.43 - 126.97 \pm 1.64$ nm. Thus, the average dispersed droplet size of **(NE3)** were smaller than that of **(NE1)** and **(NE2)**. Hence, all the nano-emulsions (**(NE1)**, **(NE2)** and **(NE3)**) had droplet size values within the desired nano-metric range of 50 – 200 nm (ideal droplet size for transdermal delivery (Tadros *et al.*, 2004:303)). The Malvern Zetasizer reports revealed that the results for all three dispersions (**(NE1)**, **(NE2)** and **(NE3)**) were “good,” confirming the acceptability of the data.

Pdl can be defined as the standard deviation to mean droplet size ratio and gives an indication of the uniformity of the droplet size that the emulsion contains (Shakeel *et al.*, 2007:E6). Stability of the nano-emulsion was measured by means of Pdl and droplet size. Pdl of a dispersion ranging from 0 – 1.0 gives an indication of the quality of dispersions and must be kept below 0.2 to be suitable for measurement of quality (Gaumet *et al.*, 2008:4; Liew *et al.*, 2010:572). If the Pdl is lower than 0.1, it can be said that the dispersion is highly homogenous. If the Pdl values are higher than 1.0, it gives an indication of the broad size distribution within particle population (Gaumet *et al.*, 2008:5). In Tables B.8 – B.10, the Pdl values was measured and it is observed that all dispersions (**(NE1)**, **(NE2)** and **(NE3)**) had Pdl values within the desired range (between 0 – 1.0). Dispersions of **(NE2)** and **(NE3)** are in the range of 0.2 and are therefore closer to the value of 0, which is an indication of a monodispersed emulsion. Dispersions of **(NE1)** were in range of 0.3 and more polydispersed. When the Pdl value is closer to 0 the nano-emulsions can be less prone to sedimentation and aggregation. According to Abolmaali *et al.* (2011:140) and Chime *et al.* (2014:77), most of the nano-emulsions can be classified as monodispersed, but **(NE1)** with a Pdl value of 0.3 emulsions can be characterised as heterogeneous.

B.7.3 Zeta-potential

If the surface charge of a nano-emulsion is determined, it is known as the zeta-potential (Eid *et al.*, 2014:2; Thakur *et al.*, 2012:223). Due to the Brownian motion, particles are in constant collision with each other and because of Van der Waals forces, these particles have the ability to aggregate (Cao & Wang, 2011:43; Larsson *et al.*, 2012:209). A barrier is needed to prevent coagulation and is indicated by the zeta-potential (Larsson *et al.*, 2012:209); a low zeta-potential can be an indication of stability, whereas a high zeta-potential can be seen as unstable (Huo *et al.*, 2019:574). Nano-emulsions with a zeta-potential of + 30 mV and - 30 mV are considered strongly cationic and anionic and can stay stable over time (Eid *et al.*, 2014:2; Marsalek, 2014:13; Silva *et al.*, 2012:860). The charge of nanoparticles can influence the permeability through the skin (Clogston & Patri, 2010:10). It is important to determine the zeta-potential of a formulation, as it can give an indication of both the stability and drug entrapment of the formulation (Bhatt & Madhav, 2011:2296; Eid *et al.*, 2014; Klang *et al.*, 2015:262).

The zeta-potential of **(NE1)**, **(NE2)** and **(NE3)** was measured with a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) (Figure B.5). Dilutions was prepared by mixing 10 ml of Milli-Q® water and one drop of the emulsion. When the mixture was dissolved it was transferred by means of a clear disposable syringe into the clear disposable DTS1070 folded capillary zeta-cell.

Table B.11: Average zeta-potential (mV) of selected statins in **(NE1)**

(NE1)	Zeta-potential (reading 1)	Zeta-potential (reading 2)	Zeta-potential (reading 3)	Average zeta- potential (mV)
(NEF1)	- 66.80	- 65.10	- 67.70	- 66.53 ± 1.32
(NEL1)	- 56.90	- 57.00	- 57.60	- 57.17 ± 0.38
(NER1)	- 31.80	- 31.80	- 32.10	- 31.90 ± 0.75
(NES1)	- 41.80	- 42.20	- 44.20	- 42.73 ± 1.30
(NEP1)	- 54.00	- 51.50	- 52.30	- 52.60 ± 1.28

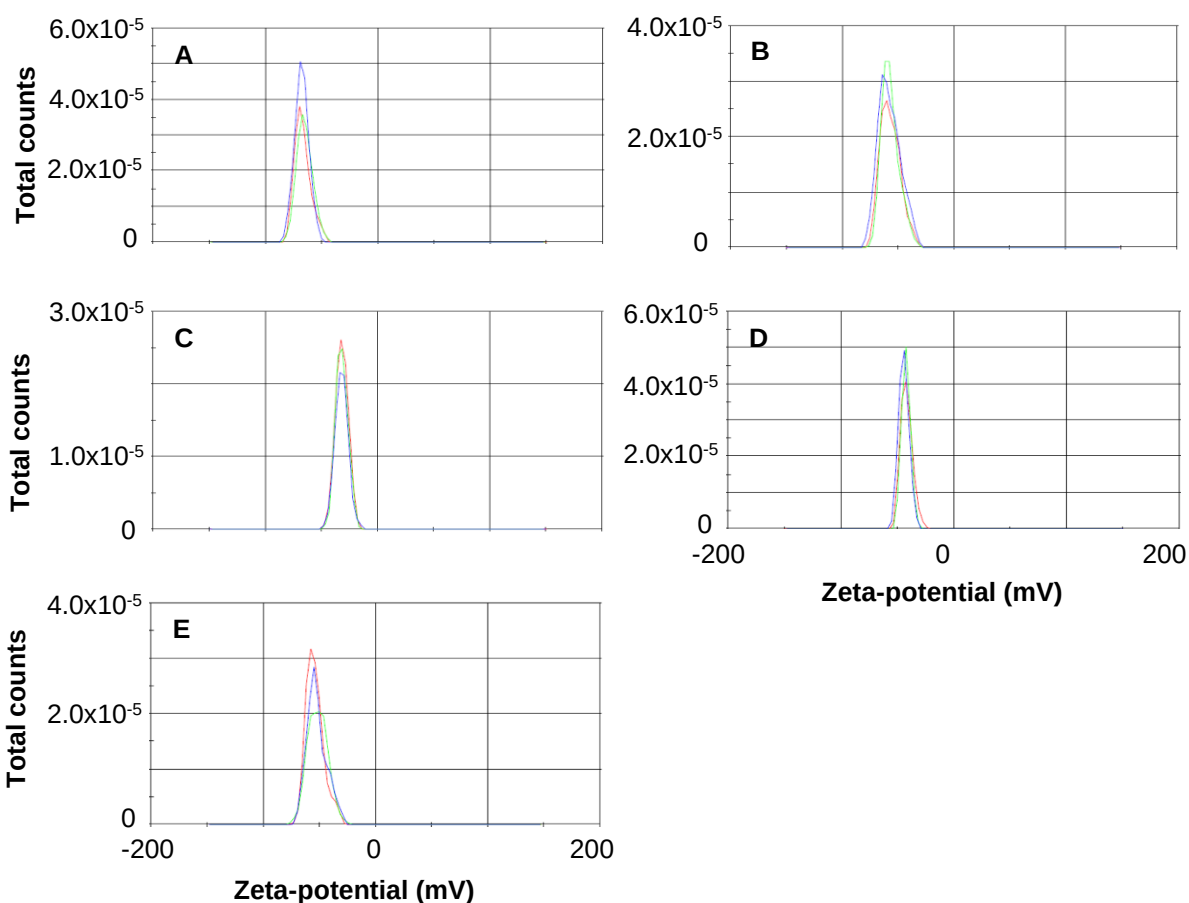


Figure B.9: Average zeta-potential (mV) of a) **(NEF1)**, b) **(NEL1)**, c) **(NER1)**, d) **(NES1)**, and e) **(NEP1)**

Table B.12: Average zeta-potential (mV) of selected statins in **(NE2)**

(NE2)	Zeta-potential (reading 1)	Zeta-potential (reading 2)	Zeta-potential (reading 3)	Average zeta- potential (mV)
(NEF2)	- 68.10	- 69.00	- 69.40	- 68.83 $\pm$ 0.67
(NEL2)	- 34.30	- 35.20	- 36.30	- 35.27 $\pm$ 1.00
(NER2)	- 33.20	- 32.60	- 34.90	- 33.57 $\pm$ 1.19
(NES2)	- 53.20	- 53.50	- 56.10	- 54.27 $\pm$ 1.59
NEP2)	- 0.28	- 0.11	- 0.51	- 0.30 $\pm$ 0.20

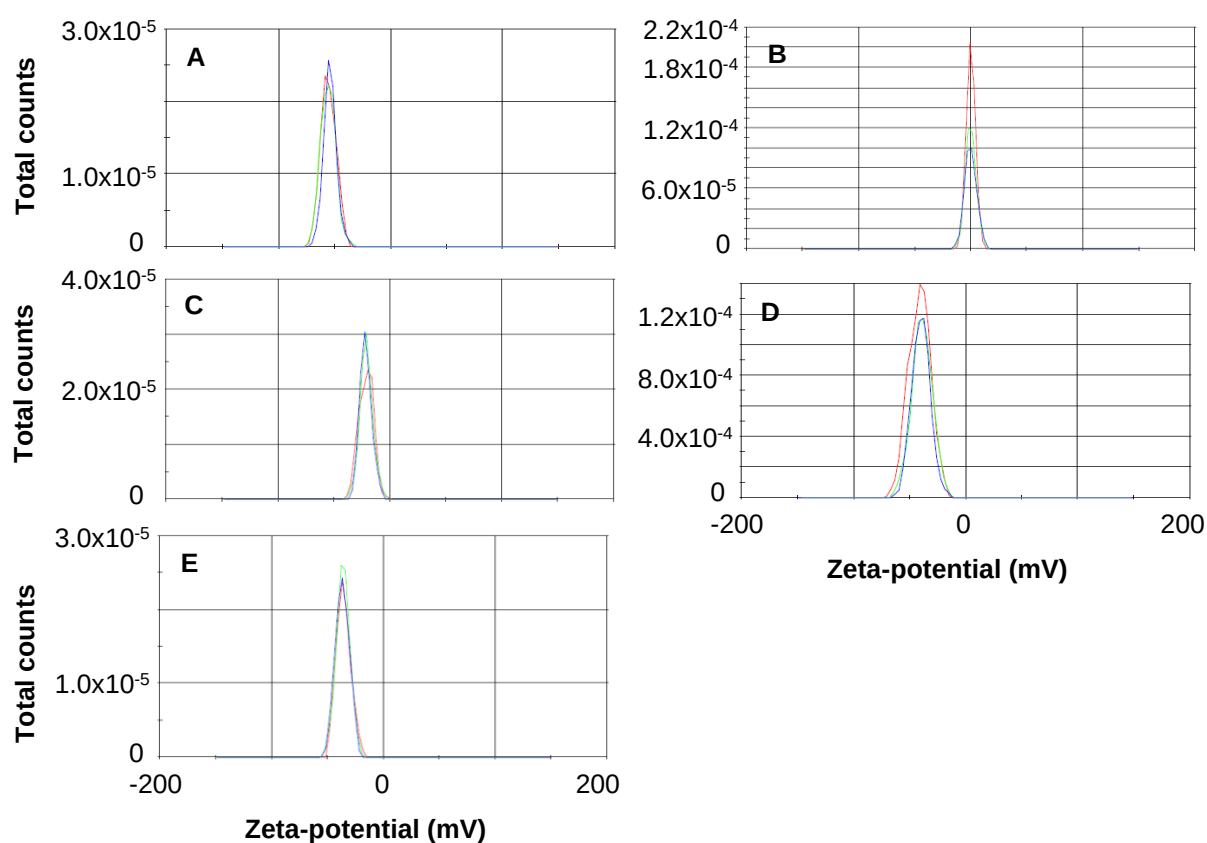


Figure B.10: Average zeta-potential (mV) of a) **(NEF2)**, b) **(NEL2)**, c) **(NER2)**, d) **(NES2)**, and e) **(NEP2)**

Table B.13: Average zeta-potential (mV) of selected statins in **(NE3)**

(NE3)	Zeta-potential (reading 1)	Zeta-potential (reading 2)	Zeta-potential (reading 3)	Average zeta- potential (mV)
(NEF3)	- 55.60	- 55.90	- 54.30	- 55.27 $\pm$ 0.85
(NEL3)	- 0.55	- 0.11	- 0.05	- 0.24 $\pm$ 0.28
(NER3)	- 21.20	- 21.00	- 21.60	- 21.27 $\pm$ 0.31
(NES3)	- 41.30	- 39.00	- 40.00	- 40.10 $\pm$ 1.15
(NEP3)	- 35.50	- 36.00	- 36.70	- 36.07 $\pm$ 0.60

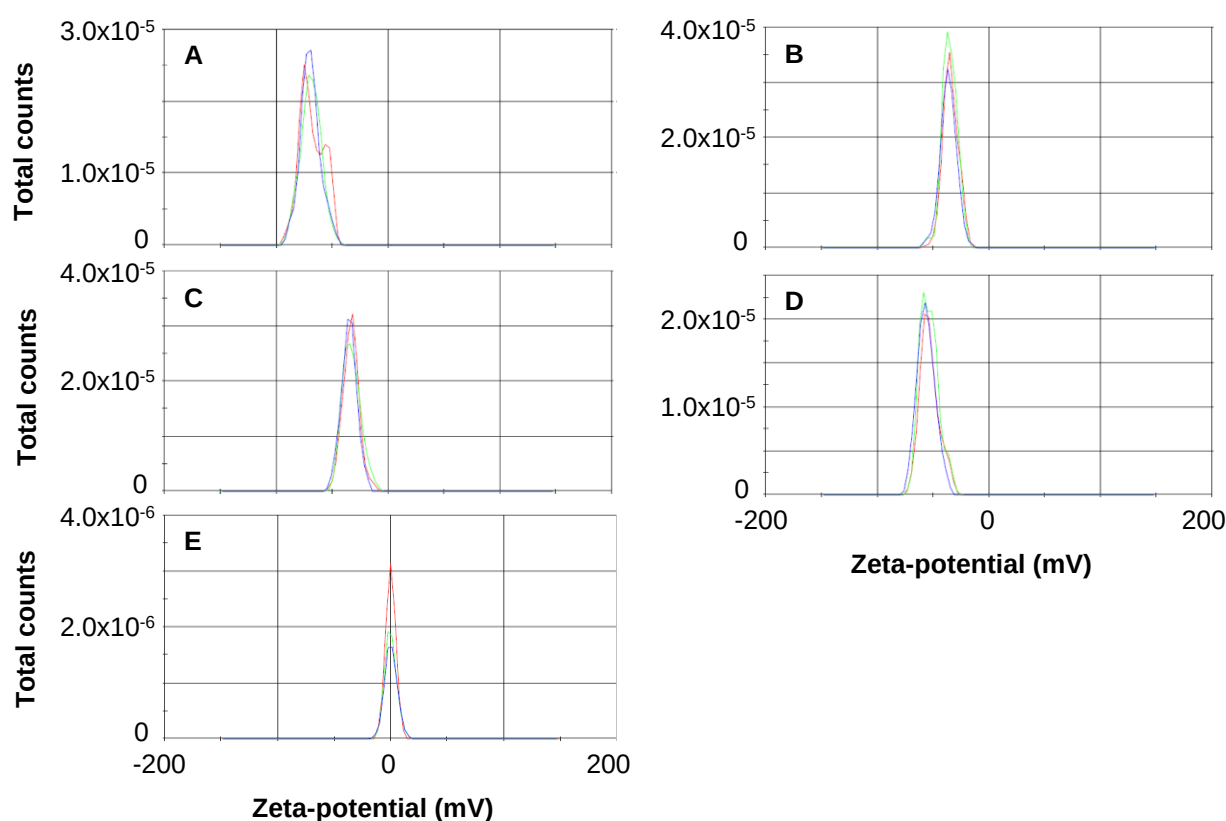


Figure B.11: Average zeta-potential (mV) of a) **(NEF2)**, b) **(NEL2)**, c) **(NER2)**, d) **(NES2)**, and e) **(NEP3)**

Tables B.9 to B.11 illustrate the dispersions of **(NE1)** and **(NE2)** all displayed negative zeta-potential values below - 30 mV (except for **(NEP2)**) and fell within the acceptable criteria according to Marsalek (2014:13). For the dispersions of **(NE3)**, it can be seen that **(NEL3)** and **(NER3)** did not adhere to the standards and therefore classified as unstable emulsions. According to Bhattacharjee (2016:347), it can be stated that **(NE1)** and **(NE2)** are stable dispersions with less probability of aggregation.

B.7.4 Viscosity

When examining a nano-emulsion it appears watery, as the largest component of an o/w emulsion is the water phase. Viscosity consists of the water, oil and surfactant components, thus when the water component increases, the viscosity decreases (Chiesa *et al.*, 2008:71; Chime *et al.*, 2014:97) and when the surfactant and oil ratios decrease, the viscosity increases by means of interfacial tension between oil and water (Chime *et al.*, 2014:97; Lovelyn & Attama, 2011:632). Faster release of APIs takes place when the viscosity of the emulsion is lower (Lovelyn & Attama, 2011:632).



Figure B.12: A Brookfield Viscometer DV2T LV Ultra connected to a water bath

Measurement of the viscosity for **(NE1)**, **(NE2)** and **(NE3)** was done with a Brookfield Viscometer DV2T LV Ultra (Middleboro, United States), which is connected to a thermostatic water bath (Figure B.12), used to acclimatise the emulsions to a temperature of 25°C. The nano-emulsions were transferred into a sample chamber, where they were fitted with a small sample adapter to the viscometer. The programme used to measure the viscosity was Rheocalc T1.2.19 and was preprogrammed to take a measurement every 10 s for 3 min. For these emulsions a SC4-18 cylindrical spindle was used. Viscosity was then measured at a speed of 200 rpm and centipose (cP) was measured at room temperature. The average was calculated for all the readings taken after 3 min, and these are presented in Tables B.14 to B.16.

Table B.14: Viscosity readings of **(NE1)**

(NE1)	Viscosity (cP)	Torque (%)	Temperature (°C)	Speed (RPM)
(NEF1)	5.78 ± 0.02	38.52 ± 0.10	24.2 ± 0.00	200.00 ± 0.00
(NEL1)	3.43 ± 0.02	22.89 ± 0.05	24.27 ± 0.05	200.00 ± 0.00
(NER1)	3.86 ± 0.01	25.69 ± 0.08	24.10 ± 0.00	200.00 ± 0.00
(NES1)	5.66 ± 0.02	37.74 ± 0.12	24.13 ± 0.05	200.00 ± 0.00
(NEP1)	3.75 ± 0.02	25.03 ± 0.11	24.30 ± 0.00	200.00 ± 0.00

Table B.15: Viscosity readings of **(NE2)**

(NE2)	Viscosity (cP)	Torque (%)	Temperature (°C)	Speed (RPM)
(NEF2)	7.17 ± 0.02	47.78 ± 0.15	20.90 ± 0.00	200.00 ± 0.00
(NEL2)	4.41 ± 0.03	29.43 ± 0.22	20.67 ± 0.05	200.00 ± 0.00
(NER2)	3.87 ± 0.02	25.80 ± 0.16	20.90 ± 0.00	200.00 ± 0.00
(NES2)	5.86 ± 0.03	39.12 ± 0.23	20.90 ± 0.05	200.00 ± 0.00
(NEP2)	4.98 ± 0.03	33.20 ± 0.17	20.80 ± 0.00	200.00 ± 0.00

Table B.16: Viscosity readings of **(NE3)**

(NE3)	Viscosity (cP)	Torque (%)	Temperature (°C)	Speed (RPM)
(NEF3)	6.25 ± 0.04	41.65 ± 0.22	17.96 ± 0.05	200.00 ± 0.00
(NEL3)	3.32 ± 0.02	22.11 ± 0.15	18.00 ± 0.00	200.00 ± 0.00
(NER3)	3.19 ± 0.03	21.26 ± 0.19	17.90 ± 0.00	200.00 ± 0.00
(NES3)	6.61 ± 0.02	44.06 ± 0.17	17.90 ± 0.00	200.00 ± 0.00
(NEP3)	3.33 ± 0.03	22.20 ± 0.20	17.90 ± 0.00	200.00 ± 0.00

From Tables B.14 to B.16, it can be observed that the viscosity values of **(NE1)** are much lower than **(NE2)** when the respective statins are compared. When the respective statins of **(NE1)** and **(NE3)** are compared, it is observed that certain dispersions (**(NEF1)** and **(NES1)**) had lower viscosity values than their respective statin in **(NE3)**, while other dispersions (**(NEL1)** and **(NER1)**) had higher viscosity values than their respective statins in **(NE3)**. This may be due to the different ratios of surfactant used when the nano-emulsions were formulated. According to Chime *et al.* (2014:97), differences in viscosity values can be ascribed to the different surfactant and oil ratios used.

B.7.5 Drug entrapment efficiency

Drug entrapment efficiency (%EE) was performed to calculate the amount of each selected statin in different formulations to determine the amount of drug entrapped in the nanoparticles (Boonsongrit *et al.*, 2006:269). HPLC was utilised to determine the %EE of the **(NE1)**, **(NE2)** and **(NE3)** dispersions. All the dispersions of **(NE1)**, **(NE2)** and **(NE3)** were prepared, whereafter 20 ml of each dispersion was transferred into an Eppendorf® centrifuge tube and centrifuged with an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) at a speed of 25 000 rpm for 45 min at room temperature. After the samples were centrifuged, the unentrapped API (supernatant) and fluid that contains the API were visible. The supernatant (200 µl) was extracted and dissolved in a 5 ml volumetric flask with THF. A small volume was transferred into an HPLC vial for analysis. A linear curve was injected by HPLC for each of the selected statins, as described in Section A.3.1. The %EE was determined with Equation B.1 (Boonsongrit *et al.*, 2006:269).

$$\%EE = \frac{(\text{Total amount of drug loading} - \text{free drug in supernatant})}{\text{Total amount of drug loading}} \times 100$$

Equation B.1



Figure B.13: Illustration of an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa).

Table B.17: Drug entrapment efficiency (%EE) as calculated for **(NE1)**, **(NE2)** and **(NE3)**

Nano-emulsion	Entrapment efficacy (%EE)
(NEF1)	90.35
(NEF2)	89.23
(NEF3)	88.79
(NEL1)	99.67
(NEL2)	99.62
(NEL3)	99.85
(NER1)	99.65
(NER2)	99.68
(NER3)	99.73
(NES1)	95.30
(NES2)	93.24
(NES3)	93.70

According to Song *et al.* (2008:321), it was reported that nanoparticles with a low molecular weight would include nanoprecipitation. From the Table B.17, it can be observed that the %EE for the **(NE1)** and **(NE3)** dispersions were higher than that of the **(NE2)** dispersion when compared with each other. According to Kurakula *et al.* (2012:37), if the %EE yield is high, it can be an indication that a larger amount of selected API can be delivered to the wanted target-site. The %EE will be close to 100% when the API is a lipophilic molecule (Loureiro *et al.*, 2015:96). Each of the selected statins formulated in the different dispersions showed near 100% entrapment.

B.7.6 Morphology

The transmission electron microscope (TEM) was operated and images were captured by Dr A. Jordaan (Electron Microscopy Laboratory of the North-West University (NWU), Potchefstroom) 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. TEM was only performed on the preferred nano-emulsion (which was **(NE1)**) due to the cost of the experiment and was used to investigate the morphological characteristics (such as the size range of droplets) of the nano-emulsion (Klang *et al.*, 2012:85). Dilutions were made with Milli-Q® water; a small volume was transferred with a micropipette to a microscopic carbon-coated 300 mesh copper grid and left to dry for 10 min (Vatsraj *et al.*, 2014:1). Osmium tetroxide was used to stain the diluted drop of nano-emulsion to provide high-contrast quality images. Osmium tetroxide provides preservation of lipids such as unsaturated fatty acids of phospholipids, and helps to view organelles at a high-electron density (Klang *et al.*, 2012:88 & Nomaki *et al.*, 2015:33). After staining, microscopic samples were left to dry for 20 – 30 min and then viewed under the TEM at a voltage of ± 200 kV. A Gatan bottom mount camera and digital micrograph software were used to capture the micrographs of the nano-emulsions.



Figure B.14: Demonstration of TEM used to capture micrographs of nano-emulsions

Nano-emulsions can be classified under the droplet nanometric size range of 100 – 500 nm and up to 1000 nm (Kela & Kaur, 2013:9203; Klang *et al.*, 2012:86; Tadros *et al.*, 2004:303). Figure B.15 exhibits the results of the HRTEM for the **(NE1)** dispersions (fluvastatin, lovastatin, rosuvastatin and simvastatin). These micrographs display the oil droplets dispersed in the water phase and consequently the droplet size could be determined.

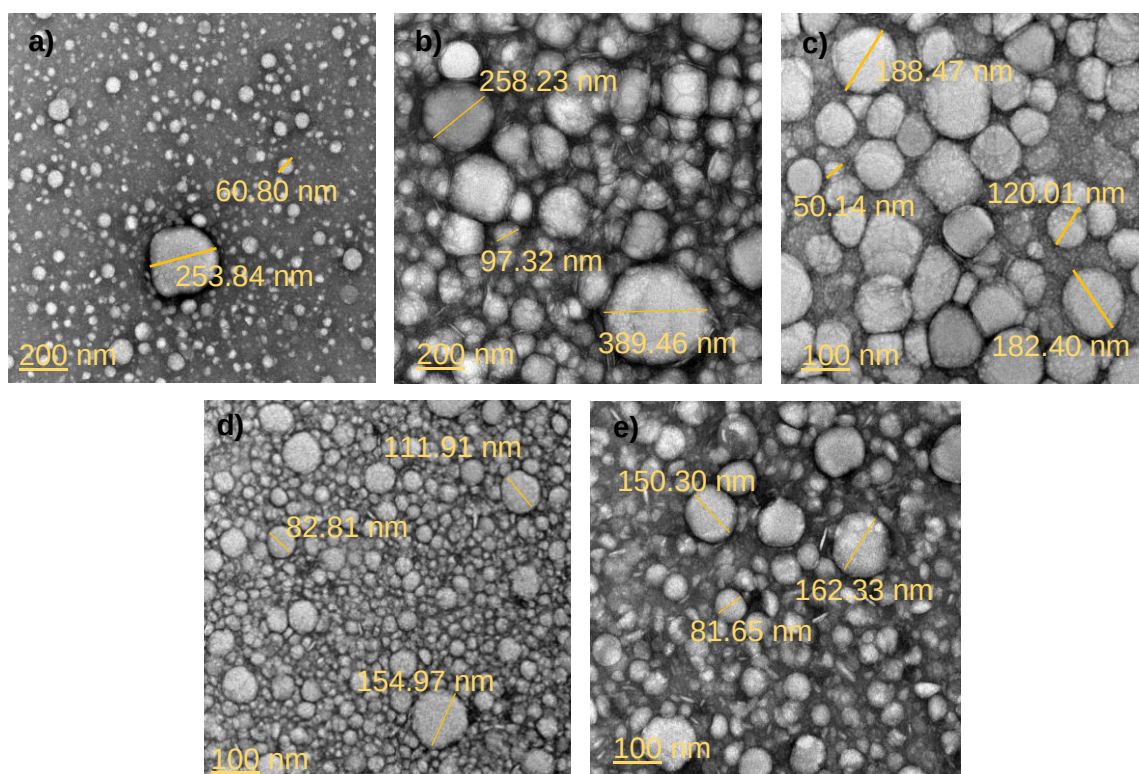


Figure B.15: TEM micrographs of oil droplets captured: a) **(NEF1)**, b) **(NEL1)**, c) **(NER1)**, d) **(NES1)** and e) **(NEP1)**

The micrographs show that all the nano-emulsions had spherical droplets. Dark shadows were observed in Figures B.15.a to B.15.e, which could be an effect of the staining through osmium tetroxide and coalescence of the droplets. It can be observed from Figure B.15 that the dispersions of **(NE1)** were in the desired range of 100 – 500 nm, as mentioned by Klang *et al.*, (2012:86) and Tadros *et al.* (2004:303): Figure B.15.a 60.80 – 253.84 nm, Figure B.15.b 97.32 – 389.46 nm, Figure B.15.c 50.14 – 188.47 nm, Figure B.15.d 82.81 – 154.97 nm and Figure B.15.e 81.65 – 162.33 nm. Therefore, these nano-emulsions were successfully formulated.

B.8 Decision on final formula to be used and conclusion

Various methods were used to characterise the dispersions of **(NE1)**, **(NE2)** and **(NE3)** separately to determine which formula would be the optimum formulation for this study. Three formulations were made where morphology was only done on **(NE1)** for cost effectiveness, all the droplet sizes were within acceptable ranges (within 100 – 500 nm) (Klang *et al.*, 2012:86). This can be an indication that this formulation is more stable.

Droplet size and zeta-potential were determined with a Malvern Zetasizer and **(NE1)** can be described as polydispersed. The Pdl value for the **(NE1)** dispersions were 0.3, whereas the Pdl for the **(NE2)** and **(NE3)** dispersions were closer to 0, which means the **(NE1)** formulation is less prone to sedimentation, according to Abolmaali *et al.* (2011:140) and Chime *et al.* (2014:77). The zeta-

potential of the nano-emulsions should be between - 30 mV and + 30 mV. Dispersions of **(NE1)** have zeta-potential values of less than - 30 mV, while the zeta-potential values of the **(NE2)** and **(NE3)** dispersions are not as low as those of **(NE1)**, hence the dispersions of **(NE1)** are more stable and suitable for use on the skin, since the skin is more negatively charged.

The pH of the dispersion must range between 3 and 9 to prevent it from irritating the skin (Barry, 2006:7; Barry, 2007:576; Malan *et al.*, 2002:388). All the dispersions (**(NE1)**, **(NE2)** and **(NE3)**) had pH values in range and would be suitable for transdermal use, since there will be minimal irritation to the skin.

From the viscosity results it was observed that the viscosity of the **(NE1)** was much lower than **(NE2)** when the respective statins were compared, and certain dispersions (**(NEF1)** and **(NES1)**) had lower viscosity values than their respective statin in **(NE3)**, which may be ascribed to the different surfactant and oil ratios used (Chime *et al.*, 2014:97).

Table B.18: Summary of the characteristics of the **(NE1)**, **(NE2)** and **(NE3)** dispersions

Formula	TEM (nm)	pH	Droplet size (nm)	Zeta-potential (mV)	Viscosity (cP)	(%EE)
(NEF1)	60.80 – 164.55	7.35 (± 0.02)	162.60 (± 1.47)	-66.53 (± 1.32)	5.78 (± 0.02)	90.35
(NEL1)	58.80 – 389.46	6.17 (± 0.01)	154.23 (± 0.68)	-57.17 (± 0.38)	3.43 (± 0.02)	99.66
(NER1)	50.14 – 575.09	6.11 (± 0.02)	151.20 (± 1.91)	-31.90 (± 0.75)	3.86 (± 0.01)	99.65
(NES1)	42.82 – 171.19	7.13 (± 0.01)	151.73 (± 2.28)	-42.73 (± 1.29)	5.66 (± 0.02)	95.30
(NEF2)	-	7.55 (± 0.00)	155.20 (± 0.69)	-68.83 (± 0.67)	7.17 (± 0.02)	89.23
(NEL2)	-	6.35 (± 0.01)	160.30 (± 1.04)	-35.27 (± 1.00)	4.41 (± 0.03)	99.62
(NER2)	-	6.37 (± 0.00)	153.53 (± 1.65)	-33.57 (± 1.19)	3.87 (± 0.02)	99.68
(NES2)	-	7.33 (± 0.00)	160.33 (± 0.85)	-54.27 (± 1.60)	5.86 (± 0.03)	93.24
(NEF3)	-	7.65 (± 0.00)	126.97 (± 1.64)	-55.27 (± 0.85)	6.25 (± 0.04)	88.79
(NEL3)	-	6.29 (± 0.01)	111.90 (± 0.96)	-0.24 (± 0.27)	3.32 (± 0.02)	99.85
(NER3)	-	6.35 (± 0.00)	99.45 (± 0.43)	-21.27 (± 0.31)	3.19 (± 0.03)	99.73
(NES3)	-	7.5 (± 0.00)	110.93 (± 0.85)	-40.10 (± 1.15)	6.61 (± 0.03)	93.69

The %EE should be as close to 100% as possible. The %EE was high for all the dispersions ((**NE1**), (**NE2**) and (**NE3**)), hence drug entrapment was successful. Each of the formulations, (**NE1**), (**NE2**) and (**NE3**) were compared, and results showed (**NE1**) and (**NE3**) obtained the most optimal %EE compared to (**NE2**) formulation.

When all the characteristics are considered, and considering that directly after formulation (**NE3**) presented with phase separation, the dispersions of (**NE1**) presented with the most optimal characteristics. (**NE1**) presented with a desired Pdl, droplet size and had the most negative zeta-potential. The (**NE1**) dispersions (8.3% (w/v) grapeseed oil and a 2% (w/v) API) were selected to perform further studies. Studies on the formulation containing a thickening agent to produce a nano-emulgel will be characterised and discussed in Appendix C.

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Appendix C

FORMULATION AND CHARACTERISATION OF A SEMI-SOLID DOSAGE FORM OF AN O/W NANO-EMULSION SEPARATELY CONTAINING THE SELECTED STATINS AND GRAPESEED OIL

C.1 Introduction

During the pre-formulation of the nano-emulsions (Appendix B), the characterisation was done on the different dispersions and the optimal nano-emulsion was chosen, which contained 8.0% (w/w) grapeseed oil and equal ratios of surfactants (Span® 60 and Tween® 80). Nano-emulsions are a good alternative option when the permeability through skin must be optimised (Sengupta & Chatterjee, 2017:354). Although nano-emulsions exert ideal properties for penetration, they consist of a low viscosity (Ali *et al.*, 2014:1128).

The obstacle of low viscosity can be overcome with the transformation of a nano-emulsion into a semi-solid dosage form with the addition of a gelling agent to the **(NE1)** formulation (Mahalingham *et al.*, 2008:268). Semi-solid dosage forms can overcome the obstacles set out by nano-emulsions and increase the stability by enhancing the permeability and lowering the interfacial tension (Gupta & Garg, 2002:144). By increasing the viscosity of a formulation, it enhances penetration for transdermal delivery.

Currently there is no treatment for statins through the transdermal route. With the transdermal route of administration, the API gets deposited directly into the bloodstream (Guy, 1996:1765). According to Wei *et al.* (2013:475), patients discontinue their treatment because of the side-effects occurring with the use of statins. The transdermal route possesses more advantages in terms of bypassing the gastrointestinal tract and reducing side-effects caused by statin therapy (Münch *et al.*, 2017:236), this will have a positive effect on the patient compliance. Different routes of administration are explored in order to have an advantageous effect with regard to statins therapy.

The main objective for this section is to incorporate the optimal nano-emulsion **(NE1)** into a semi-solid dosage form to increase the viscosity and to act as a carrier system for selected statins across the barrier of the skin (Mahalingham *et al.*, 2008:268).

C.2 Intended purpose of the formulation

When choosing a formulation for semi-solid dosage forms, the optimal efficiency must be chosen for topical application. The rate at which the skin metabolises the API applied to the skin differs for every API, and depends on the absorption, distribution, metabolism and excretion (MacKay *et al.*, 2013:475). During this study, a gelling agent was added to the **(NE1)** to formulate a semi-solid

dosage form. If the formulation is not permeable enough, it may lead to poor patient compliance (Elmataeeshy *et al.*, 2018:18). Semi-solids are more stable and the added gelling agent causes a better permeation, viscosity and extended release of the API through the skin (Azeem *et al.*, 2012:437; Chellapa *et al.*, 2015:45).

C.2.1 Semi-solid dosage form selection

Various types of formulations are available for topical and transdermal delivery, such as sprays/solutions, gels, ointments, creams, patches and emulsions. These formulations each present with their own advantages and disadvantages (Allen *et al.*, 2011:272; Bora *et al.*, 2014:3594; Kathe & Kathpalia, 2017:488). Particles in a suspension must be uniformly dispersed to prevent premature release of the drug from the particles (Freiberg & Zhu, 2004:2). Semi-solid dosage forms are formulations that contain both a solid and a liquid component, where the API is dissolved in the oil or water phase (Valentine, 2014:153). For this study, the transdermal delivery of selected statins was investigated and the formulation of choice was a nano-emulgel.

C.2.2 Gels as a semi-solid dosage form

Gels consist of a better dissolution rate and bioavailability, therefore are a better delivery option for poorly water-soluble drugs (Shadab & Shamsi, 2018:2). Although gels have a higher aqueous component, they enhance dissolution and migration of the API (Khullar *et al.*, 2012:63). Advantages of these formulations include being thixotropic, greaseless, easily spreadable, non-staining and have a pleasant appearance (Stanos, 2007:342).

C.2.2.1 Emulgel

When a gel and emulsion are combined, it is known as an emulgel (Khullar *et al.*, 2012:63). Gels are created for the entrapment of an aqueous liquid into a network of colloidal solid particles. Gels have a limitation in the delivery of hydrophobic drugs, but are better in delivering lipophilic drugs such as the APIs used in this study (Khullar *et al.*, 2012:64; Mohammed, 2004). Emulgel formulations can consist of an o/w phase or w/o phase emulsion with the addition of a gelling agent creating the emulgel formulation (Kute & Saudagar, 2013:368). Although emulgels have advantages that increase patient compliance, they still hold a challenge for permeation through the skin, since they usually consist of large droplet sizes (Baibhav *et al.*, 2011:66).

C.2.2.2 Nano-emulgel

The obstacle emulgel formulations possess is having a larger droplet size, however this can be overcome by using high energy emulsification methods to obtain droplet sizes in nano-metric ranges, which leads to the formation of nano-emulgels. Nano-emulgels are formulated to improve systemic delivery of lipophilic drugs and can be used for their non-invasive application method and lower

gastrointestinal side-effects (Sengupta & Chatterjee, 2017:1). Nano-emulgels give controlled release of the API out of the gelling system and achieve increased penetration (Eid *et al.*, 2014:1). Although nano-emulgels are advantageous, they possess other obstacles, such as low viscosity and restricted spreadability; these can be overcome by altering the amounts of the aqueous bases and by incorporating gelling agents into the formulation (Dhawan *et al.*, 2014:65; Eid *et al.*, 2014:1). This formulation consists of two phases, namely the water phase, where the gelling agent is added, and the oil phase containing the natural oil, the API and the emulsifiers (Dev *et al.*, 2015:64).

C.2.3 Suitable semi-solid dosage form

Although nano-emulgel formulations present with both advantages and disadvantages for use as a transdermal delivery system, the formulation may enhance delivery of lipophilic molecules such as the statins used in this study.

The formulation of **(NE1)** was utilised to formulate a nano-emulgel for each of the selected statins, characterisation was performed on each of the selected statins in the nano-emulgel formulation, respectively, to limit disadvantages of the **(NEG)** formulation. From this point onwards, the nano-emulgel formulation **(NEG)** for each statin will be referred to as:

- * Fluvastatin nano-emulgel **(NEGF)**
- * Lovastatin nano-emulgel **(NEGL)**
- * Rosuvastatin nano-emulgel **(NEGR)**
- * Simvastatin nano-emulgel **(NEGS)**

Table C.1 lists the excipients used during the formulation of the nano-emulgels (**(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)**).

C.3 Excipients used to formulate the nano-emulgels

C.3.1 General excipients used for nano-emulgel

The general excipients used to formulate a nano-emulgel includes the aqueous material, oil, emulsifier, API and a gelling agent (Kute & Saudagar, 2013:370). Formulations of nano-emulgels were made using a gelling agent and penetration enhancer, formulated in different phases (oil phase and water phase). The gel phase is prepared by dispersing Carbopol® into the water phase with constant stirring (Khullar *et al.*, 2012:64). Aqueous phases are emulsified by using high or low energy methods to obtain desired particle sizes (Drais & Hussein, 2017:13).

C.3.2 Excipients used to formulate a nano-emulgel

During the formulation of the nano-emulgels ((**NEGF**), (**NEGL**), (**NEGR**) and (**NEGS**)) the following excipients as tabulated in Table C.1 were used.

Table C.1: Excipients used in the formulation of nano-emulgels ((**NEGF**), (**NEGL**), (**NEGR**), (**NEGS**))

Excipients	Batch number	Supplier	Function
Milli-Q® water	Direct Pure UP	In-Lab	Water phase/solvent
Carbopol® Ultrez 20	0102052576	Sigma-Aldrich	Gelling agent/thickening agent
Tween® 80	BCBT9142	Sigma-Aldrich	Hydrophilic surfactant/emulsifier
Span® 60	SLBN0127V	Sigma-Aldrich	Lipophilic surfactant/emulsifier
Grapeseed oil	1512E036197	CJP Chemicals	Oil phase and penetration enhancer
Statins	Fluvastatin (1701108447) Lovastatin (20170423) Rosuvastatin (ROC/1512B/0008J1) Simvastatin (IF-SI-170913)	DB FINE chemicals	API

C.3.2.1 Oil (grapeseed)

Grapeseed oil serves as the oil phase in the formulation. Oils are utilised in formulations as penetration enhancers (Kute & Saudagar, 2013:370). According to Kute & Saudagar. (2013:370), penetration enhancers have two mechanisms of action for penetration through the skin, which includes disruption of the structure of the stratum corneum lipids and interaction with cellular proteins in the stratum corneum. Oils contains fatty acids, which are used as a lipid bilayer disrupting agent in a formulation. Long chain fatty acids show better penetration enhancing effects (Williams & Barry, 2012:128). Fatty acids consist of linoleic acid and oleic acid in high percentages that help increase transdermal delivery (Van Zyl *et al.*, 2016:192). Grapeseed oil contains 69.0 – 78.0% linoleic acid (omega 6), 15.0 – 20.0% oleic acid (omega 9) and 0.1 – 3.0% linolenic acid (omega 3) (Oronsky *et al.*, 2016:9), and was used for this study as it contains the highest amount of linoleic acid and also acts as a penetration enhancer.

C.3.2.2 Emulsifiers

Emulsifying agents are used as stabilisers during the formulation of nano-emulgels and promote stability over a longer term (Baibhav *et al.*, 2011:68; Kute & Saudagar, 2013:370). The main role of

emulsifiers is to prevent the formation of larger droplets by preventing smaller droplets from coalescing with neighbouring droplets, in turn this reduces interfacial tension (Dickinson, 2009:1475; Oronsky *et al.*, 2016:4). Tween® 80 and Span® 60 was selected as the hydrophilic emulsifier and the lipophilic surfactant, respectively during the formulation of the nano-emulgel.

C.3.2.3 Gelling agent

Gelling agents are thickening agents that swell or grow in the nano-emulsion and enhance the characteristics of the formulation (Mahalingam *et al.*, 2008:293). By incorporating a gelling agent into a formulation, it converts the formulation into a colloidal solution, which acts as a precursor for the network of particles or polymers (Beshkar *et al.*, 2017:66); this can increase the stability by preventing separation of particles (Mitsui, 1997:138). For this study Carbopol® Ultrez 20 was utilised as gelling agent.

Carbopol® is an acrylic acid polymer and once stabilised in an aqueous medium, it becomes a gelling agent, which will increase the viscosity of the emulsion (Fresno *et al.*, 2002:335). Carbopol® is classified as a water-soluble base and can optimise non-polar drug delivery. Some advantages may include higher viscosity in low concentrations. This polymer consists of bio-adhesive properties and has a wide interval for viscosity and flow behaviour (Fresno Contreras *et al.*, 2001:437).

C.3.2.4 Water

Milli-Q® water was used and makes up the largest part of the nano-emulgel. Tween® 80 (surfactant) was added to the Milli-Q® water, followed by the Carbopol® Ultrez 20 (gelling agent to serve as the hydrogel base in combination with the water mixture).

C.4 Formulation of a nano-emulgel

The reason for formulating a nano-emulgel was to enhance penetration through the skin (Chellapa *et al.*, 2015:44). A nano-emulgel formulation was chosen because of the higher viscosity and causes a decrease in surface and interfacial tension, which makes permeation easier (Chellapa *et al.*, 2015:45; Dhawan *et al.*, 2014:66); this will lead to an increase in delivery of the API, since the delivery system now acts as a reservoir (Mou *et al.*, 2008:270).

C.4.1 Formulation method

The formula used in Appendix B for the formulation of **(NE1)** was utilised to create the nano-emulgel formulation by adding a gelling agent (Carbopol® Ultrez 20, Sigma & Aldrich, batch-0102052576). Incorporation of the gelling agent into the water phase of the nano-emulsion was done as suggested by Purrushottam *et al.* (2013:233) and Singla *et al.* (2012:486).

C.4.2 Formula used for preparation (NEG)

In Table C.2 the formula to manufacture the (NEG) is illustrated.

Table C.2: Formula and excipients used to formulate nano-emulgels (NEG)

Phase	Ingredient	Function	Amount (%m/m)
Water phase	Tween® 80	Hydrophilic surfactant	6.00% (2.80 ml)
	Milli-Q® water	Water phase	77.54% (40.00 ml)
	Carbopol® Ultrez 20 polymer	Gelling agent/thickening agent	0.46%
Oil phase	Grapeseed oil	Oil phase and penetration enhancer	8.00% (4.35 ml)
	Span® 60	Lipophilic surfactant	6.00% (3 g)
	Statin	API	2.00% (1 g)

C.4.3 Formulation method used for (NEG)

During this study, an o/w nano-emulgel was formulated with 8% grapeseed oil and 2% API. The method used to formulate the nano-emulgel for the different statins is displayed in Figure C.1. The first step in formulating the nano-emulgel was to weigh the excipients of both the oil and the water phase. Tween® 80 was dissolved in the Milli-Q® water by placing it on the hotplate while stirring with a magnet. After the Tween® 80 was dissolved in the Milli-Q® water, the Carbopol® Ultrez 20 was added to the water phase and stirred for 10 min to ensure proper dissolution. The grapeseed oil was heated (between 70 and 75 °C) in a separate container, where after Span® 60 was added to dissolve in the oil; the API was dissolved in the Span® 60 and oil mixture. After 10 min of dissolution of the gelling agent, the water phase was ultrasonicated for 3 min to free any trapped air caused by the gelling agent, and transferred to an overhead mechanical stirrer for 15 min at 850 rpm, thereafter the oil phase was added to the water phase whilst continually. After the nano-emulgel was left to stir for 15 min, it was ultrasonicated again for 5 min. Although the viscosity of the emulgel is higher, the ultrasonicator was still used to form nano-sized particles. The pH of the nano-emulgel was then adjusted, according to Choi *et al.* (1999:16), with NaOH to reduce the pH of the gel, which in turn then reduces the viscosity. Literature from the Lubrizol (2009:1) data sheet suggests that the pH of a nano-emulgel varies between 2.5 and 3.5 before neutralisation and therefore is not safe to use on the skin since it is too acidic, thus it needed to be adjusted. For the (NEG) the pH was adjusted with NaOH.



Figure C.1: Method of formulating a nano-emulgel: a) water phase preparation by adding Milli-Q® water with Tween® 80; b) mixing of Milli-Q® water and Tween® 80; c) dissolving Tween® 80 in Milli-Q® water; d) addition of the gelling agent (Carbopol® Ultrez 20) to the water phase; e) high speed mixing of gelling agent into the water phase for dissolution; f) dissolving Span® 60 into the grapeseed oil, after which API is added and dissolved; g) ultrasonication of the water phase with gelling agent (3 min); h) water phase is then placed in the overhead mechanical stirrer; i) oil phase is added to the water phase while stirring with the overhead mechanical stirrer; j) oil and water phase is left on the mechanical stirrer for 15 min; k) after 15 min on the overhead mechanical stirrer, the emulgel formulation is ultrasonicated for 5 min; l) adjustment of the pH to that of the nano-emulsion to obtain a emulgel formulation

C.5 Outcome

The formulation of a nano-emulgel was prepared for the development of a possible transdermal delivery system. Texture of the **(NEG)** can be explained as a thick and homogenous gel with a smooth, pleasant texture that can be applied to the skin with ease. The colours of the nano-emulgel formulations can be described as **(NEGF)** having a light-yellow colour gel, **(NEGS)** a slight beige colour, **(NEGL)** and **(NEGR)** a white colour. Characterisation was done on the different gels to determine if the formulations were suitable for further studies with the skin. The results were then compared to those of the nano-emulsions.

C.6 Characterisation of the nano-emulgels (semi-solid)

Characterisation had to be performed on **(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)**. These nano-emulgels were investigated in terms of:

- * morphology (light microscopy);
- * visual inspection;
- * pH;
- * droplet size and distribution;
- * zeta-potential;
- * viscosity.

C.6.1 Light microscopy

Light microscopy is carried out to examine any changes in the formulation, such as homogeneity and phase separation, but also to analyse the size of the droplets individually (Yapar *et al.*, 2013:531). According to Silva *et al.* (2012:862), particle aggregation occurrence in the formulations can also be seen with the use of a light microscope.

Light microscopy of **(NEG)** was performed by utilising a Nikon Eclipse 50i microscope, fitted with a Nikon DSfi1 camera (Nikon, Japan Linkam THMS600), equipped with a T95 LinkPad temperature controller (Surry, England), as displayed in Figure C.2. Micrographs were captured using NIS-elements D (4.00.01, 64-bit) software. Samples were prepared for microscopy by transferring a small amount of the formulation (**(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)**) to a microscope slide, covered by a 16 mm cover slip. Microscopy was used to determine the droplet size and morphology by implementing different magnifications.

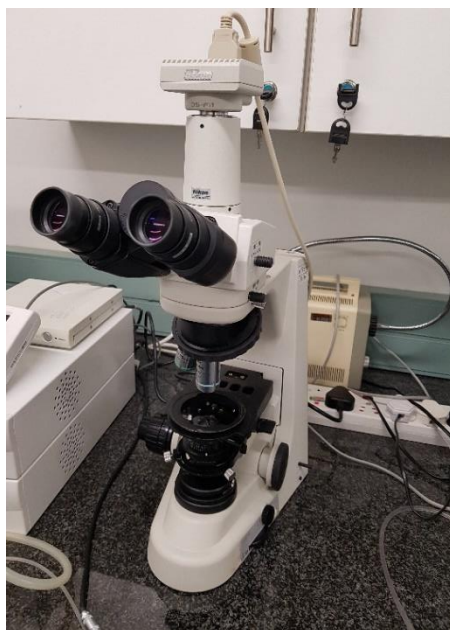


Figure C.2: Image of a Nikon Eclipse 50i microscope

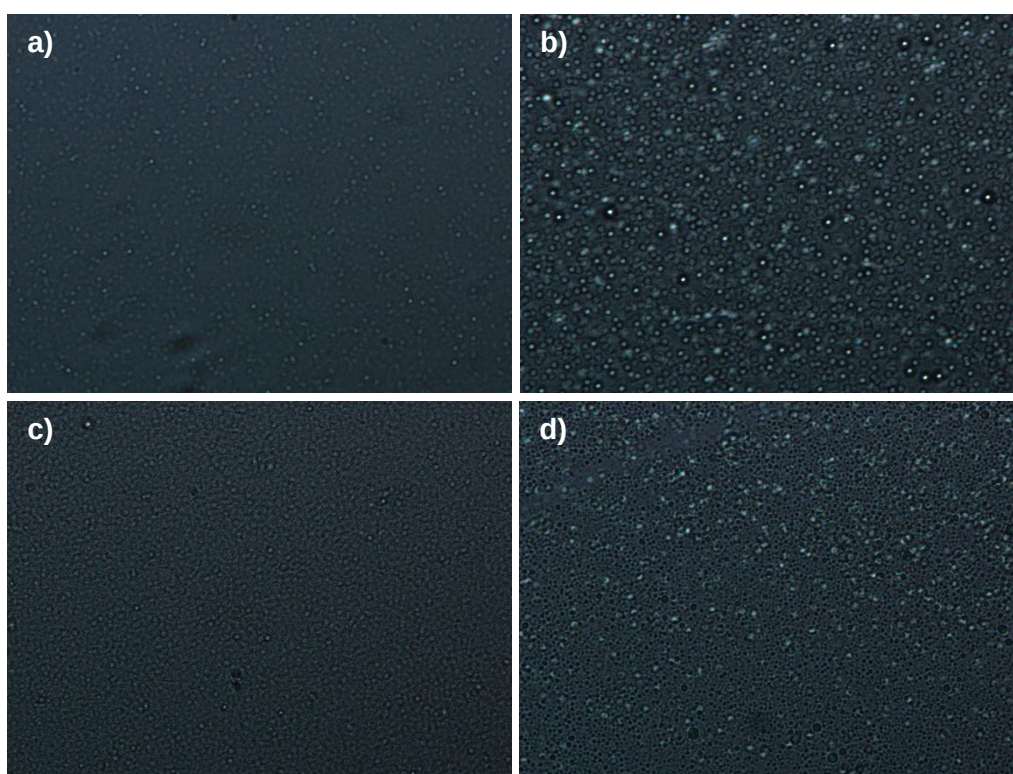


Figure C.3: Micrographs of: a) **(NEGF)**; b) **(NEGL)**; c) **(NEGR)**, and d) **(NEGS)** taken with the light microscope

When light microscopy is compared to TEM, these micrographs appear much smaller, since TEM has a higher magnification capability. In Figure C.3, it can be observed that the droplets of **(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)** are visible and that the **(NEG)** formulations had smaller droplets than the **(NE1)** dispersions, as confirmed by Baibhav *et al.* (2011:369), which states that the droplet size

of nano-emulgels are relatively smaller than the droplet sizes of nano-emulsions. These micrographs of **(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)** show no indication of aggregation or coalescence. The use of TEM micrographs could not be performed due to the nano-emulgels having a higher viscosity (which can be harmful to the TEM), and would require alteration to the sample. When samples are altered, they are dissolved in ethanol that destroys the nanoparticles. If the nanoparticles are damaged, it will change the properties of the sample (Klang *et al.*, 2013:117).

C.6.2 pH

For nanocarriers to facilitate drug delivery through tissue for effective use, the pH must be close to the pH 7.4 of the normal tissue and blood stream (Tamura *et al.*, 2012:241). Carbopol® Ultrez 20 is a polyacrylic acid (PAA) polymer that transforms from a gel to an aqueous solution if the pH is raised (Gupta & Vyas, 2012:959). According to Lubrizol (2009:1), the pH of a formulation before neutralisation varies between 2.5 – 3.5; this is because of the incorporation of Carbopol® Ultrez 20.

The pH was measured using a Mettler Toledo® pH meter (Mettler, Toledo, United States) fitted with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, United States). Measurements were taken by placing the electrode into the nano-emulgel where after a reading was obtained. Due to the Carbopol® Ultrez 20 causing the pH to be acidic, NaOH was used to adjust the pH values to the desired values of between 3 and 9. Readings were taken in triplicate and averages given, as illustrated in Table C.3.

Table C.3: Average pH values of the respective nano-emulsions and nano-emulgels

Formula	Average pH value
(NEF1)	7.35 ± 0.017
(NEGF)	6.44 ±0.000
(NEL1)	6.17 ± 0.010
(NEGL)	5.98 ±0.000
(NER1)	6.11 ± 0.020
(NEGR)	5.96 ±0.000
(NES1)	7.13 ± 0.010
(NEGS)	6.06 ±0.020

The pH values given in Table C.3 show that the **(NEG)** values varies between 5 – 6, which was lower than the values of **(NE1)** (pH values between 6 – 7). According to Barry (2002:512), formulas for skin penetration should be between 3 – 9 to avoid irritation. These pH values fall within range and it can be concluded that no irritation will be caused to the skin when applied topically.

C.6.3 Droplet size and distribution

Small droplet sizes result in better absorption of the drug, which means the target-site is easily reached. Droplet sizes allow formulations to be more accurate when it comes to dosing and ensure better bioavailability of the API (Sutradhar & Amin, 2013:97). Lower droplet sizes are thus ideal, since there is a smaller chance for creaming, coagulation, flocculation and sedimentation of the formulation (Kumar & Rajeshwarrao, 2012:5; Solans *et al.*, 2005:105).

Droplet size was determined using a Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom) (Figure B.5). The Zetasizer operates using PCS, which functions on the light fluctuations that occur between droplets caused by Brownian motion (Chime *et al.*, 2014:96; Gaur *et al.*, 2014:40; Malvern Instruments Limited, 2015:15). Dilutions were made in a polytop with a drop of each formulation and 2 ml of Milli-Q® water. It was thoroughly mixed and transferred to a clear disposable zeta-cell (DTS1070 folded capillary cell) by means of a syringe. Each measurement was done in triplicate.

PdI values are measured in varieties of 0 – 1; a higher PdI gives an indication of lower homogeneity, according to Elmataeeshy *et al.* (2018:24), while a PdI value close to 0 gives an indication that the particles nearly have the same particle size and uniform distribution (Divsalar *et al.*, 2012:81). A PdI value close to 0.2 gives an indication of a narrow size distribution of particles (Baspinar & Borchert, 2012:249). The PdI is measured as a degree for the width and size distribution (Baspinar & Borchert, 2012:249). The ideal PdI range for nano-emulgels will be < 0.5 and was determined for each of the **(NEG)** formulations; results are given in Table C.4.

Small droplet sizes give the formulation the ability to flow easily and have good rheological properties, which enhances the physical stability for delivery of high concentrations API through the stratum corneum (Leong *et al.*, 2009:721). The preferred range for nano-emulgel formulations are 100 – 500 nm. For this study, these ranges will be used as the acceptable criteria (Drais & Hussein, 2017:10; Nalini *et al.*, 2017:1453; Rambharose *et al.*, 2017:221).

Table C.4: Average droplet size and Pdl values of the **(NE1)** dispersions and the **(NEG)** formulations

(NE1)	Average droplet size (nm)	Average Pdl
(NEF1)	162.60 ± 1.47	0.31 ± 0.02
(NEGF)	146.10 ± 3.41	0.24 ± 0.01
(NEL1)	154.23 ± 0.68	0.40 ± 0.01
(NEGL)	117.96 ± 4.99	0.46 ± 0.06
(NER1)	151.20 ± 1.91	0.30 ± 0.04
(NEGR)	183.16 ± 4.08	0.40 ± 0.01
(NES1)	151.73 ± 2.28	0.30 ± 0.04
(NEGS)	200.83 ± 4.05	0.39 ± 0.02

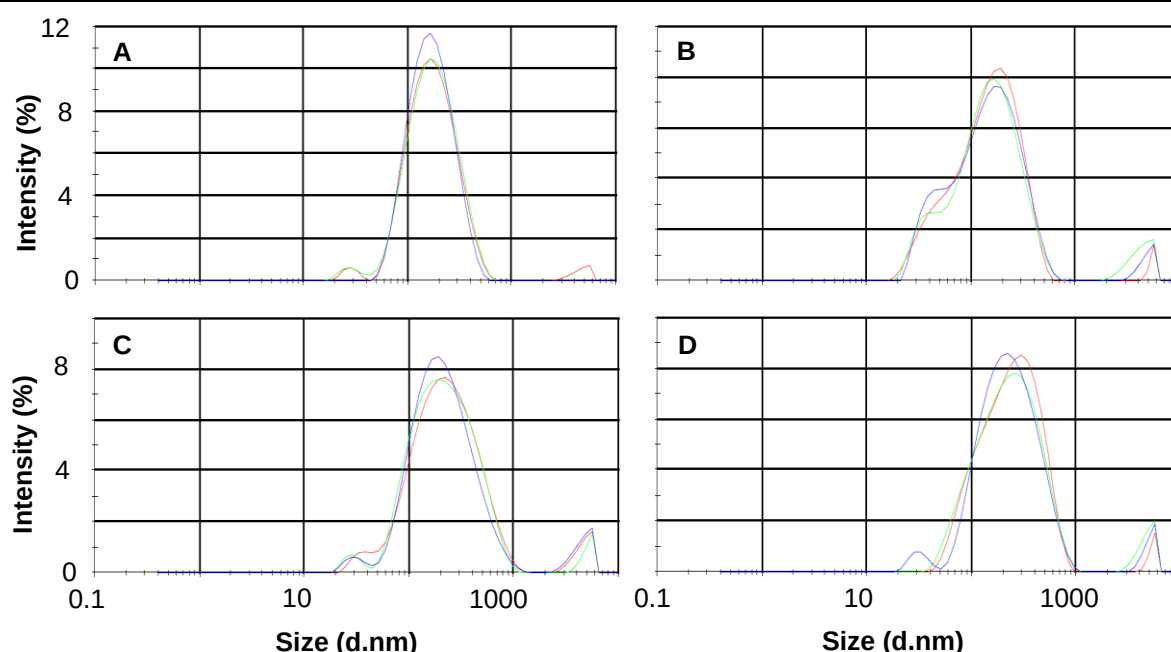


Figure C.4: Average droplet size per droplet radius for a) **(NEGF)**, b) **(NEGL)**, c) **(NEGR)** and d) **(NEGS)**

Table C.4 compares the **(NE1)** and **(NEG)** results for droplet size. When the results are inspected, it is evident that **(NE1)** and **(NEG)** values are within the acceptable criteria with little to no change in the **(NEG)**, which contains Carbopol® Ultrez 20. When the **(NEG)** results are compared, it can be observed that **(NEGF)** and **(NEGL)** had the lowest average droplet size values, which can be a result of increased stirring speed during manufacturing of the nano-emulgels. It can be observed that the droplet size of **(NEGR)** and **(NEGS)** are larger than those of **(NE1)** counterparts, which can be due to increased formation of cross-linking (Eid *et al.*, 2014:5).

The Pdl values of the **(NE1)** and **(NEG)** were between 0.2 – 0.5. After examination, it was evident that the Pdl value of **(NEGF)** was lower than that of **(NEF1)**. Pdl values for **(NEGL)**, **(NEGR)** and

(NEGS) were higher than the Pdl values of their **(NE1)** counterparts, which agrees with the literature of Eid *et al.* (2014:5) that Carbopol® Ultrez 20 has no significant effect on Pdl values of formulations.

C.6.4 Zeta-potential

Zeta-potential can be described as ionised solid particles that surrounds particles of opposite charge (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860; Thakur *et al.*, 2012:223). Zeta-potential was determined by utilising the Malvern Zetasizer Nano ZS (Malvern Instruments, United Kingdom), which uses laser Doppler electrophoresis for measurements (Tantra *et al.*, 2010:280). Nano-emulgels were diluted by using one drop of the nano-emulgel and diluting it with 100 ml of Milli-Q® water in a volumetric flask. A syringe was used to extract the sample out of the volumetric flask into the clear disposable zeta-cell (DTS 1070 folded capillary cell). The sample (2 ml) was carefully transferred to avoid air bubbles forming in the zeta-cell. Zeta-potential measurements took place in triplicate and the data obtained are shown in Table C.5.

Table C.5: Zeta-potential readings (mV) of the **(NE1)** in comparison to readings of the **(NEG)**

Formulation	Zeta-potential (reading 1)	Zeta-potential (reading 2)	Zeta-potential (reading 3)	Average zeta- potential (mV)
(NEF1)	- 66.80	- 65.10	- 67.70	- 66.53 ± 1.32
(NEGF)	- 87.50	- 88.70	- 89.40	- 88.53 ± 0.96
(NEL1)	- 56.90	- 57.00	- 57.60	- 57.17 ± 0.38
(NEGL)	- 46.60	- 47.80	- 43.10	- 45.83 ± 2.44
(NER1)	- 31.80	- 31.80	- 32.10	- 31.90 ± 0.75
(NEGR)	- 42.80	- 42.30	- 42.90	- 42.67 ± 0.32
(NES1)	- 41.80	- 42.20	- 44.20	- 42.73 ± 1.29
(NEGS)	- 71.10	- 71.40	- 69.10	- 70.53 ± 1.25

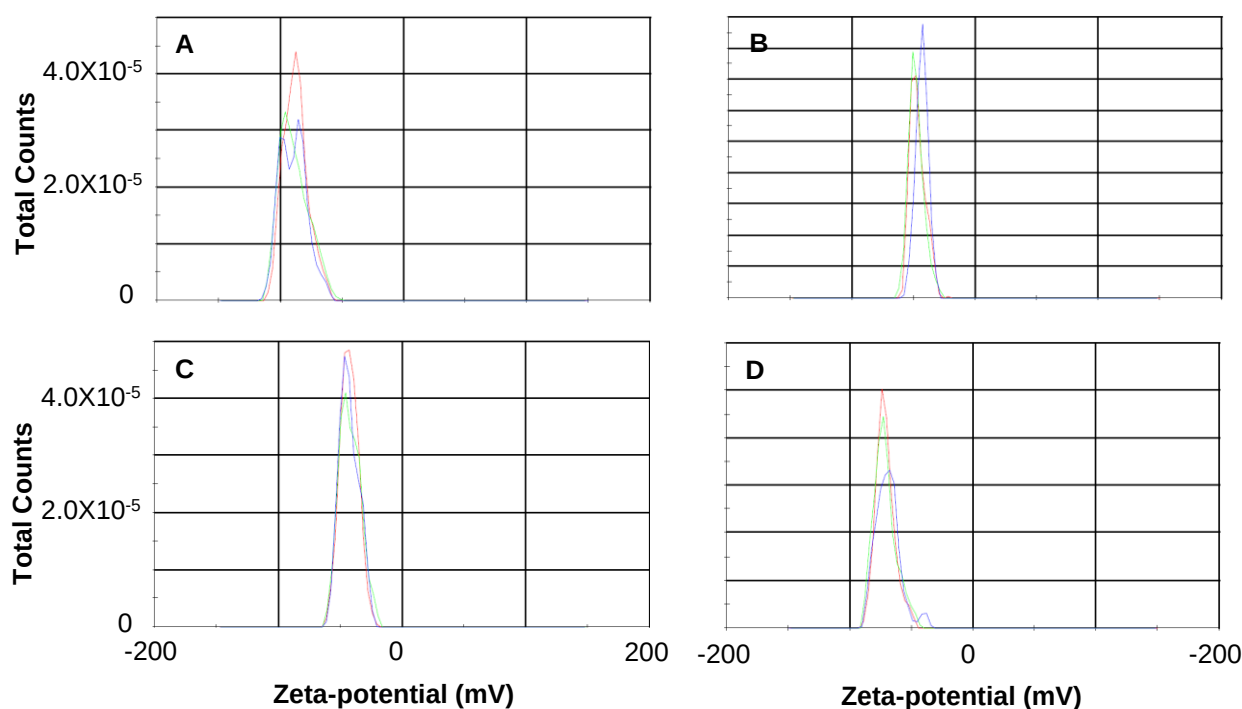


Figure C.5: Average zeta-potential (mV) for a) **(NEGF)**, b) **(NEGL)**, c) **(NEGR)** and d) **(NEGS)**

Nano-emulgels containing Carbopol® Ultrez 20 in the water phase can cause an increase in zeta-potential. It can be proposed that the increase in zeta-potential is a measurement of higher stability of the formulation, where dispersions with a zeta-potential of 0 – 30 mV is highly unstable, while values > - 30 is classified as highly stable (Bhattacharjee, 2016:347; Eid *et al.*, 2014:5; Silva *et al.*, 2012:860). It can be observed that **(NEGF)**, **(NEGL)**, **(NEGR)** and **(NEGS)** all have a zeta-potential of lower than - 30 mV (Eid *et al.*, 2014:5; Silva *et al.*, 2012:860). When comparing the results of **(NEGF)**, **(NEGR)** and **(NEGS)** to their **(NE1)** counterpart, it was observed they had lower zeta-potential values than that of **(NE1)**; except **(NEGL)**. **(NEGL)** had a higher zeta-potential than **(NE1)**, but still fell within acceptable range of a stable formulation, hence it can be proposed that the **(NEG)** formulations will be more stable. Zeta-potential does not give an insight into the Van der Waals forces between particles therefore it is likely to have a formulation with a high positive zeta-potential that is stable and vice versa (Bhattacharjee, 2016:347).

C.6.5 Viscosity

Nano-emulsions have a lower viscosity and thus a lower spreadability than nano-emulgels, thus the nano-emulgel is more convenient because of its higher viscosity and spreadability (Chellapa *et al.*, 2015:44). Viscosity increases when the physical presence of a molecule changes due to a gelling agent that is added to a composition (Bowen-Leaver *et al.*, 2006:3). The consistency changed after the addition of Carbopol® Ultrez 20 to the nano-emulsion in preparation of the nano-emulgel (Shadab & Shamsi, 2018:5). If Carbopol® Ultrez 20 is added to the preparation, the pH (when neutralised)

increases the viscosity, thus the pH must be neutralised to a pH of 6.0 – 6.5 to obtain the desired viscosity (Lubrizol, 2011:1; Shokri *et al.*, 2012:226).

Measurement of viscosity was done with a Brookfield viscometer DV2T LV Ultra (Middleboro, United States), which was fitted to a thermostatic bath at a controlled temperature of $\pm 25^{\circ}\text{C}$. The appropriate spindle must be used for readings of a nano-emulgel, therefore for these measurements the T-bar spindle was utilised for fluvastatin, rosuvastatin T-A spindles, for lovastatin T-B spindles and for simvastatin a T-F spindle was adequate for measurement of viscosity. The T-bar was fitted to the Helipath Stand accessory that enables measurements of formulations with a higher viscosity. The speed of the T-bar was set at 10 rpm to rotate. Measurements were taken in cP in 10 sec intervals for a 2 min period. The software used was Rheocalc T1.2.19 to collect the data given in Table C.6 on the calculated average of the viscosity value. This method was followed to determine the viscosity for each of the nano-emulsions.

Table C.6: Viscosity readings of **(NE1)** and **(NEG)**

Formulation	Viscosity (cP)	Torque (%)	Temperature (°C)	Speed (RPM)
NEF1	5.78 ± 0.02	38.52 ± 0.10	24.20 ± 0.00	200.00 ± 0.00
NEGF	1301.83 ± 134.17	69.43 ± 7.15	24.10 ± 0.00	10.00 ± 0.00
NEL1	3.43 ± 0.02	22.89 ± 0.05	24.27 ± 0.05	200.00 ± 0.00
NEGL	2463.08 ± 254.74	65.68 ± 6.79	24.80 ± 0.00	10.00 ± 0.00
NER1	3.86 ± 0.01	25.69 ± 0.08	24.10 ± 0.00	200.00 ± 0.00
NEGR	1141.92 ± 48.51	61.90 ± 2.58	24.10 ± 0.00	10.00 ± 0.00
NES1	5.66 ± 0.02	37.74 ± 0.12	24.13 ± 0.05	200.00 ± 0.00
NEGS	56189.17 ± 6346.44	59.93 ± 6.77	24.00 ± 0.00	10.00 ± 0.00

From Table C.6, it can be observed that the viscosity values of **(NEG)** was much higher in comparison to the viscosity of the **(NE1)**. According to Chellapa (2015:45), the **(NEG)** should have a higher viscosity, because of the gelling agent that enhances the viscosity and increases the interfacial tension in the **(NEG)**. The viscosity also increased due to the pH that was neutralised before the readings were taken. Neutralisation was done with NaOH to reach the desired pH level of 6.0 – 6.5. The torque (%) was higher than the torque of the **(NE1)** and the spindle speed of the **(NEG)** was taken at a lower speed (RPM); this shows the extent of the resistance given by the **(NEG)** formulation.

C.7 Discussion and conclusion

Various methods of characterisation were performed on each of the **(NEG)**. Compared to the **(NE1)**, the **(NEG)** presented with a thickened formulation of higher viscosity due to the addition of a gelling agent Carbopol® Ultrez 20 (Mahalingam *et al.*, 2008:293), which according to Eid *et al* (2014:1) will enhance permeability and improve stability.

Table C.7: Summary of the comparisons between **(NE1)** dispersions and **(NEG)** formulations

Formula	pH	Droplet size (nm)	Zeta-potential (mV)	Viscosity (cP)
(NEF1)	7.35 ± 0.017	162.60 ± 1.47	- 66.530 ± 1.32	5.78 ± 0.02
(NEGF)	6.44 ± 0.000	146.10 ± 3.41	- 88.530 ± 0.96	1301.83 ± 134.14
(NEL1)	6.17 ± 0.010	154.23 ± 0.68	- 57.170 ± 0.38	3.43 ± 0.02
(NEGL)	5.98 ± 0.000	117.96 ± 4.99	- 45.830 ± 2.44	2463.08 ± 254.74
(NER1)	6.11 ± 0.020	151.20 ± 1.91	- 31.900 ± 0.75	3.86 ± 0.01
(NEGR)	5.96 ± 0.000	183.16 ± 4.08	- 42.667 ± 0.32	1141.92 ± 48.51
(NES1)	7.13 ± 0.010	151.73 ± 2.28	- 42.730 ± 1.29	5.66 ± 0.02
(NEGS)	6.06 ± 0.020	200.83 ± 4.05	- 70.530 ± 1.25	56189.17 ± 6346.44

The pH of formulations was tested in triplicate and with the addition of Carbopol® Ultrez 20, the pH of formulation decreased (Gupta & Vyas, 2012:959). When pH values were neutralised with NaOH the pH increased. Both **(NE1)** and **(NEG)** had pH values within acceptable ranges and should therefore not irritate the skin, which makes them suitable for transdermal delivery. When the zeta-potential was inspected, it was observed that both **(NE1)** and **(NEG)** had zeta-potential values > -30 mV and should therefore be stable (Bhattacharjee, 2016:347; Eid *et al.*, 2014:5; Silva *et al.*, 2012:860). The droplet sizes of both **(NE1)** and **(NEG)** fell within acceptable range of 100 – 500 nm (Drais & Hussein, 2017:10).

When all the results are taken into account, it can be concluded that **(NEG)** is more stable and should enhance penetration better than **(NE1)**. Further studies to determine skin permeability of **(NE1)** and **(NEG)** will be investigated in Appendix D.

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Appendix D

DIFFUSION STUDIES OF AN O/W NANO-EMULSION AND A NANO-EMULGEL CONTAINING SELECTED STATINS AND GRAPESEED OIL

D.1 Introduction

The skin is known as the largest organ of the body and acts as a defence mechanism against microorganisms (Venus *et al.*, 2011:473). The skin acts as a barrier, essential for preserving the integrity of the skin and preventing harmful substances from passing through this barrier (Lundborg *et al.*, 2018:269). The skin consists of a multi-layered stratified epidermis (Lai-Cheong & McGrath, 2017:347), where intracellular lipids are found and is the ideal pathway for drugs through the skin. These lipids in the stratum corneum consist of a lipid structure and hydrocarbon lipid chain (Barba *et al.*, 2019:95).

Transdermal drug delivery was the chosen route of delivery to avoid first-pass metabolism of the drugs. In transdermal drug delivery, a drug passes through the skin (epidermis, dermis, hypodermis) into the systemic circulation via capillary action with help of penetration enhancers. A smaller particle size of the APIs can also enhance penetration (Zhou *et al.*, 2018:1713). Nano-emulsions and nano-emulgels were the chosen drug delivery vehicles, as these kinds of formulations hold ideal characteristic properties for the transdermal delivery of APIs.

To determine if there was any release and delivery from the nano-emulsions (**NE1**) and nano-emulgels (**NEG**), both membrane release studies and *in vitro* skin diffusion studies were performed together with tape stripping to determine the amount of API topically. The formulations were characterised (Appendix B and Appendix C), and showed promising results for skin diffusion studies. To determine the delivery of these statins through the skin, Franz diffusion cells were utilised, since it provides insights of the relationship between drug, skin and formulation (Ng *et al.*, 2010a:209).

D.2 Methods

D.2.1 HPLC analysis of statin samples

The HPLC (discussed in Appendix A) was used to quantify the concentrations of the selected statins (fluvastatin, lovastatin, rosuvastatin and simvastatin) present in the (**NE1**) and the (**NEG**), after samples were collected during the membrane release studies, *in vitro* diffusion studies and tape stripping of the skin. The samples collected from the Franz cell receptor phase were transferred into HPLC vials and then analysed using a HP1100 series with a gradient pump. ChemStation Rev.A.10.03 software was used to analyse the chromatograms. A Venusil XBP C₁₈ (2) reverse phase column (150 x 4.6 mm) with a particle size of 5 µm was used (Agela Technologies, Germany).

The mobile phase consisted of acetonitrile and water with 0.005 M (0.2%) octane sulphonic acid. The laboratory temperature was controlled at 25 °C and chromatographic conditions were maintained throughout the analysis.

Table D.1: Chromatographic conditions used for the analysis of **(NE1)** and **(NEG)**

Run time	10.5 min
Injection volume	10 µl
Flow rate	1.0 ml/min
Detection wavelength	240 nm
Retention times	Fluvastatin: ± 4.090 min
	Lovastatin: ± 7.119 min
	Rosuvastatin: ± 3.654 min
	Simvastatin: ± 4.506 min

A standard solution containing 20 mg of each statin separately, was mixed with 100 ml methanol (Solution 1) and diluted twice. The first dilution was made by placing 5 ml of the Solution 1 into a 100 ml volumetric flask and making it up to 100 ml (Solution 2) and the second dilution was made by transferring 5 ml of Solution 2 into a 100 ml volumetric flask and making it up to 100 ml with methanol (Solution 3). Each solution was injected in four volumes (2.5, 5.0, 7.5 and 10 µl) to obtain a linear regression.

D.2.2 Franz cell method

The rate of penetration does not only depend on the physicochemical properties of the active compound, but also on the temperature needed for optimal diffusion (Shahzad *et al.*, 2015:2; Wissing & Müller, 2002:225). According to Williams (2013:685), the receptor phase should be placed in a water bath at 37 °C and formulations must be kept at 32 °C (this represents the temperature of the skin).

A Franz cell consists of a receptor and a donor compartment (Figure D.1). The donor compartment's function is to hold the formulation that is being tested. The donor compartment fits on top of the receptor compartment (which is filled with PBS (pH 7.4):10% ethanol). Between these two phases, a membrane or dermatomed skin is placed. The donor compartment initiates the formulation applied to the skin and the receptor phase illustrates the blood phase underneath the skin (Bolzinger *et al.*, 2012:160).

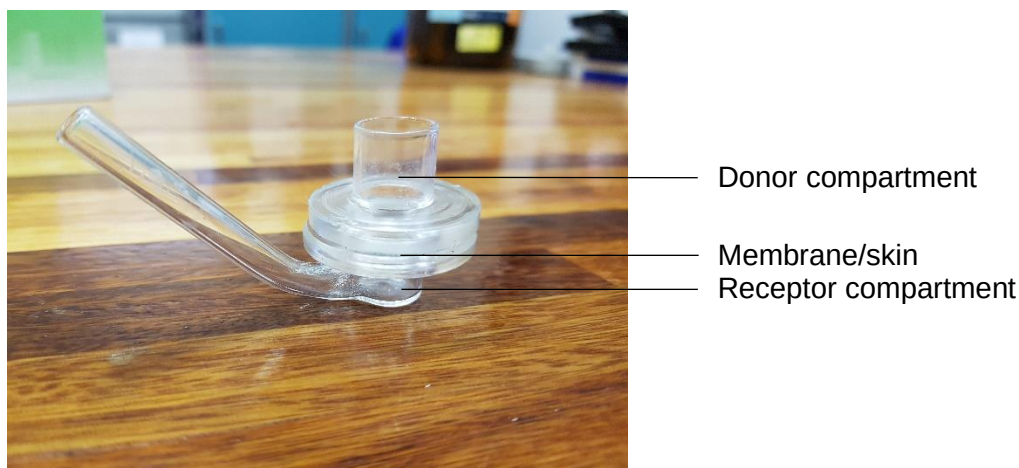


Figure D.1: Illustration of Franz cell compartments

D.2.2.1 Donor phase preparation

For the donor phase, **(NE1)** (Appendix B) and **(NEG)** (Appendix C) were formulated. Eight formulas each containing the API (**(NEF1)**, **(NEL1)**, **(NER1)**, **(NES1)** and **(NEGF)**, **(NEGL)**, **(NEGR)**, **(NEGS)**) were tested together with the placebos (control group). Each of these formulas were placed into the donor phase and tested during both membrane release and skin diffusion studies.

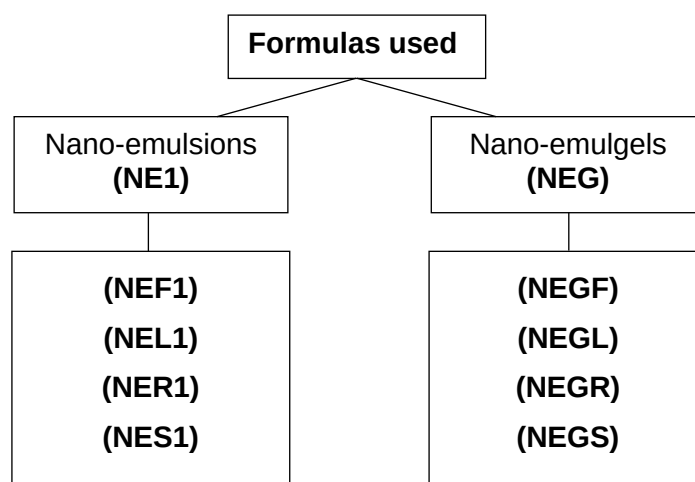


Figure D.2: Formulas used for the membrane release and skin diffusion studies

D.2.2.2 Preparation of PBS (pH 7.4) as receptor phase of the Franz cells

PBS (pH 7.4) was prepared by dissolving 1.5 g NaOH in 400 ml Milli-Q® water. While the NaOH was busy dissolving, 6.5 g potassium dihydrogen orthophosphate (KH₂PO₄) was dissolved in 250 ml Milli-Q® water. When both the products were dissolved NaOH and KH₂PO₄ were mixed together. The final volume of the solution was made up to 1 000 ml with Milli-Q® water (350 ml). The pH was then measured with a Mettler Toledo® pH meter (Mettler Toledo CU) equipped with a Mettler Toledo InLab® 410 electrode (Mettler Toledo, CU). To adjust the pH to 7.4, either NaOH or orthophosphoric

acid (H_3PO_4) were added. After the pH was adjusted, 100 ml of the solution was taken out and replaced with a 100 ml ethanol to obtain a concentration of 10% ethanol. The addition of 10% ethanol does not compromise the skin and assists with better recovery of the API from the stratum corneum (Praça *et al.*, 2018:130).

D.2.2.3 Membrane release studies

Membrane release studies were performed on **(NE1)** and **(NEG)** to investigate if the selected statins were released from the formulations made (see Figure D.3).



Figure D.3: Materials used for membrane release studies: a) Franz cell with a donor and receptor compartment; b) PVDF synthetic membranes; c) Dow Corning® high vacuum grease; d) Franz cells in a Franz cell stand on the magnetic stirring plate; placed in the water bath; e) Parafilm® used to cover the Franz cells; f) syringes used for extraction of receptor phase; g) assembled Franz cell with 1 ml formulation in the donor compartment (top) and 2 ml PBS (pH 7.4):10% ethanol in the receptor compartment (bottom) before placed in the water bath; h) horse shoe clamp used to fasten the Franz cell compartments; i) Grant® water bath

During this study, 12 Franz diffusion cells containing the formulation being tested and 2 Franz cells with the placebo (serving as a control group) were tested. The receptor phase (PBS (pH 7.4):10% ethanol) was placed in a preheated Grant® water bath at 37 °C (Grant Instruments, United Kingdom). The formulations were placed in a second preheated Grant® water bath at a temperature of 32 °C; the water baths were preheated an hour prior to experiments. A third water bath was utilised where the prepared Franz cells were placed for the duration of the experiment.

Each Franz cell (receptor and donor phase) was smeared with Dow Corning® high vacuum grease. A magnetic stirring rod was then placed into each receptor compartment, thereafter a polyvinylidene fluoride (PVDF) (Pall® Life Sciences, United States) synthetic membrane, with a pore size of 0.45 µm and a 25 mm diameter, was placed on top of the receptor phase. The donor compartment was placed on top of the receptor compartment containing the synthetic membrane and sealed together with a thick layer of vacuum grease to prevent any leakages. A horse shoe clamp was tightly secured onto the two compartments of the Franz cell to hold the two phases together. Each Franz cell was then carefully filled with the PBS (pH 7.4):10% ethanol at 37 °C to ensure no air bubbles formed inside the receptor phase. Each donor phase was then filled with either 1 ml of **(NE1)** or **(NEG)** that was preheated to 32 °C. Each donor phase was then enclosed with a piece of Parafilm® and a plastic cap to ensure the formulations stayed intact, where after the Franz cells were placed into a preheated water bath of 37 °C with a Variomag® magnetic stirring plate (Variomag, United States). After 1 hour the PBS (pH 7.4) was extracted in the same order that it was filled, and refilled with PBS (pH 7.4):10% ethanol at 37 °C. The extracted receptor phase was placed into HPLC vials, each marked for 1 h extractions; the extraction took place in 1 h intervals for a total of 6 h.

D.2.2.4 *In vitro* skin diffusion studies

In vitro skin studies and tape stripping were performed after the membrane release studies to establish whether the selected statins in the **(NE1)** or the **(NEG)** achieved transdermal delivery and if the API was delivered into the stratum corneum epidermis (SCE) or epidermis dermis (ED).

D.2.2.4.1 Skin collection and ethical aspects

The skin used for this study was obtained from a Caucasian female, post-abdominoplasty surgery. The purpose of the skin donation was explained to the patient and they were informed they could withdraw from the study at any time. Confidentiality of the patient was protected using a reference number for the skin collected. Informed consent was first obtained from the patient donating the skin and then co-signed by the plastic surgeon and a witness. Skin was collected using a cooler box when transported, and then stored in a freezer at - 20 °C. The use of female skin throughout the study will minimise the variation in results obtained. Approval to conduct the study on abdominal skin of females was granted from the Health Research Ethics Committee (HREC) of the North-West

University, ethics number (NWU-00111-17-A1-04) under the larger study “The use of human skin (biological waste) to study transdermal and topical delivery” (Ethics no: NWU-00111-17-A1).

D.2.2.4.2 Preparation of skin for *in vitro* diffusion studies

The skin collected from abdominoplasty was used to prepare dermatomed skin samples. The skin was defrosted and spread out, the fat cut off and then the skin then placed with the stratum corneum facing upwards on a piece of paper towel. The skin was removed with a Dermatome™ (Zimmer TDS, United Kingdom) kept at an angle of 30 – 45 ° with constant pressure applied. The skin used had a thickness of $\pm 400 \mu\text{m}$ and was placed on a Whatman® filter paper to dry. The skin on the filter was then wrapped in aluminium foil and placed in the freezer at - 20 °C. Prior to skin diffusion studies, the skin was cut into circles on the filter paper to fit onto the receptor phase of the Franz diffusion cells.



Figure D.4: a) Dermatome™ (Zimmer TDS, United Kingdom) and b) dermatomed skin samples on Whatman® filter paper ($\pm 400 \mu\text{m}$ in thickness)

D.2.2.4.3 *In vitro* skin diffusion studies

In vitro skin diffusion studies were done to investigate the selected statins' absorption, distribution and accumulation in the skin (Hashiguchi *et al.*, 1997:11). For the *in vitro* diffusion studies the same technique was used as with the membrane release studies (Section D.2.3.3). Except that during the membrane release studies, a PDVF synthetic membrane was used, which was replaced with dermatomed skin during the skin diffusion studies and the receptor phase was only extracted once (after a 12 h), as determined by Marais (2018:228) and Maree (2018:197). Instead of using 12 Franz diffusion cells (as with the membrane release study), only 8 – 9 Franz cells were used during the skin diffusion studies (excluding the placebos).

D.2.2.4.4 Tape stripping

Tape stripping was done after the 12 h *in vitro* skin diffusion studies to evaluate how effective the formulations delivered the APIs into the skin (Demierre *et al.*, 2012:306). This was done by removing the horse-shoe clamp and taking the donor and receptor phases apart to remove the skin. Subsequently, the dermatomed skin was removed from the filter paper and pinned onto a piece of Parafilm™ on a wooden board and dabbed with a piece of paper towel to remove any excess formulations still left on the skin. The SCE was removed by using 3M Scotch® Magic™ Tape cut into sizes small enough to fit onto the diffusion area that was covered by the formulations. To avoid contamination, the first tape strip was discarded as it may still contain formulation. Roughly 15 tape strips were used to remove the SCE, until the skin had a glistening appearance. The tape strips (known as the SCE) of each Franz cell were placed into separate polytops that contained 5 ml of PBS (pH 7.4):10% ethanol. The remainder of the skin (known as the ED) of each Franz cell was then placed into a separate polytop containing 5 ml PBS (pH 7.4):10% ethanol after being cut into pieces. Both the SCE and ED samples were left in the fridge at $\pm 4^{\circ}\text{C}$ for ± 8 h. Where after they were removed and filtered through a $0.45\ \mu\text{m}$ PTFE filter into HPLC vials for analysis; this process was done for each formulation. By analysing the data from the HPLC, the concentrations of API that remained in the skin (SCE and ED) were determined.

D.2.2.5 Data analysis

A standard solution of each of the four statins were injected into the HPLC to obtain a linear regression line; this linear line and data could be used to establish the concentration for each of the selected statins in the different formulations (Ng *et al.*, 2010a:213). The average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) that diffused through the membrane was plotted against time (h) for each statin (Shakeel *et al.*, 2007: E3). An average slope of linear regression formed, resulting in the average flux (degree to what extent statins permeates the membrane) measured in ($\mu\text{g}/\text{cm}^2\cdot\text{h}$).

During the skin diffusion studies, the amount per area diffused ($\mu\text{g}/\text{cm}^2$) after 12 h was obtained together with the %diffused through the skin (%). The average SCE and ED concentrations ($\mu\text{g}/\text{ml}$) that permeated into the skin after the 12 h diffusion study represented the tape stripping data. After membrane release studies and skin diffusion studies, an indication was given as to whether the selected statins in the **(NE1)** and the **(NEG)** were thoroughly released from the formulations, and it could be determined if the target-site was reached. The dermis can act as a barrier for permeation, but formulations can permeate through for uptake in the bloodstream (Jarvis *et al.*, 2004:918).

D.2.2.6 Statistical data analysis

Statistical data analysis was performed on the data of the membrane release studies, as well as skin diffusion studies for eight formulas (four were nano-emulsions and four were nano-emulgels containing the selected statins) by means of Statistica (Statsoft, 2008). During the skin diffusion and tape stripping (SCE and ED) studies, the median of the four statins in different formulations were compared to one another. Statistical data analysis was performed in a more-way analysis of variance (ANOVA).

ANOVA is a statistical model, testing between-mean differences in more than two conditions simultaneously (Boisgontier & Cheval, 2016:1004). The ANOVA, Welch and Kruskal-Wallis methods are three groups in a global test with null hypothesis (H_0), where with H_0 , the means of ANOVA and the medians of Welch and Kruskal-Wallis are the same versus the alternative hypothesis (Liu, 2015: ii). The Kruskal-Wallis test (performed on the *in vitro* skin diffusion data) is a non-parametric method used for testing samples that have the same distribution and the test is performed on ranked data (Liu, 2015:7). The H_0 is often the mode of choice used for statistical analysis, but it does not give an in-depth focus on the p-values. The p-value is often used to describe or summarise the results and draw a conclusion. The p-value can be used to describe statistically significance or insignificance. For a summary of data to have a statistical significance, the p-value must be < 0.05 (Consonni & Bertazzi, 2017:328); for p-values > 0.05 contains a statistical insignificance.

After the ANOVA was completed, the Levene's test for homogeneity of variances was performed. The Levene's test is done to test for equal variances of the different groups (here statins or formulations) (Shear *et al.*, 2018:1). After a one-way ANOVA on the statin-groups, the Tukey honest significance test (HSD), multiple comparison test is performed to compare all the possible mean differences. From this test the mean values can be categorised in groups (a, b, c, etc.) according to their homogeneity (Hamada, 2018:16). The groups ranked together can be seen as homogenous, as the mean values of their data show little differences (i.e. not significant on a 0.05 level).

Descriptive statistics of different groups include the use of the mean, median as location characteristics and the variance, standard deviation as a scale characteristic to describe the data variation or spread (Shear *et al.*, 2018:1). Determination of data only based on the mean can be inaccurate as the outliers and distribution of data can have an effect on the mean, while the median is less affected by these components (Driscoll *et al.*, 2000:275), thus data was analysed by median differences.

D.3 Results and discussion

D.3.1 Membrane release studies

Membrane release studies were performed on the **(NE1)** and the **(NEG)** for all four selected statins. The results are tabulated in Table D.2 and summarise the average %release together with the average and median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) values. The drug release over 6 h performed in 1 h intervals are presented in Figures D.5 – D.20.

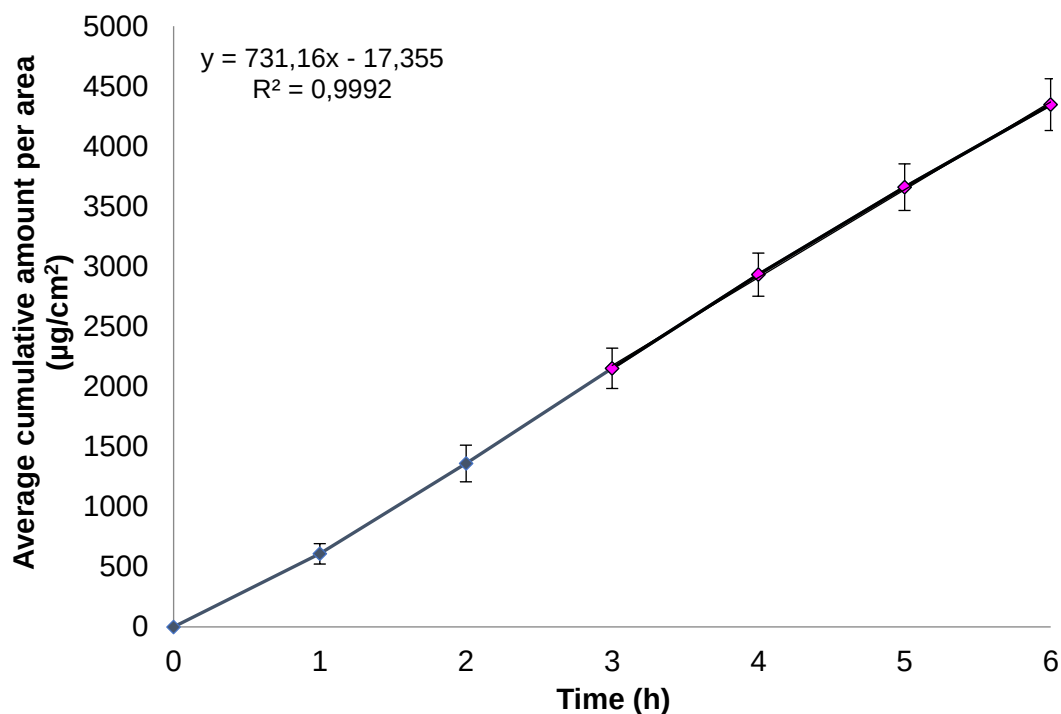


Figure D.5: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEF1** that was released through the membranes to indicate the average flux from 3 – 6 h ($n = 12$)

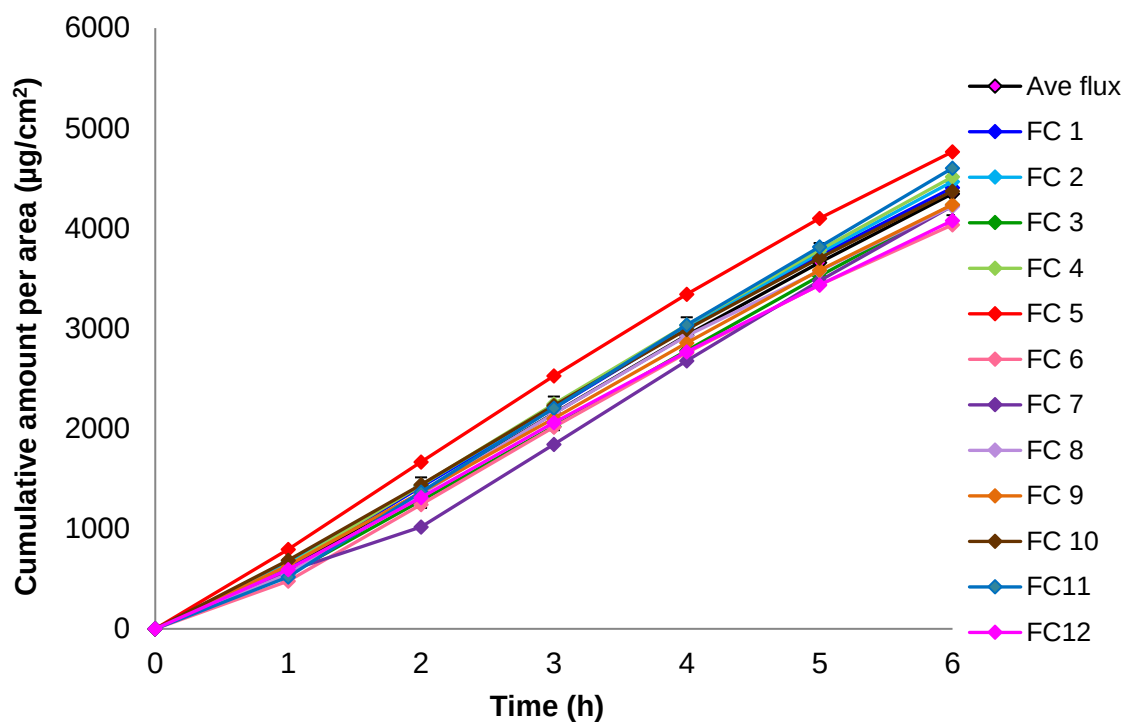


Figure D.6: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEF1** that was released through the membranes of each individual Franz cell over 6 h ($n = 12$)

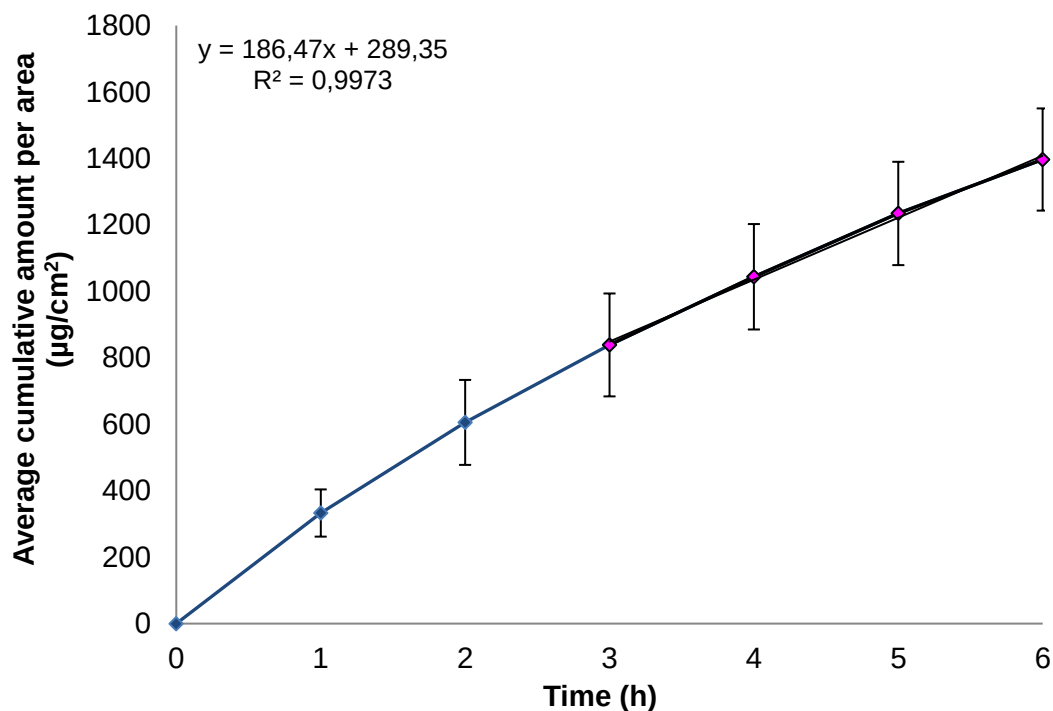


Figure D.7: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEGF** that was released through the membranes to indicate the average flux from 3 – 6 h ($n = 11$)

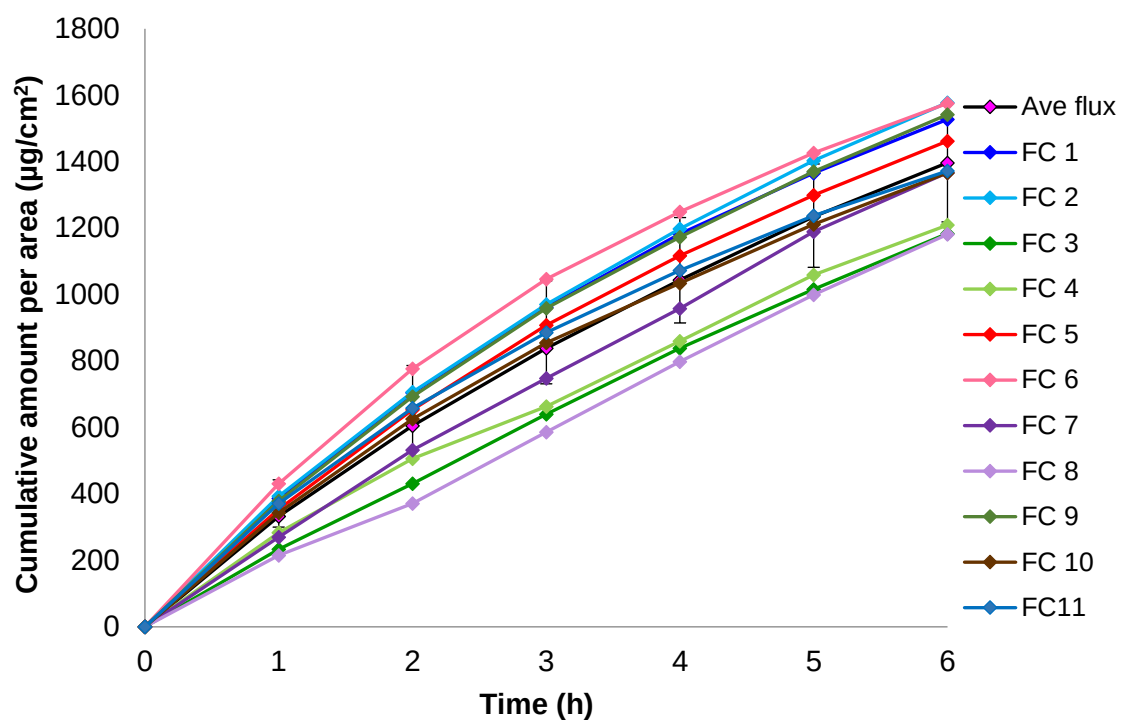


Figure D.8: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEGF** that was released through the membranes of each individual Franz cell over 6 h (n = 11)

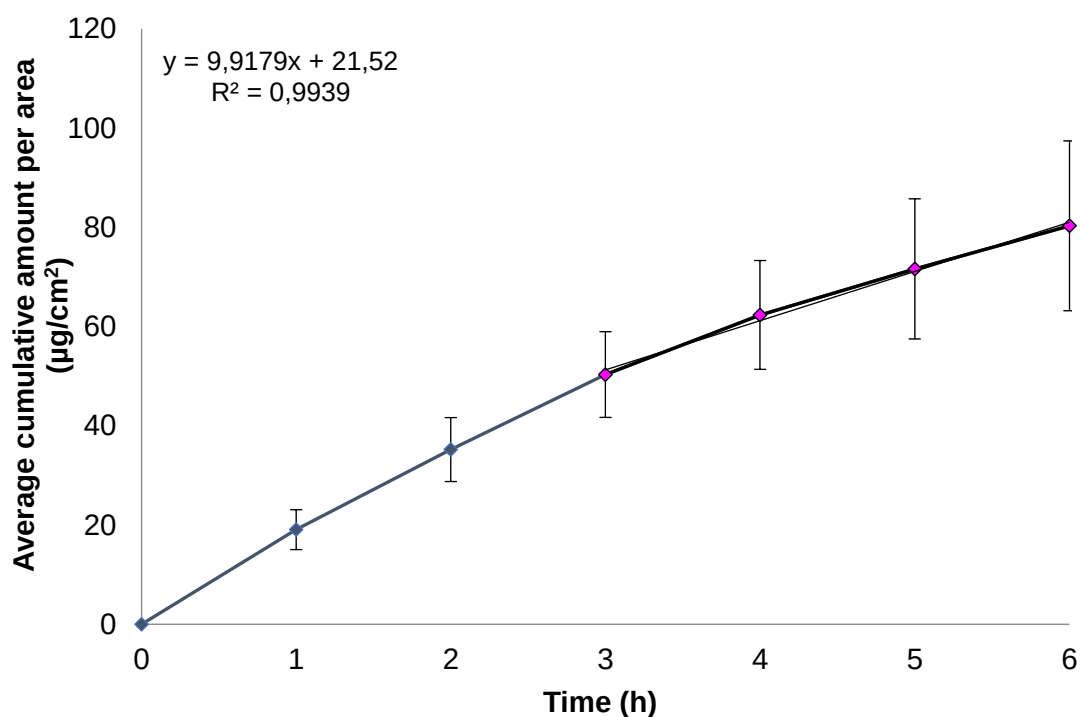


Figure D.9: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEL1** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 12)

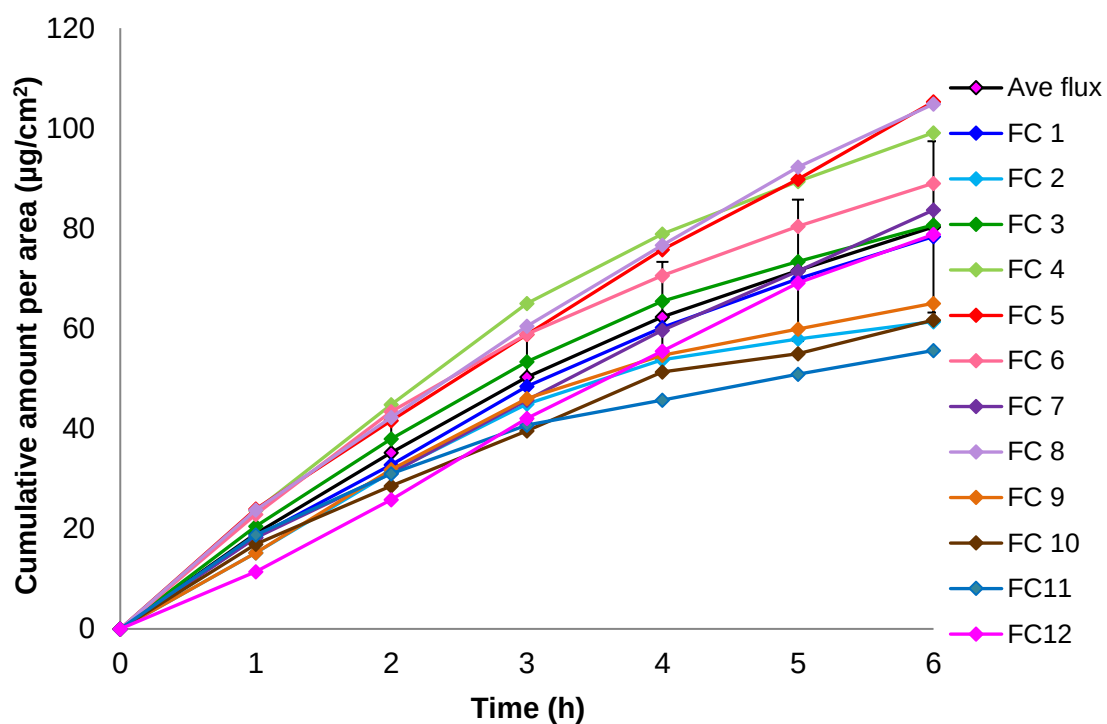


Figure D.10: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEL1** that was released through the membranes of each individual Franz cell over 6 h (n = 12)

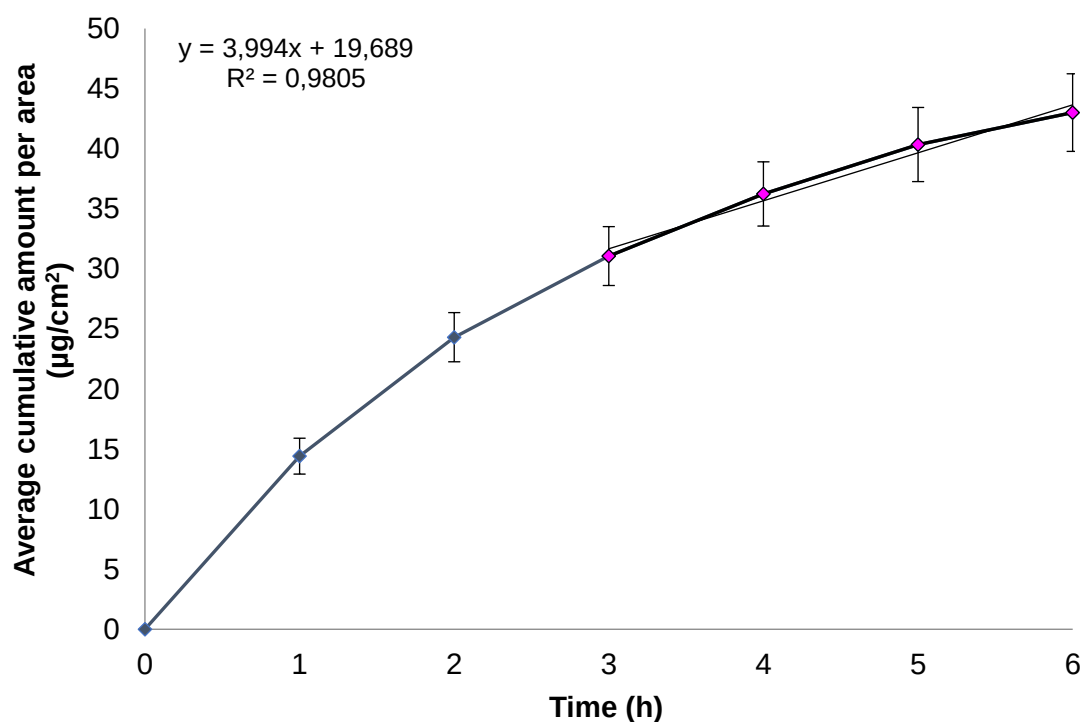


Figure D.11: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEGL** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 10)

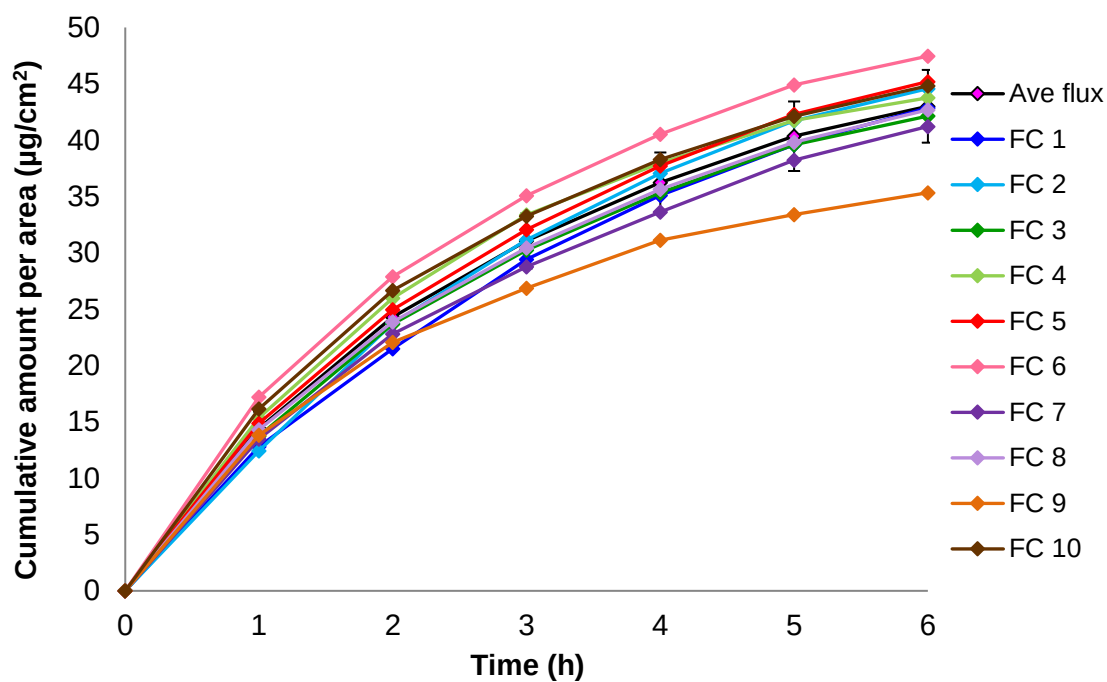


Figure D.12: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEGL** that was released through the membranes of each individual Franz cell over 6 h (n = 10)

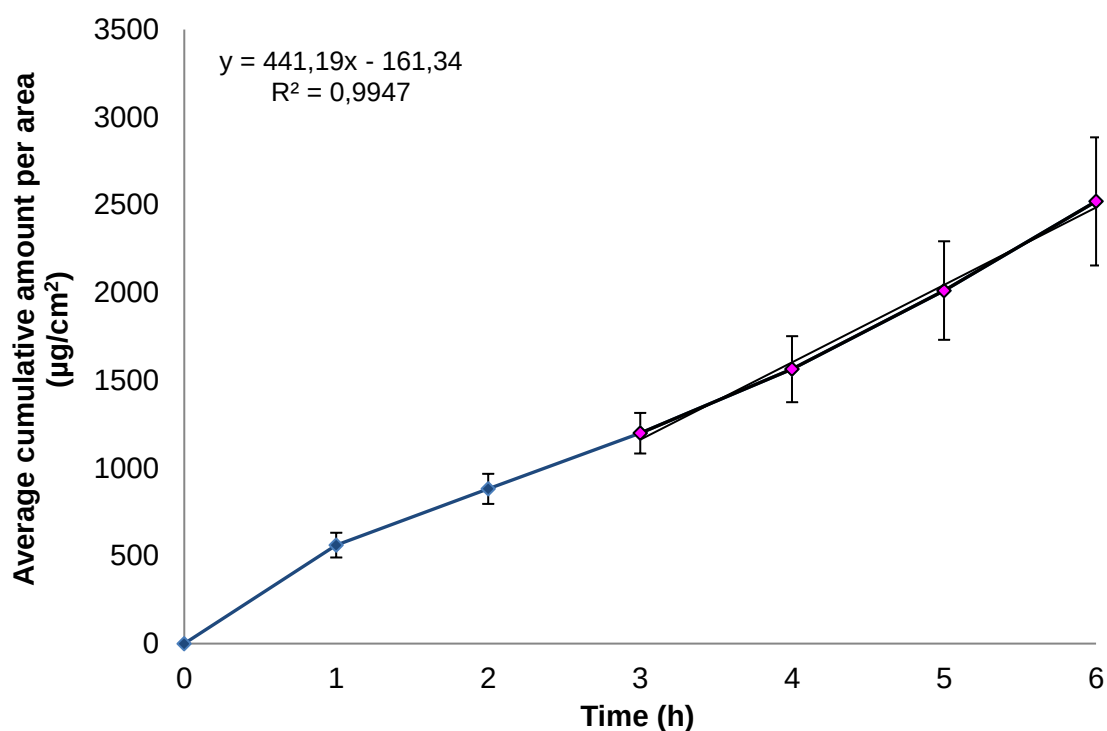


Figure D.13: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NER1** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 11)

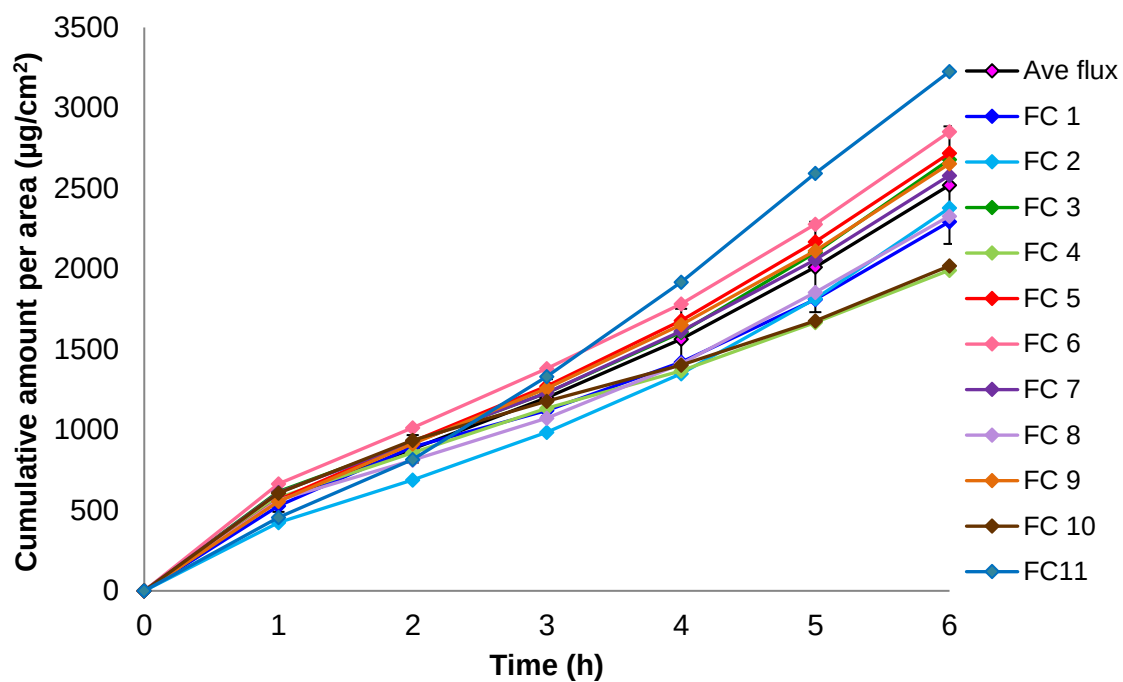


Figure D.14: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NER1** that was released through the membranes of each individual Franz cell over 6 h (n = 11)

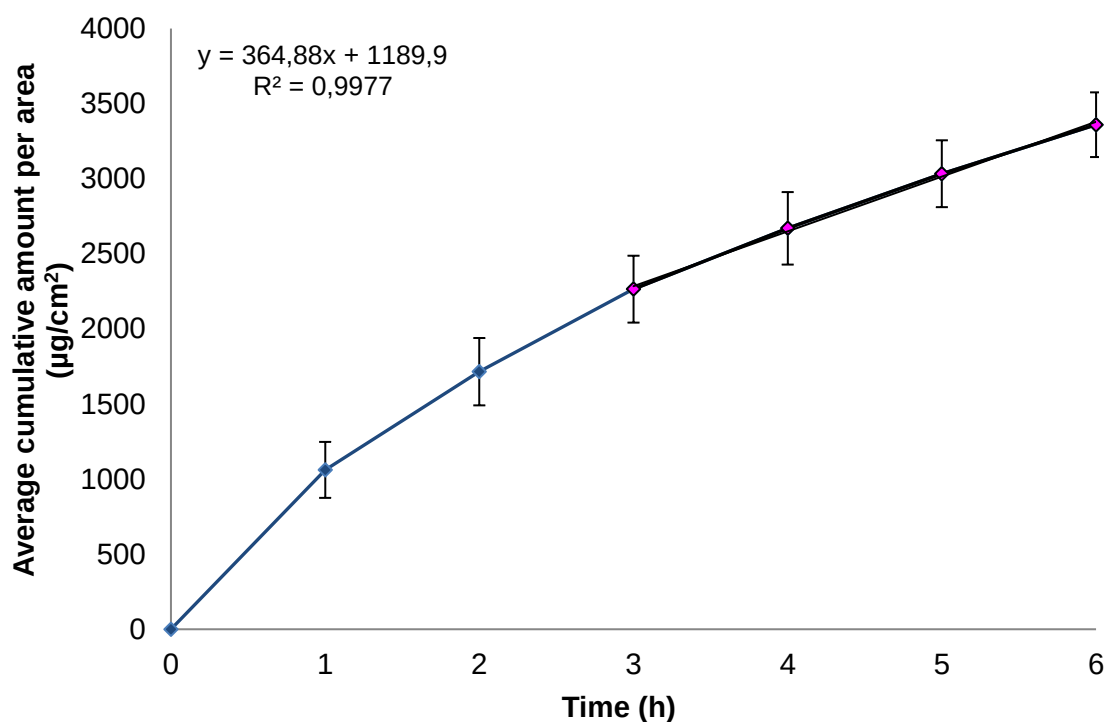


Figure D.15: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NEGR** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 12)

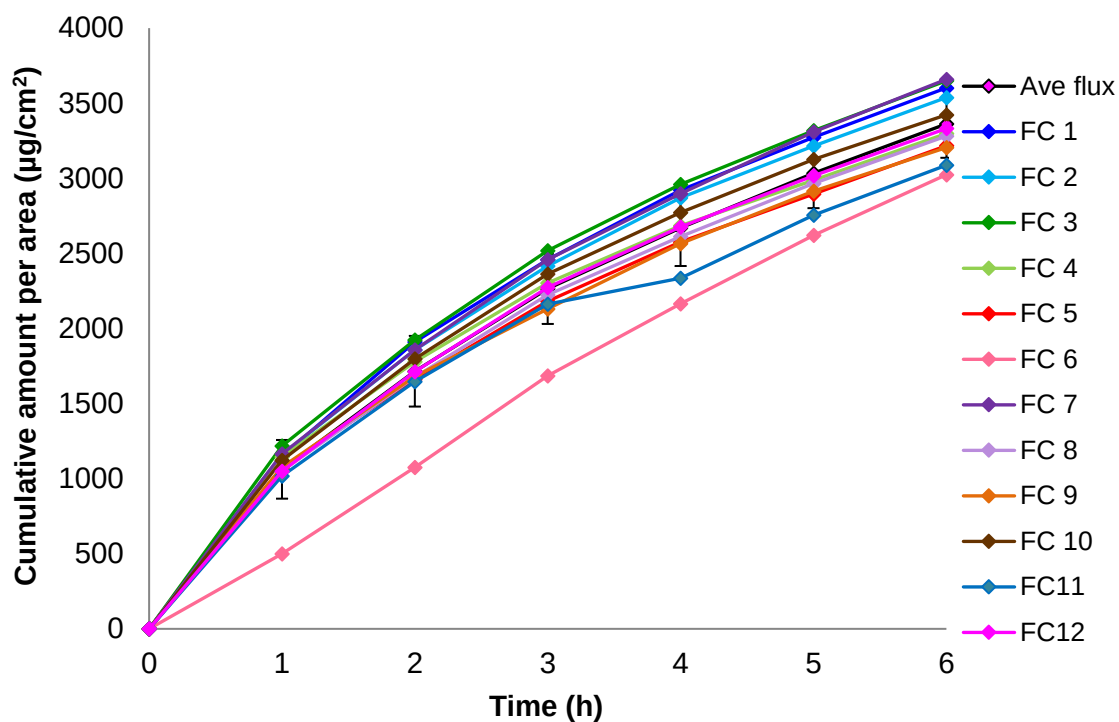


Figure D.16: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NEGR** that was released through the membranes of each individual Franz cell over 6 h (n = 12)

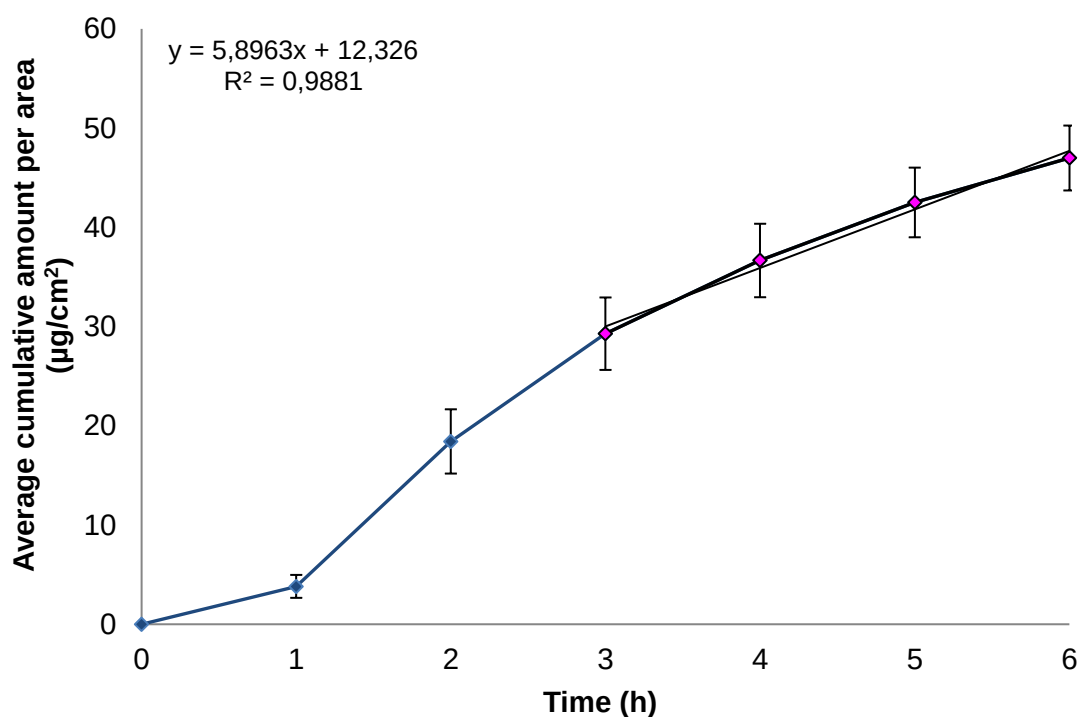


Figure D.17: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NES1** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 12)

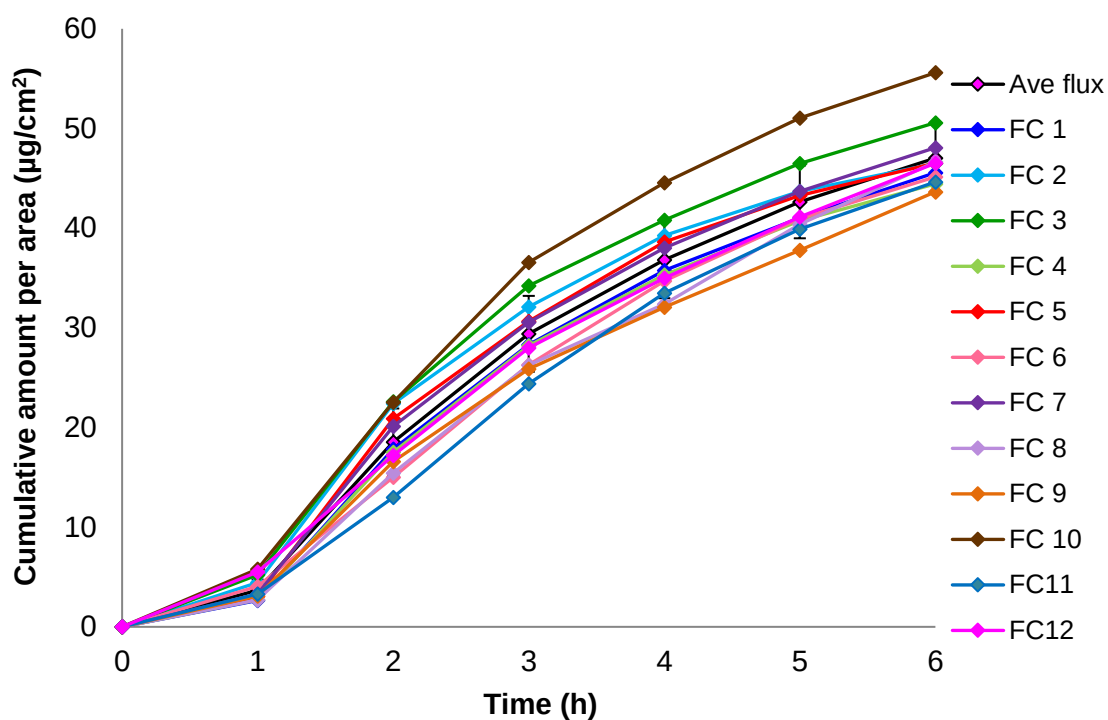


Figure D.18: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NES1** that was released through the membranes of each individual Franz cell over 6 h (n = 12)

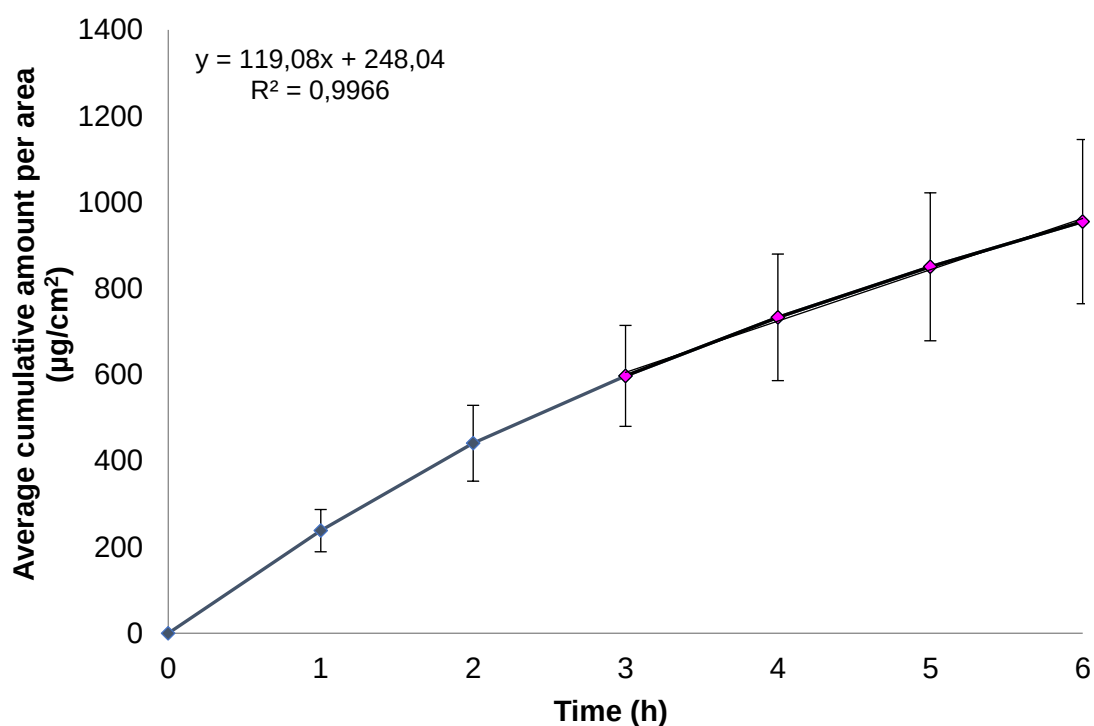


Figure D.19: Average cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NEGS** that was released through the membranes to indicate the average flux from 3 – 6 h (n = 9)

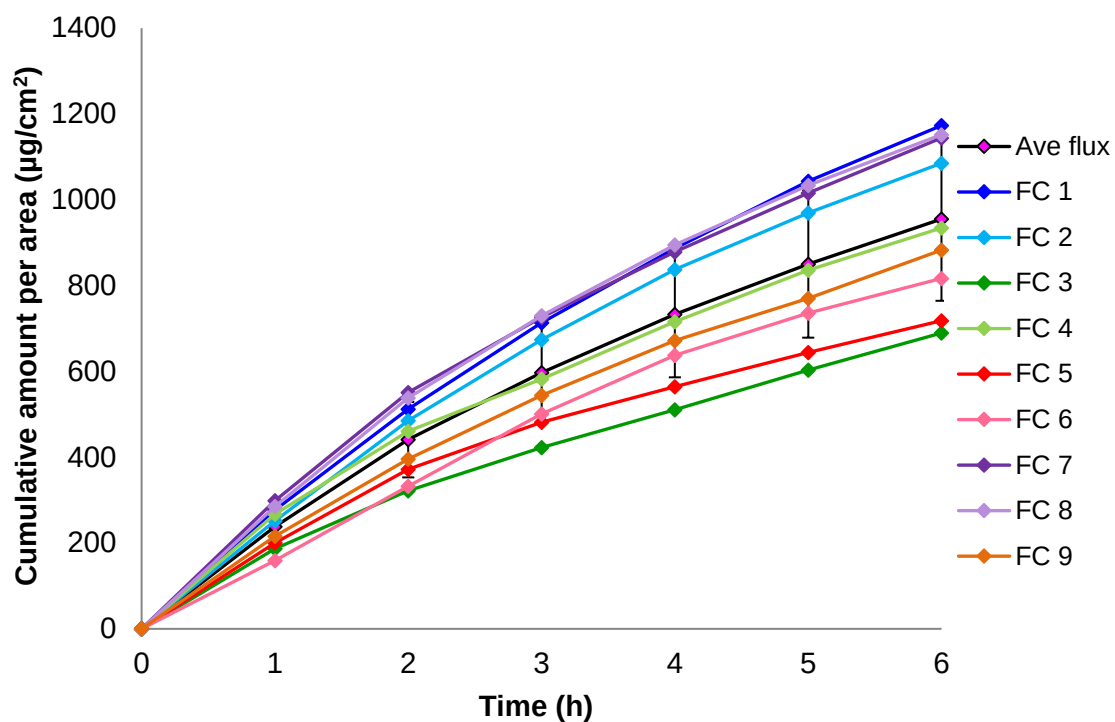


Figure D.20: Cumulative amount per area ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NEGS** that was released through the membranes of each individual Franz cell over 6 h ($n = 9$)

Table D.2: Average %released together with the average and median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) for each of the statins from the **(NE1)** and the **(NEG)** after 6 h, where n is the number of cells used

Formula	n	Average %release (%)	Average flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)	Median flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$)
(NEF1)	12	11.44 ± 0.57	731.16 ± 126.18	603.18
(NEGF)	11	3.76 ± 0.41	186.47 ± 28.74	210.36
(NEL1)	12	0.19 ± 0.04	9.92 ± 2.75	10.80
(NEGL)	10	0.12 ± 0.01	3.99 ± 4.15	12.11
(NER1)	11	6.52 ± 0.95	441.19 ± 126.84	350.80
(NEGR)	12	9.03 ± 0.58	364.88 ± 33.72	451.79
(NES1)	12	0.13 ± 0.01	5.90 ± 0.50	8.34
(NEGS)	9	2.57 ± 0.51	119.08 ± 36.34	131.85

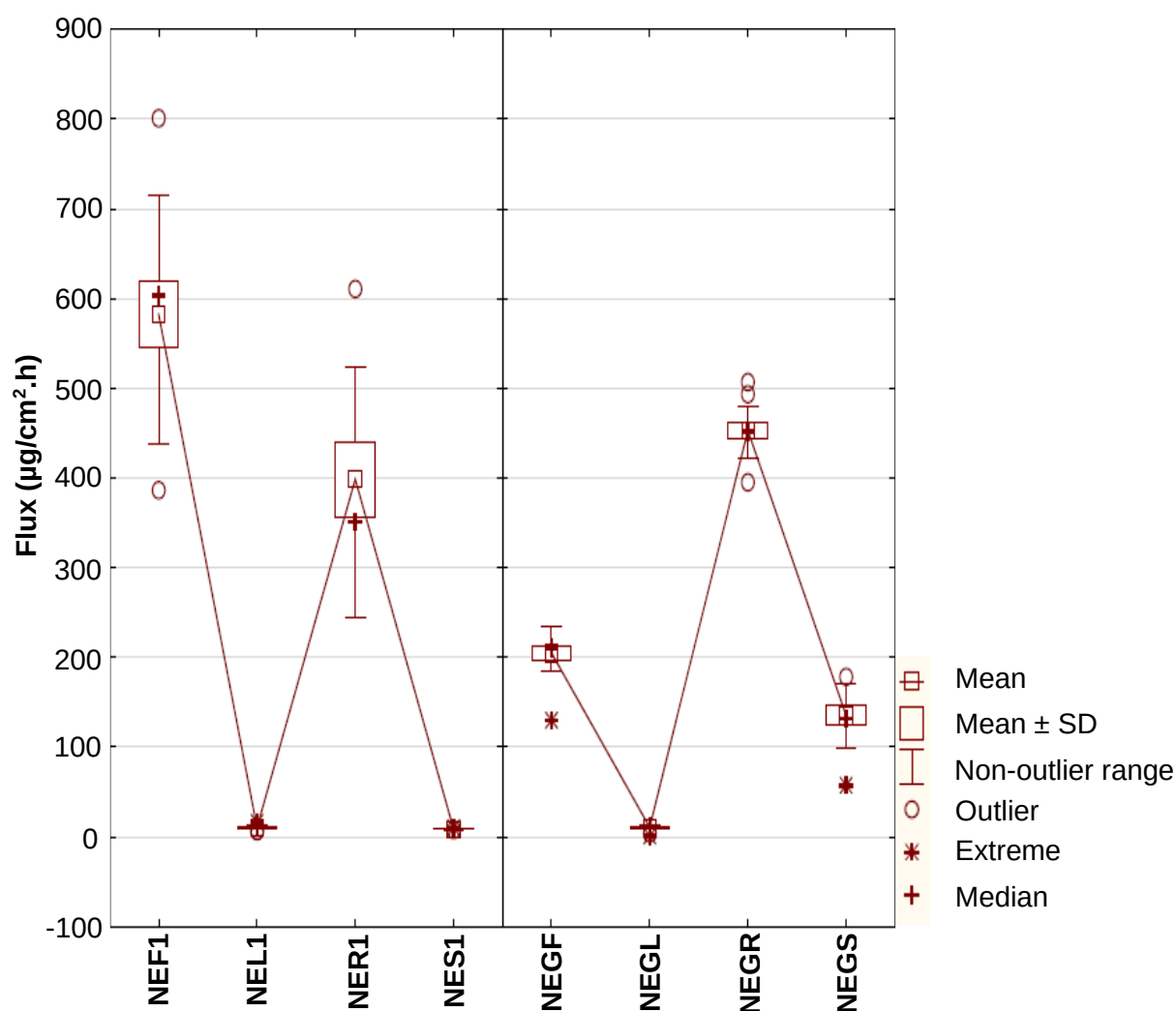


Figure D.21: Box-plots indicating the flux ($\mu\text{g}/\text{cm}^2\cdot\text{h}$) of the **(NE1)** and the **(NEG)** of all four statins during the membrane release studies over 6 h

When Table D.2 is compared with Figure D.21, it is evident that a difference between average and mean could be observed, since the average of the linear regression slope was determined by the best fit (flux determined between 3 and 6 h), while the mean flux values (determined during the statistical analysis) were determined between 1 and 6 h.

The average %released together with the average and median flux of all four statins in the different formulations were determined and analysed after membrane release studies were performed. During this study, the median flux values will be discussed, since the average/mean flux values are affected by outliers (Driscoll *et al.*, 2000:275). It can be observed that when the median flux values for the **(NE1)** were compared, **(NEF1)** had the highest median flux value ($603.18 \mu\text{g}/\text{cm}^2\cdot\text{h}$), followed by **(NER1)** ($350.80 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(NEL1)** ($10.80 \mu\text{g}/\text{cm}^2\cdot\text{h}$) and lastly, **(NES1)** ($8.34 \mu\text{g}/\text{cm}^2\cdot\text{h}$). When the median flux values for the **(NEG)** were compared, it was detected that **(NEGR)** obtained the highest median flux value ($451.79 \mu\text{g}/\text{cm}^2\cdot\text{h}$), followed by **(NEGF)** ($210.36 \mu\text{g}/\text{cm}^2\cdot\text{h}$), **(NEGS)** ($131.85 \mu\text{g}/\text{cm}^2\cdot\text{h}$) and lastly, **(NEGL)** with a median flux value of ($12.11 \mu\text{g}/\text{cm}^2\cdot\text{h}$). From the

membrane release study (Figures D.5 – D.21 and Table D.2), it was evident that both the **(NE1)** and the **(NEG)** released the APIs (statins) from the formulas over a period of 6 h.

D.3.2 *In vitro* skin diffusion studies

Skin diffusion studies were done on both the **(NE1)** and the **(NEG)** and the results for all eight formulas presented in Table D.3. The data was collected by means of HPLC analysis, where after it was processed. Results are given according to average %diffused, average concentration diffused ($\mu\text{g/ml}$), average amount per area diffused ($\mu\text{g/cm}^2$) and the median amount per area diffused ($\mu\text{g/cm}^2$) after 12 h.

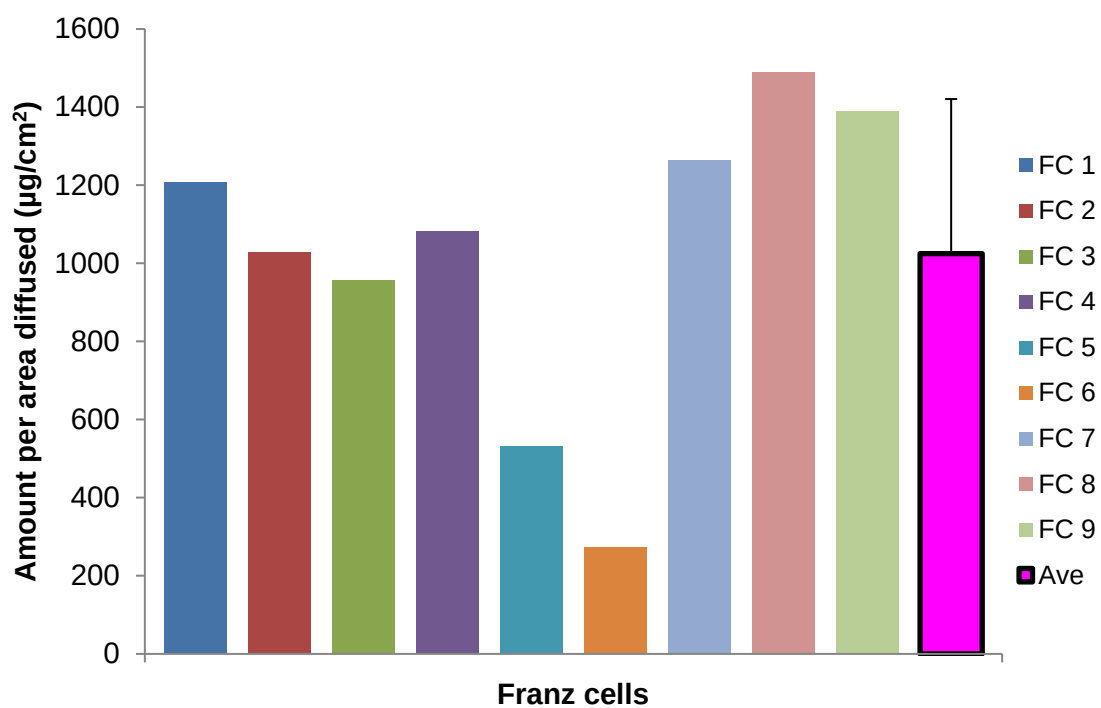


Figure D.22: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEF1** after the 12 h diffusion study ($n = 9$)

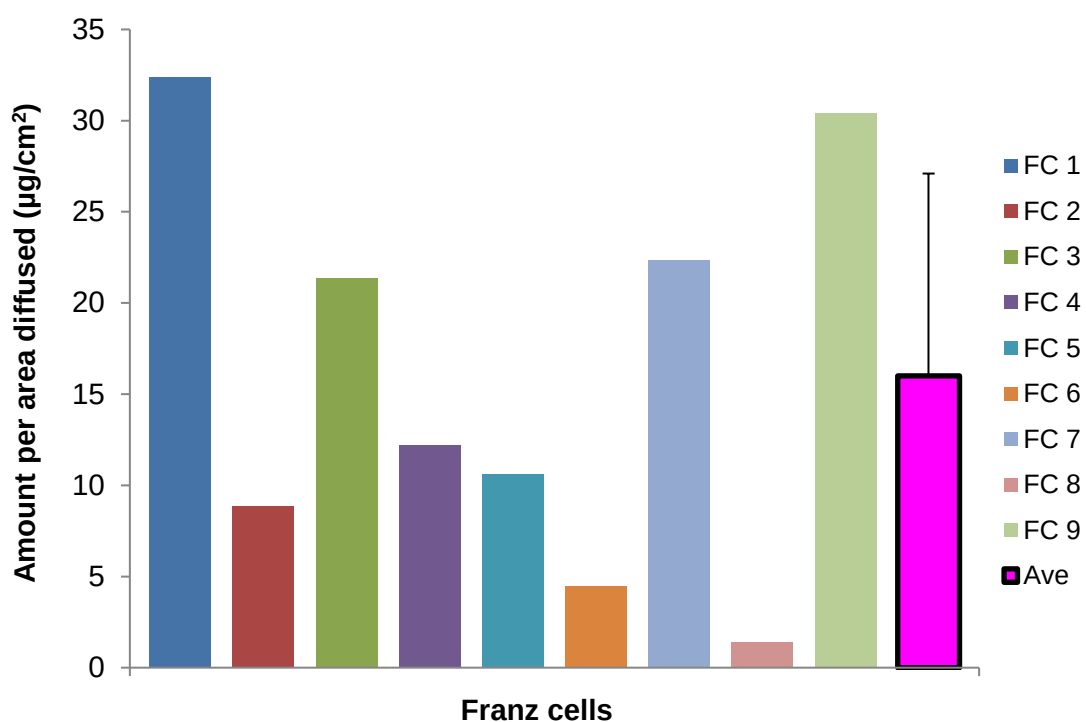


Figure D.23: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of fluvastatin from **NEGF** after the 12 h diffusion study ($n = 9$)

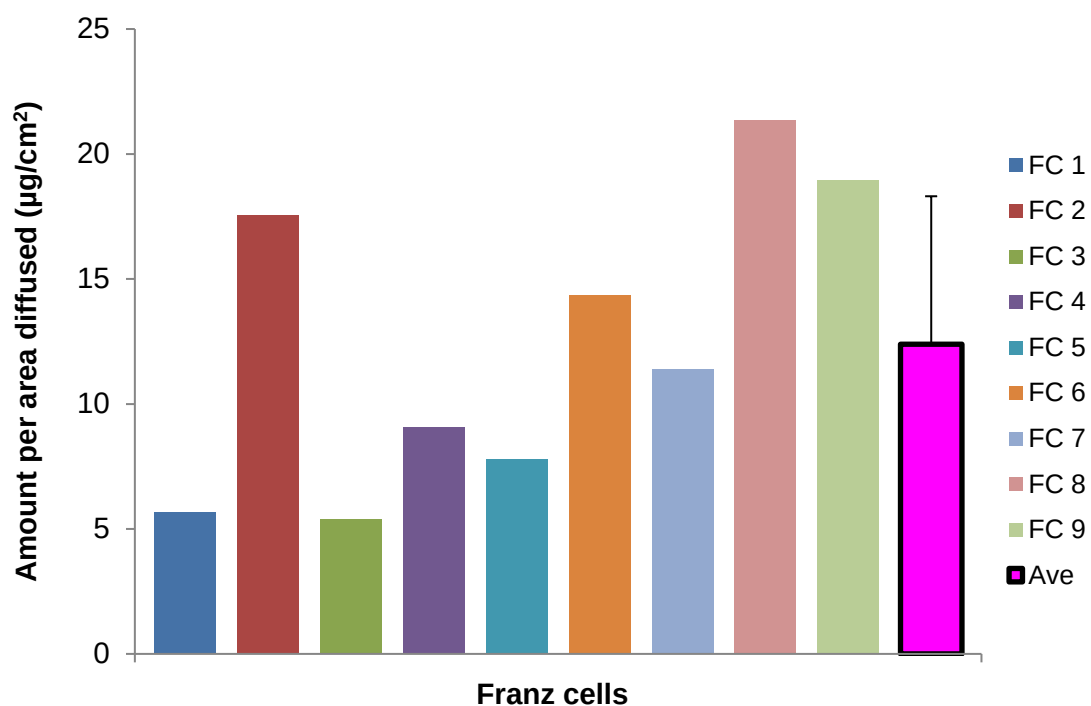


Figure D.24: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEL1** after the 12 h diffusion study (n = 9)

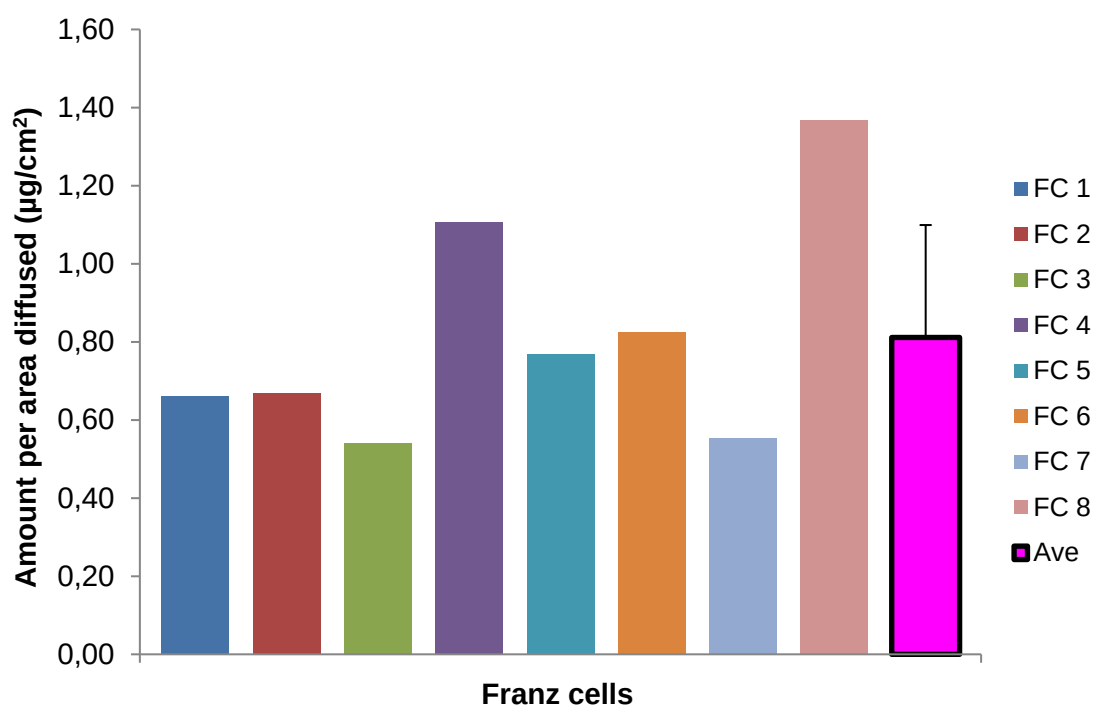


Figure D.25: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of lovastatin from **NEGL** after the 12 h diffusion study (n = 8)

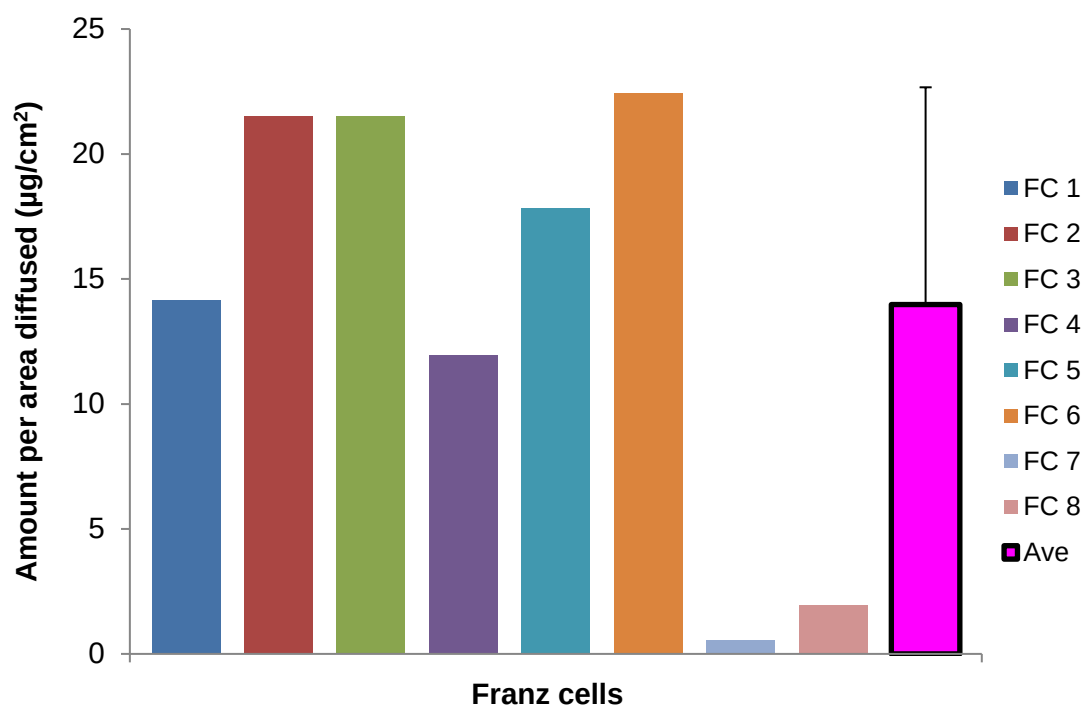


Figure D.26: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NER1** after the 12 h diffusion study (n = 8)

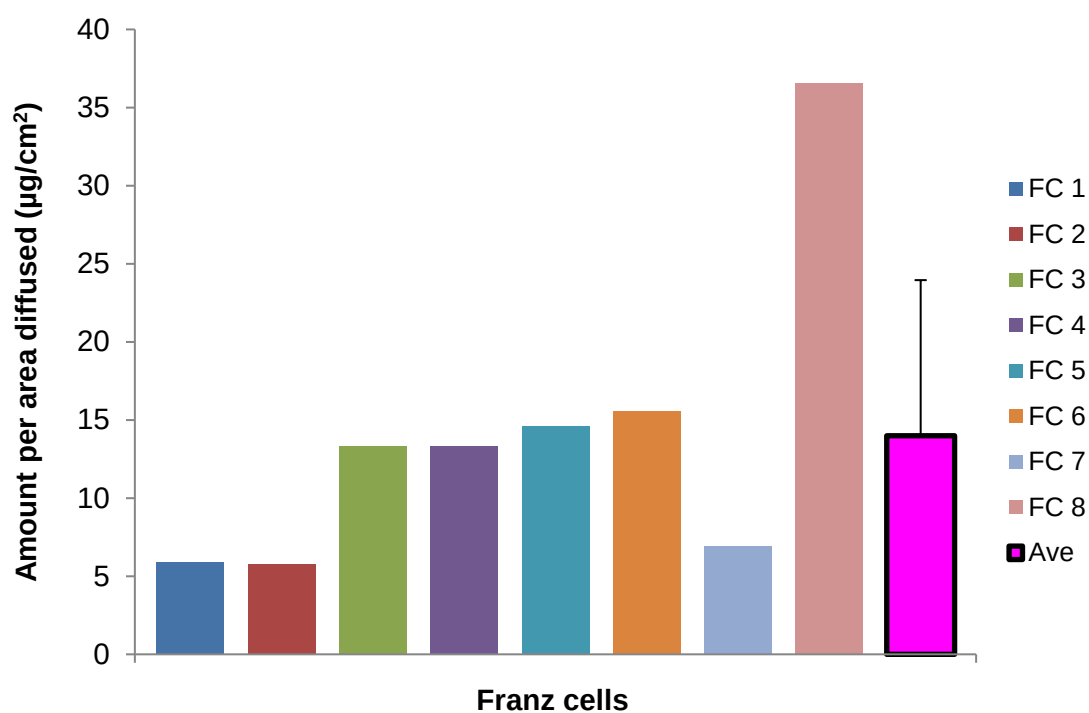


Figure D.27: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of rosuvastatin from **NEGR** after the 12 h diffusion study (n = 8)

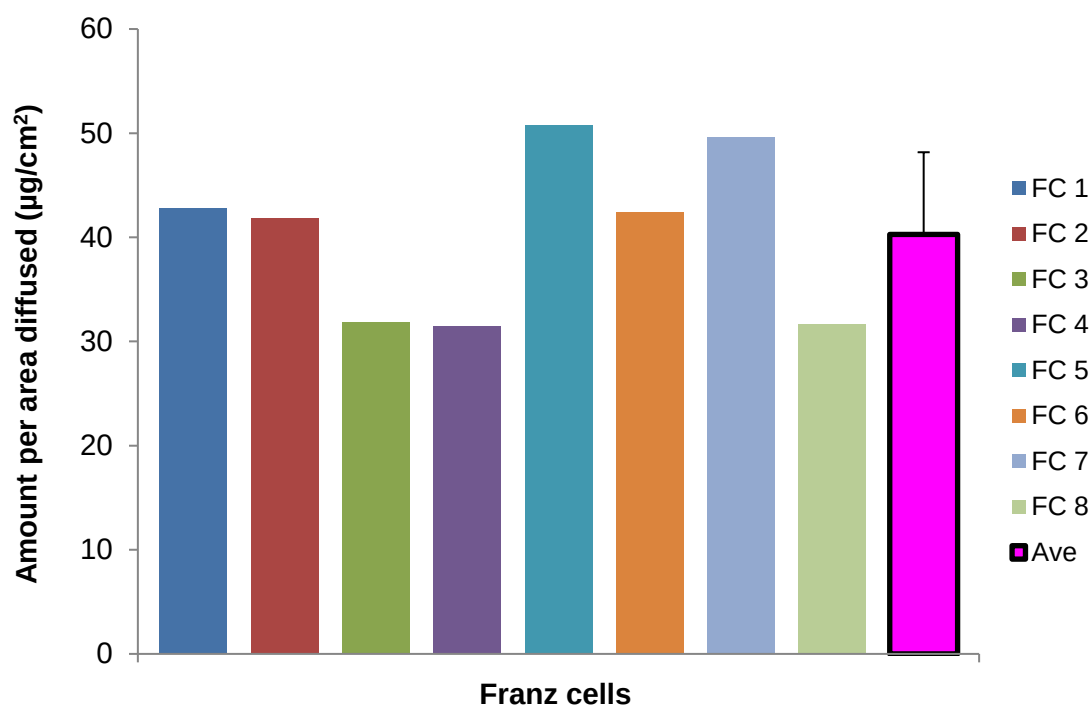


Figure D.28: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NES1** after the 12 h diffusion study ($n = 8$)

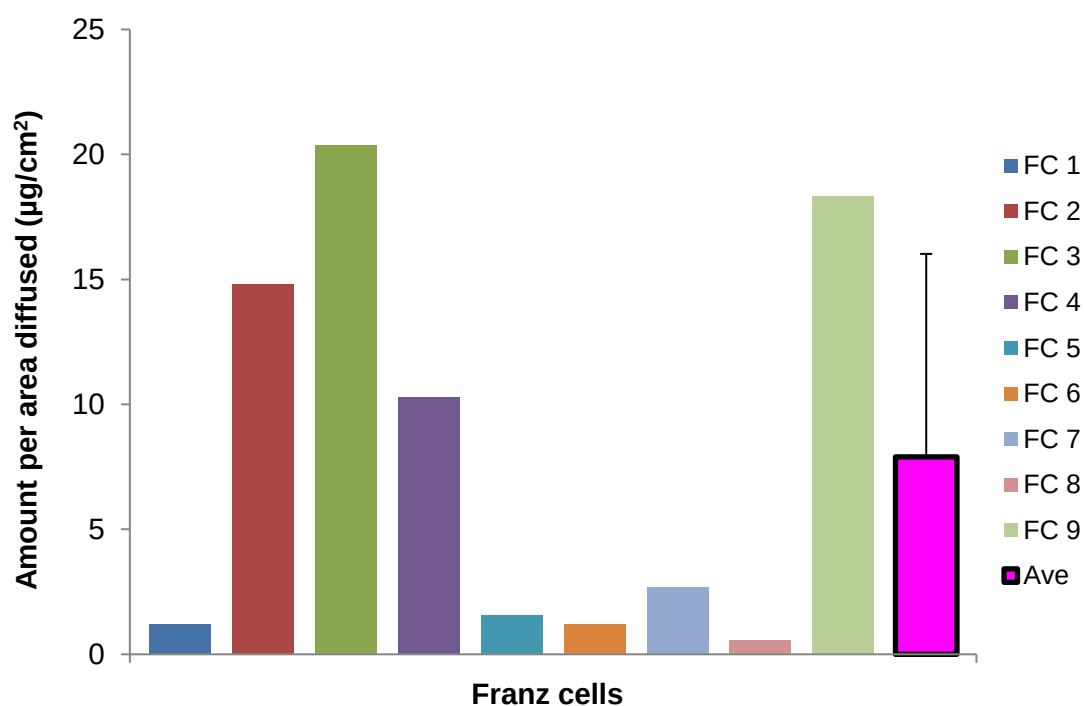


Figure D.29: The amount per area diffused ($\mu\text{g}/\text{cm}^2$) of simvastatin from **NEGS** after the 12 h diffusion study ($n = 9$)

Table D.3: The average percentage diffused (%), average concentration diffused ($\mu\text{g/ml}$), average amount per area diffused ($\mu\text{g/cm}^2$) and the median amount per area diffused ($\mu\text{g/cm}^2$) for the **(NE1)** and the **(NEG)** (n = number of Franz cells used)

Formula	n	Average %diffused	Average concentration diffused ($\mu\text{g/ml}$)	Average amount per area diffused ($\mu\text{g/cm}^2$)	Median amount per area diffused ($\mu\text{g/cm}^2$)
(NEF1)	9	2.74 ± 1.06	551.06 ± 212.95	1024.70 ± 395.97	1082.18
(NEGF)	9	0.04 ± 0.03	62.88 ± 5.97	16.00 ± 11.10	12.19
(NEL1)	9	0.03 ± 0.02	6.66 ± 3.19	12.39 ± 5.92	11.38
(NEGL)	8	0.00 ± 0.00	0.44 ± 0.16	0.81 ± 0.30	0.72
(NER1)	8	0.04 ± 0.02	7.52 ± 4.68	13.98 ± 8.69	15.98
(NEGR)	8	0.04 ± 0.03	7.52 ± 5.36	14.00 ± 10.00	13.32
(NES1)	8	0.11 ± 0.02	21.67 ± 4.24	40.29 ± 7.88	42.12
(NEGS)	9	0.02 ± 0.02	4.25 ± 4.37	7.9 ± 8.124	2.68

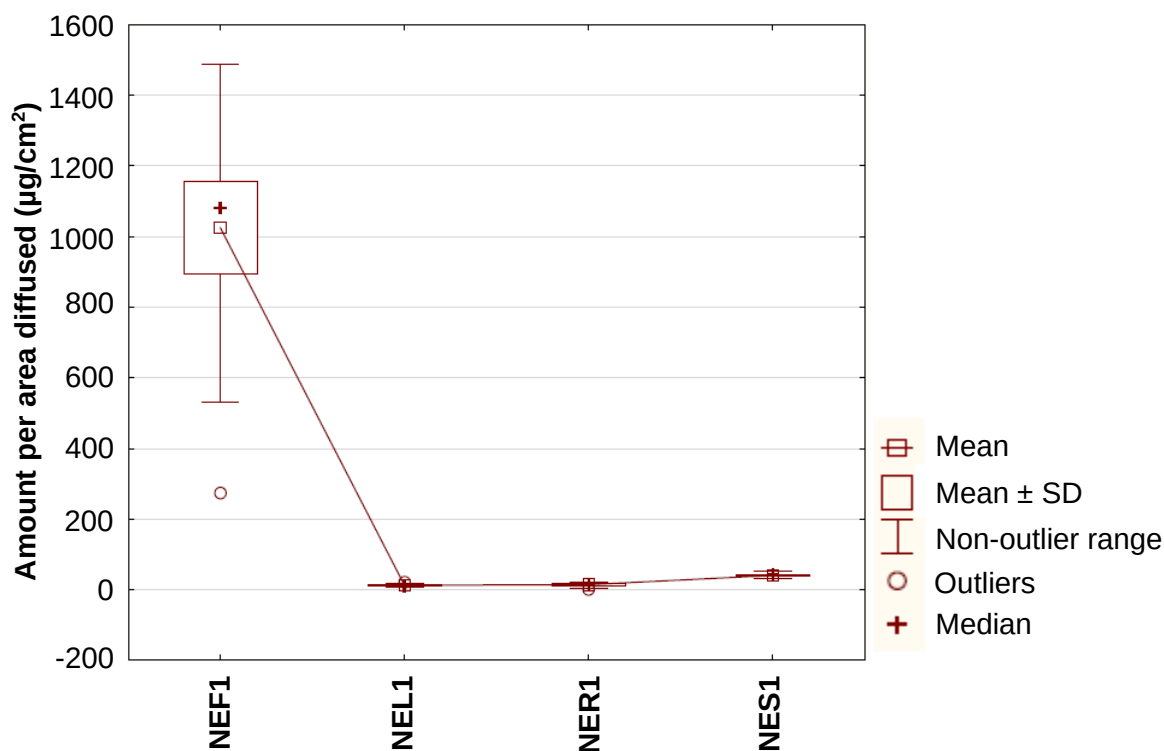


Figure D.30: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g/cm}^2$) for each of the selected statins in the **(NE1)** ((**NEF1**) and (**NEL1**) both had $n = 9$, (**NER1**) and (**NES1**) had $n = 8$ after 12 h

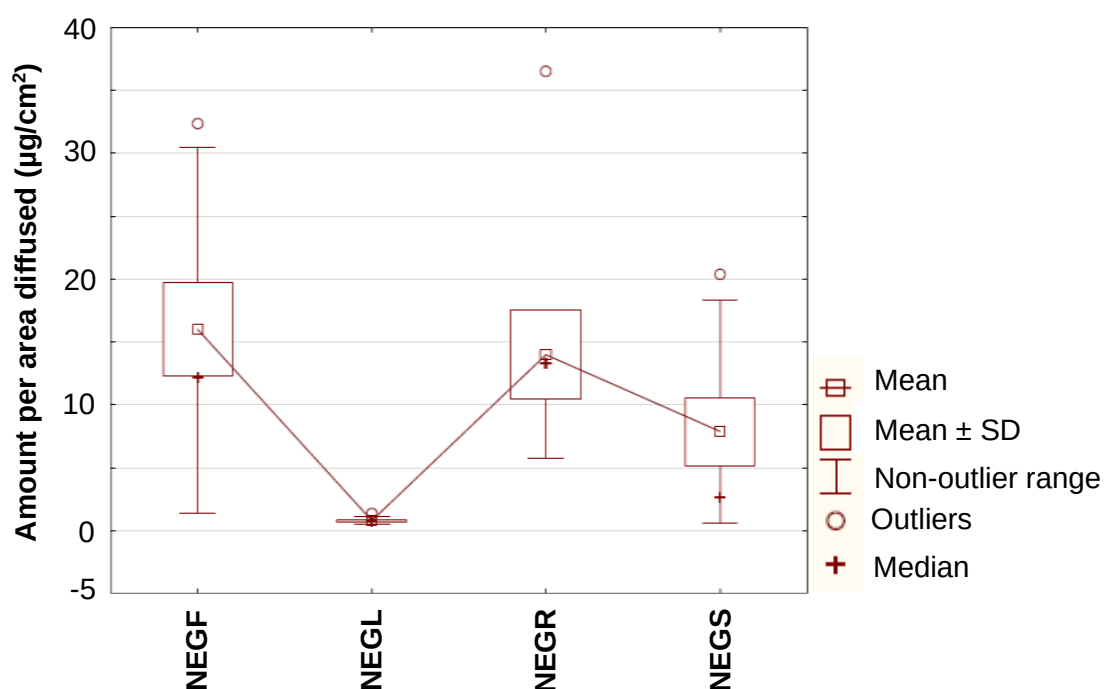


Figure D.31: Box-plot indicating the mean and median amount per area diffused ($\mu\text{g}/\text{cm}^2$) for each of the selected statins in the **(NEG)** (**(NEGL)** and **(NEGR)** both had $n = 8$, **(NEGF)** and **(NEGS)** had $n = 9$ after 12 h

During the HPLC analysis (Section A.3.7), the LOD and LOQ for all the statins were determined. It can be stated that all the statins delivered transdermally, fell in the LOD and LOQ ranges, except for **(NEGL)** which had an average concentration diffused of $0.44 \pm 0.16 \mu\text{g}/\text{ml}$. Although the average concentration **(NEGL)** diffused was higher than the LOD ($0.15 \mu\text{g}/\text{ml}$) value for lovastatin, it was lower than the LOQ value ($0.62 \mu\text{g}/\text{ml}$); nonetheless, the diffusion results for lovastatin were still quantified. **(NEL1)** had an average concentration diffused of $6.66 \pm 3.19 \mu\text{g}/\text{ml}$, which was higher than both the LOD and LOQ for lovastatin.

During this study, the median amount per area diffused will be discussed, since the average amount per area diffused are affected by outliers (Driscoll *et al.*, 2000:275). When the results of the **(NE1)** were compared, **(NEF1)** presented with the highest median amount per area diffused ($1082.18 \mu\text{g}/\text{cm}^2$), followed by **(NES1)** ($42.12 \mu\text{g}/\text{cm}^2$), **(NER1)** ($15.98 \mu\text{g}/\text{cm}^2$) and lastly, **(NEL1)** ($11.38 \mu\text{g}/\text{cm}^2$). The **(NEG)** had different results than those of the **(NE1)** as observed from Table D.3. **(NEGR)** obtained the highest median amount per area diffused with a value of $13.32 \mu\text{g}/\text{cm}^2$, followed by **(NEGF)** ($12.19 \mu\text{g}/\text{cm}^2$), **(NEGS)** ($2.68 \mu\text{g}/\text{cm}^2$), and lastly **(NEGL)** ($0.72 \mu\text{g}/\text{cm}^2$). When comparing the results of **(NE1)** and **(NEG)**, it was evident that **(NE1)** had higher median amounts per area diffused than the **(NEG)** counterpart, which can be because the diffusion of the nano-emulgels were more controlled (Gopalasatheeskumar *et al.*, 2017:240).

When comparing the different statins in the formulas ((**NE1**) and (**NEG**)), it was evident that the log D value of fluvastatin (1.36), like simvastatin (1.80) (Marais, 2018:245; Maree, 2018:232) was more ideal (log D between 1 and 3; Williams, 2003:36) than that of rosuvastatin (4.50) and lovastatin (-0.20) (Marais, 2018:245). The aqueous solubility of fluvastatin (0.53 mg/ml) was higher than the other statins (simvastatin (0.24 mg/ml), rosuvastatin (0.31 mg/ml) and lovastatin (0.00 mg/ml)) (Marais, 2018:245; Maree, 2018:233), and although the aqueous solubility values of the statins were not ideal (1 mg/ml or more; Williams, 2003:36), it was expected that fluvastatin would diffuse better than simvastatin and the other statins (due to its physicochemical properties) and that lovastatin would have the lowest diffusion of all the statins. Hence, fluvastatin diffused better than simvastatin, and lovastatin had the lowest diffusion of all the statins for both the (**NE1**) and the (**NEG**). It should be noted that rosuvastatin had the highest median amount per area diffused of all the (**NEG**), but also that the median amount diffused of rosuvastatin (13.32 $\mu\text{g}/\text{cm}^2$) did not differ greatly from that of fluvastatin (12.19 $\mu\text{g}/\text{cm}^2$) in the (**NEG**).

D.3.3 Tape stripping

The tape stripping technique was applied as discussed in Section D.2.3.4.4. Table D.4 gives a representation of the average and median concentration ($\mu\text{g}/\text{ml}$) for both the SCE and ED for selected statins in the different formulas. Results are discussed according to median values. Median values give a representative value or centre value of data, where the average values measure the midrange and distribution (Bakker & Gravemeijer, 2004:147).

Table D.4: Average and median concentration ($\mu\text{g}/\text{ml}$) of the selected statins in SCE and ED

Formula	<i>n</i>	Average concentration in the SCE ($\mu\text{g}/\text{ml}$)	Median concentration in the SCE ($\mu\text{g}/\text{ml}$)	Average concentration in the ED ($\mu\text{g}/\text{ml}$)	Median concentration in the ED ($\mu\text{g}/\text{ml}$)
(NEF1)	9	1684.28 $\pm$ 152.14	1744.78	138.63 $\pm$ 135.19	87.72
(NEGF)	9	66.19 $\pm$ 26.43	67.45	2.66 $\pm$ 5.67	0.53
(NEL1)	9	62.52 $\pm$ 4.55	60.76	0.39 $\pm$ 0.08	0.39
(NEGL)	8	104.30 $\pm$ 6.89	104.05	0.24 $\pm$ 0.06	0.24
(NER1)	8	75.05 $\pm$ 8.41	75.31	7.14 $\pm$ 4.33	6.36
(NEGR)	8	119.27 $\pm$ 10.66	119.98	13.46 $\pm$ 12.11	7.01
(NES1)	8	67.34 $\pm$ 9.38	70.08	13.03 $\pm$ 9.52	11.19
(NEGS)	9	81.76 $\pm$ 7.86	80.06	2.32 $\pm$ 1.96	1.70

D.3.3.1 Stratum corneum-epidermis

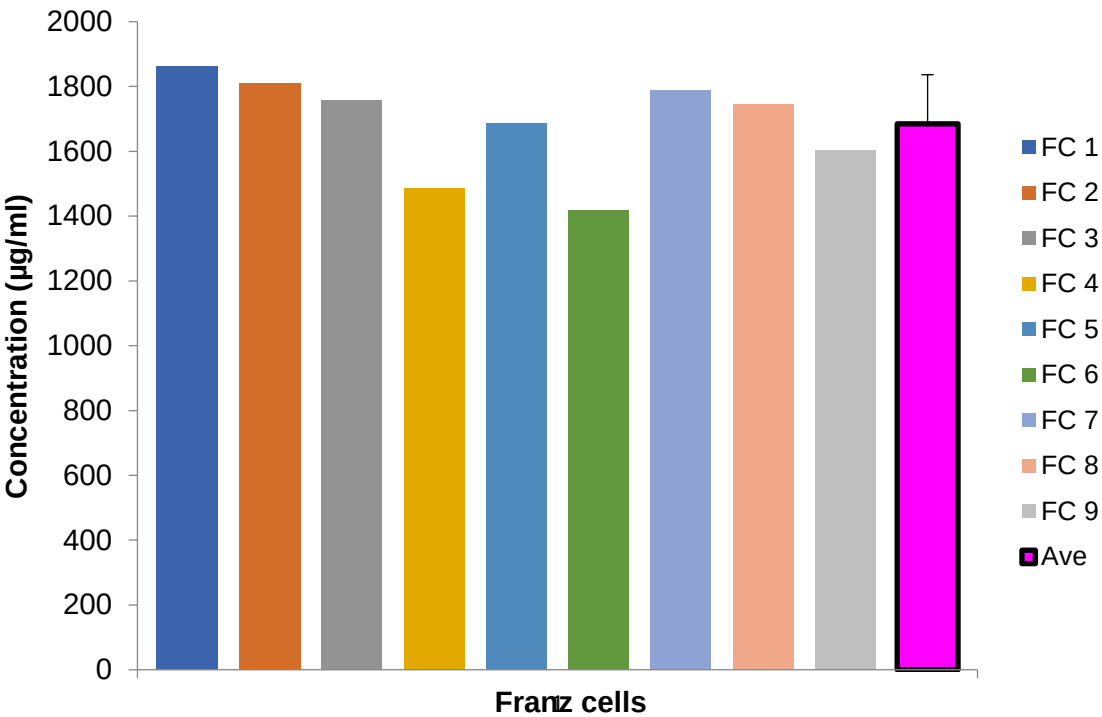


Figure D.32: Fluvastatin concentration (µg/ml) from the **NEF1** present in the SCE (n = 9)

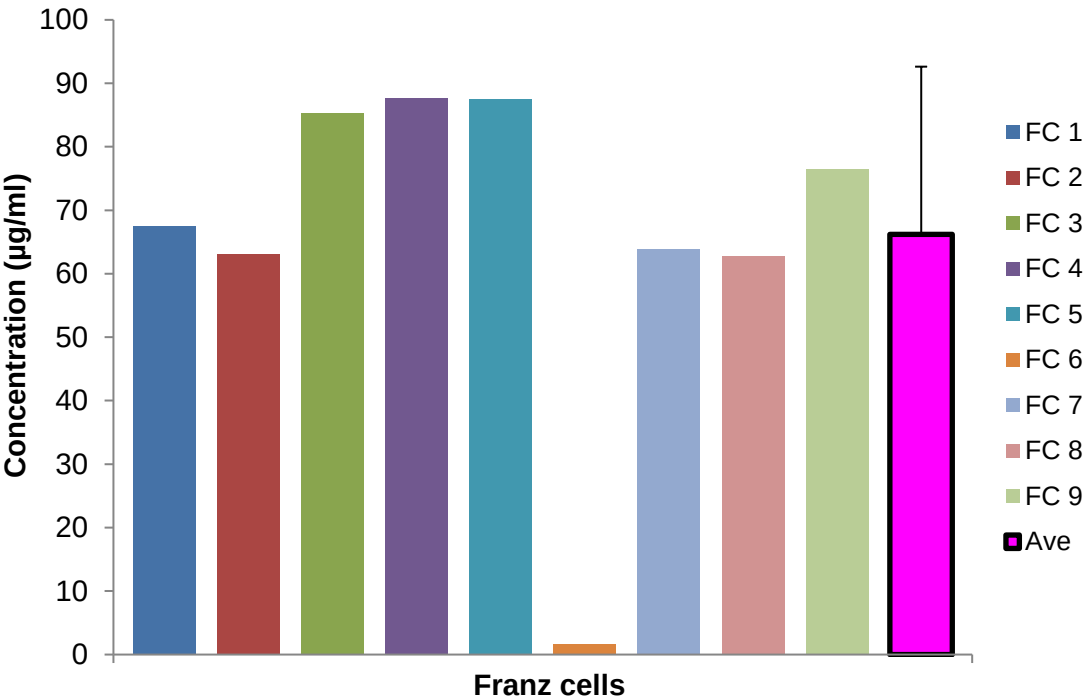


Figure D.33: Fluvastatin concentration (µg/ml) from the **NEGF** present in the SCE (n = 9)

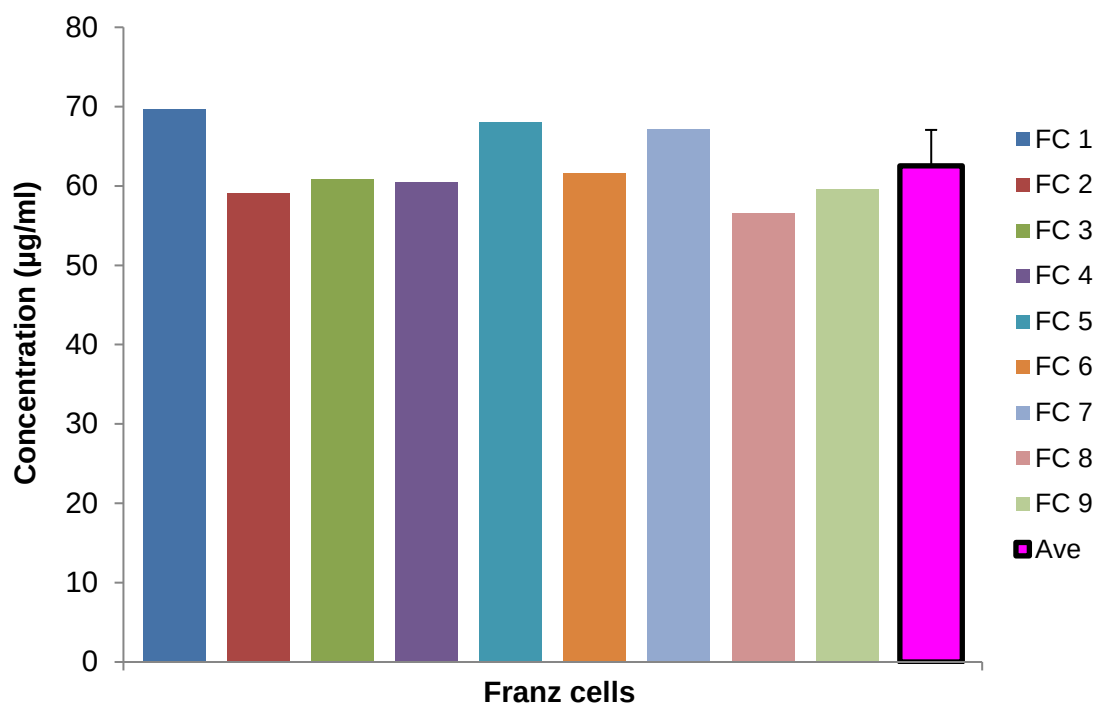


Figure D.34: Lovastatin concentration (µg/ml) from the **NEL1** present in the SCE (n = 9)

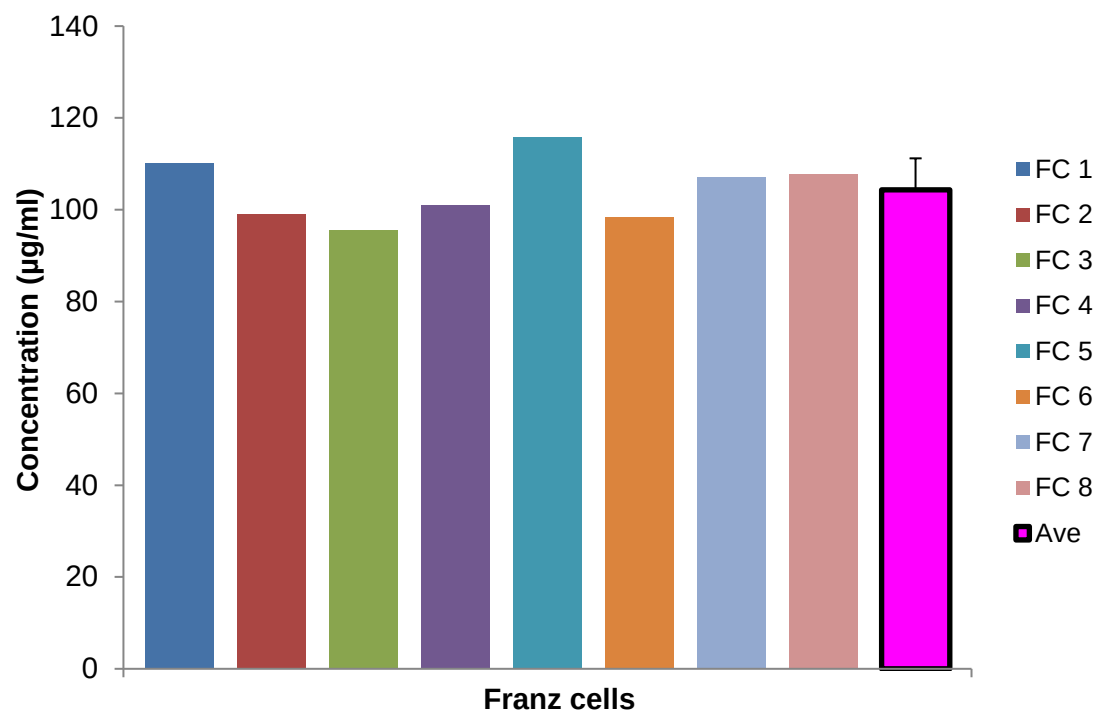


Figure D.35: Lovastatin concentration (µg/ml) from the **NEGL** present in the SCE (n = 8)

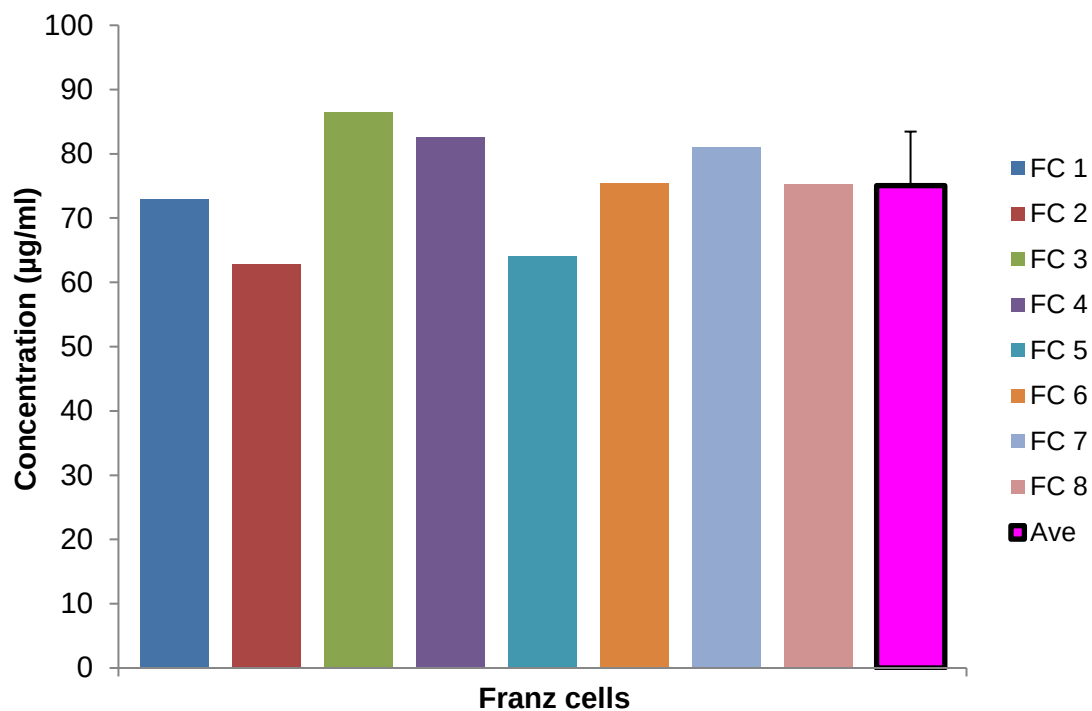


Figure D.36: Rosuvastatin concentration (µg/ml) from the **NER1** present in the SCE (n = 8)

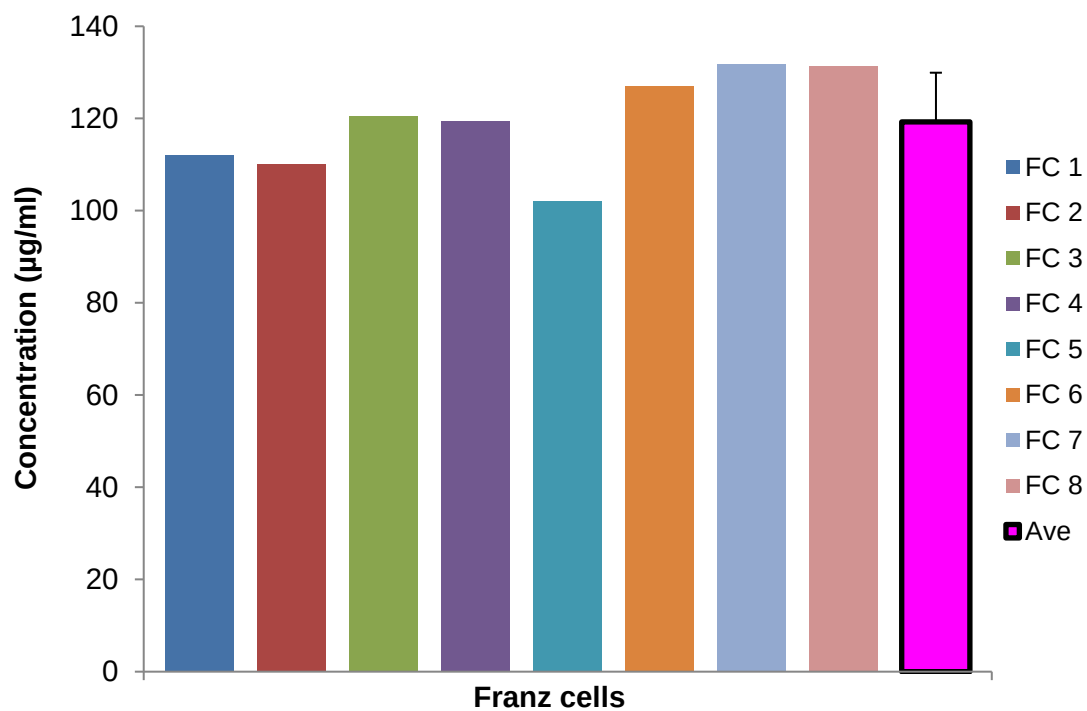


Figure D.37: Rosuvastatin concentration (µg/ml) from the **NEGR** present in the SCE (n = 8)

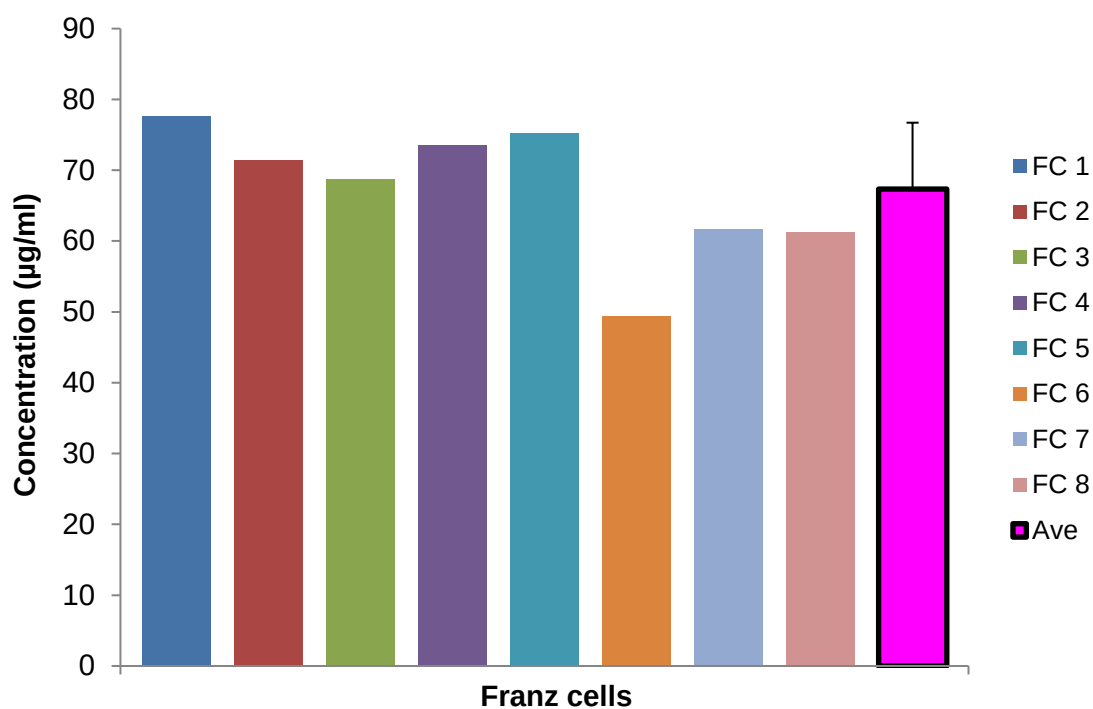


Figure D.38: Simvastatin concentration (µg/ml) from the **NES1** present in the SCE (n = 8)

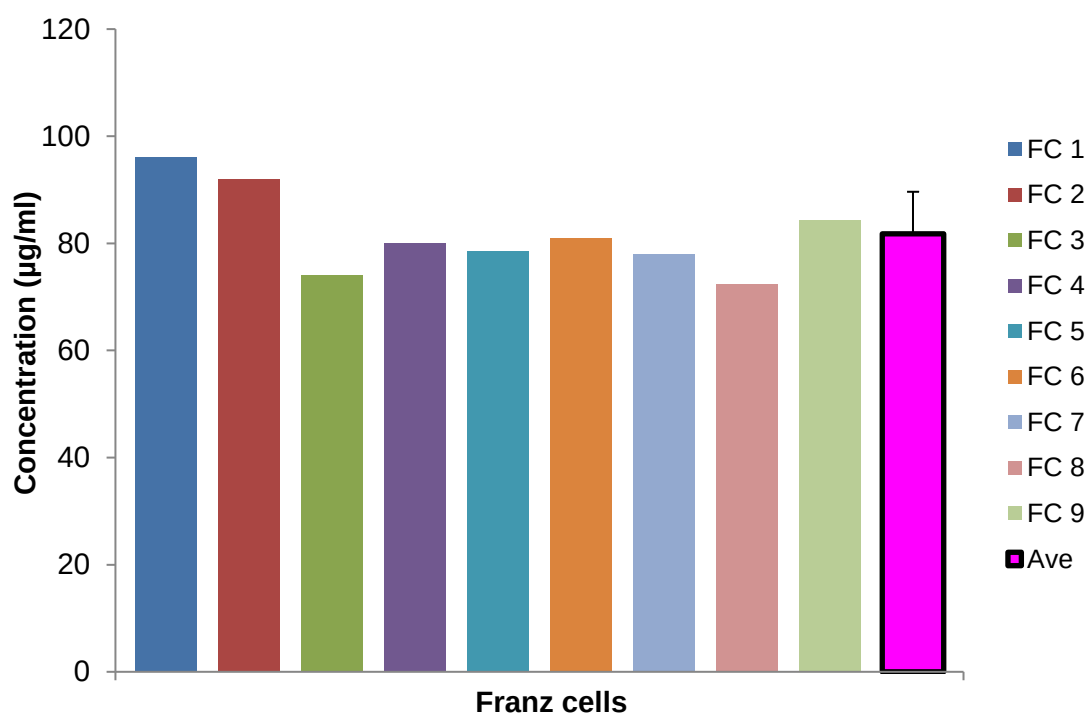


Figure D.39: Simvastatin concentration (µg/ml) from the **NEGS** present in the SCE (n = 9)

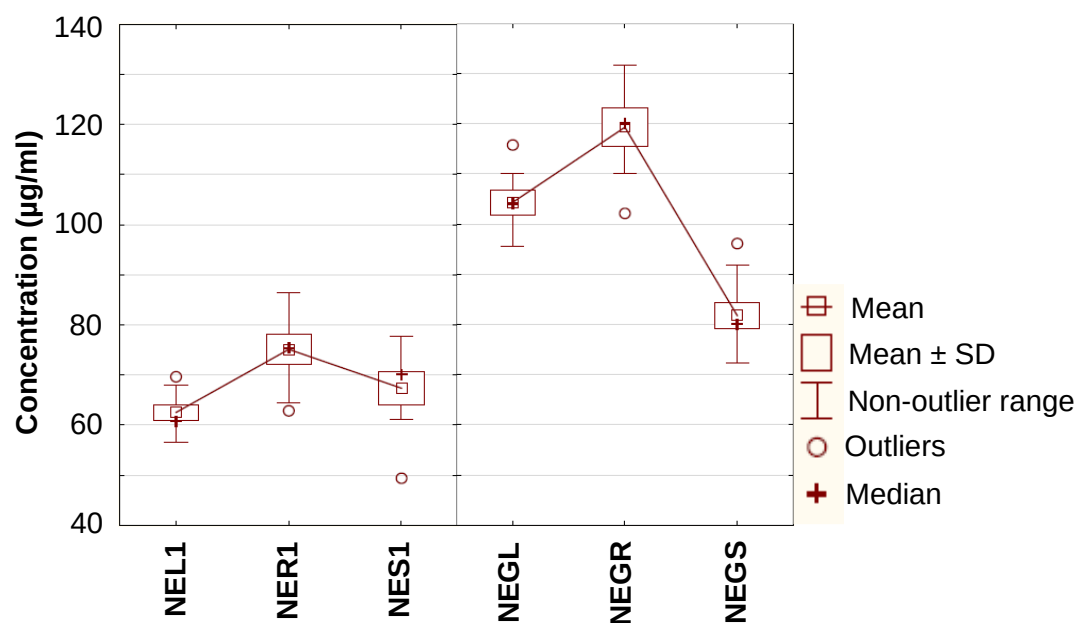


Figure D.40: Box-plots indicating the mean and median concentration (µg/ml) of the **(NE1)** and the **(NEG)** for each of the selected statins (excluding fluvastatin) present in the SCE

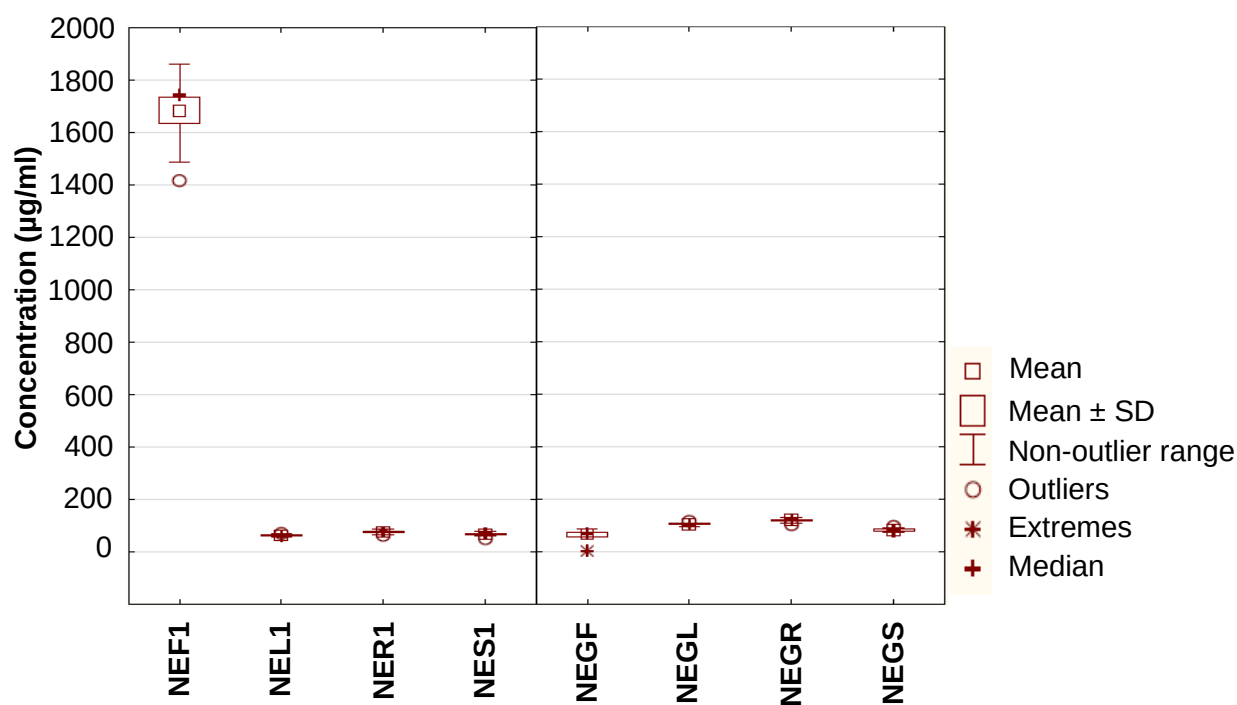


Figure D.41: Box-plots indicating the mean and median concentration (µg/ml) of the **(NE1)** and the **(NEG)** for each of the selected statins (including fluvastatin) present in the SCE

Results were discussed by means of median concentrations, since average concentrations are influenced by outliers (Driscoll *et al.*, 2000:275). After comparing the median concentrations of the different **(NE1)** in the SCE, it was found that **(NEF1)** had the highest median concentration value (1744.78 µg/ml), followed by **(NER1)** (75.31 µg/ml), **(NES1)** (70.08 µg/ml) and lastly, **(NEL1)**

(60.76 µg/ml). A comparison was drawn between the **(NEG)** in the SCE, and **(NEGR)** presented with the highest median concentrations (119.98 µg/ml), followed by **(NEGL)** (104.05 µg/ml), **(NEGS)** (80.06 µg/ml) and lastly, **(NEGF)** (67.45 µg/ml).

In the SCE when the median concentrations of the **(NE1)** and the **(NEG)** were compared, it was evident that the **(NEG)** showed an overall higher median statin concentration than the **(NE1)**, except for **(NEF1)**, which had a higher median concentration than its **(NEG)** counterpart **(NEGF)**. The nano-emulgels showed a higher affinity for delivery through the skin's surface (SCE) for deeper penetration (Chang *et al.*, 2013:41). This can be ascribed to the fact that the gelling agent (Carbopol® Ultrez 20) was incorporated in the **(NEG)** and since it has a film-forming effect on the skin; permeation of the APIs from the **(NEG)** increased and penetration was more controlled (Kathe & Kathpalia, 2017:491).

When comparing the different statins in the formulas (**(NE1)** and **(NEG)**), it should be kept in mind that nano-emulsions are more hydrophilic, while nano-emulgels are more lipophilic in nature (Katteboina *et al.*, 2009:241; Rangsimawong *et al.*, 2014:18). Therefore, it could be presumed that lovastatin (the more lipophilic statin) in the **(NEG)** would deliver the highest concentration in the lipophilic SCE, but this was not the trend observed. Instead, rosuvastatin obtained the highest concentration in the SCE for **(NEG)**; although the concentration difference between rosuvastatin and lovastatin did not differ greatly. It was also anticipated that the delivery of fluvastatin (due to its more ideal physicochemical properties than the other statins) would be good; this was true for the **(NE1)**. It can be stated that the delivery of fluvastatin from **(NEGF)** may have been enhanced by the nano-emulgel, which may have improved permeation through the SCE to the more hydrophilic environment, such as the ED and the receptor.

D.3.3.2 Epidermis dermis

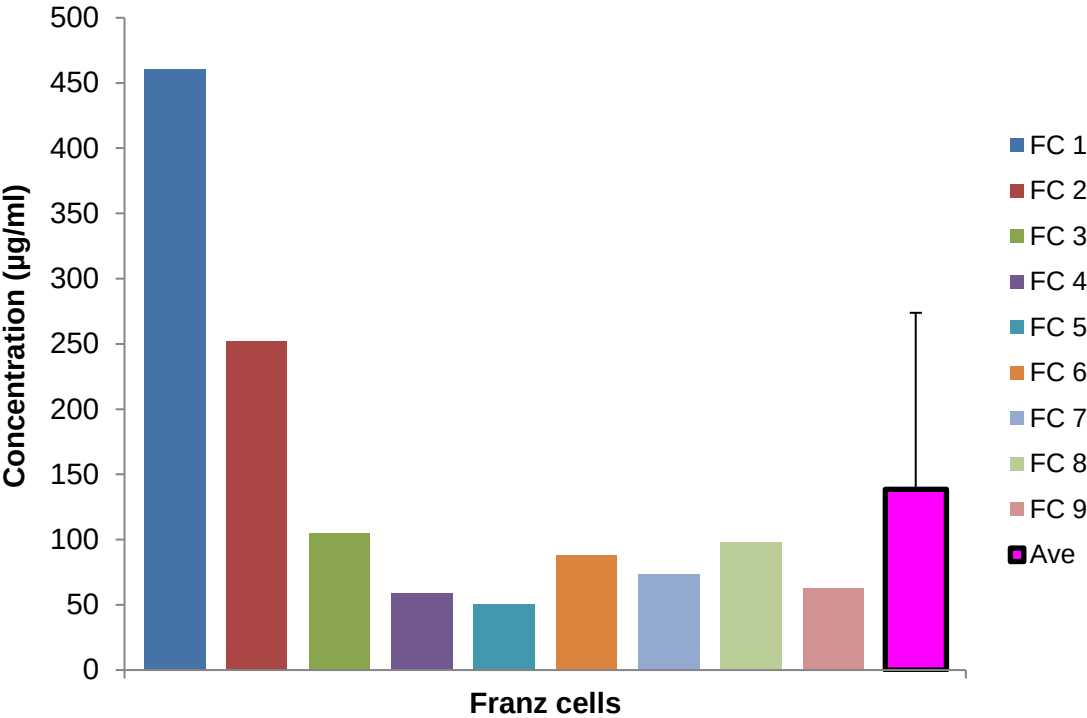


Figure D.42: Fluvastatin concentration (µg/ml) from the **NEF1** present in the ED (n = 9)

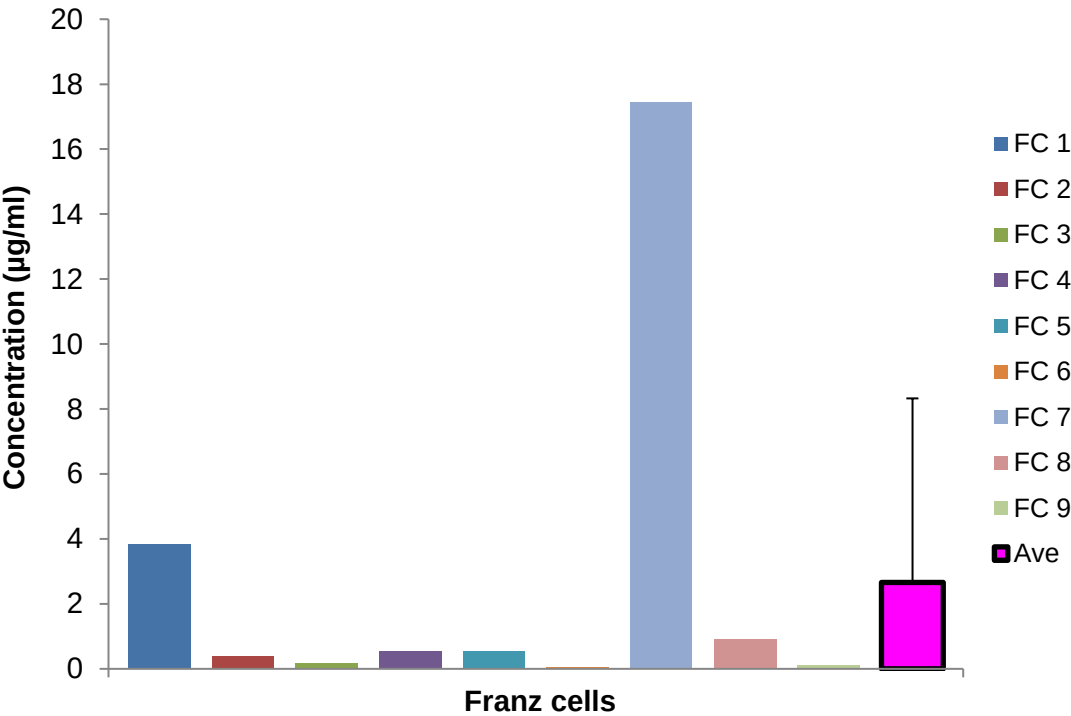


Figure D.43: Fluvastatin concentration (µg/ml) from the **NEGF** present in the ED (n = 9)

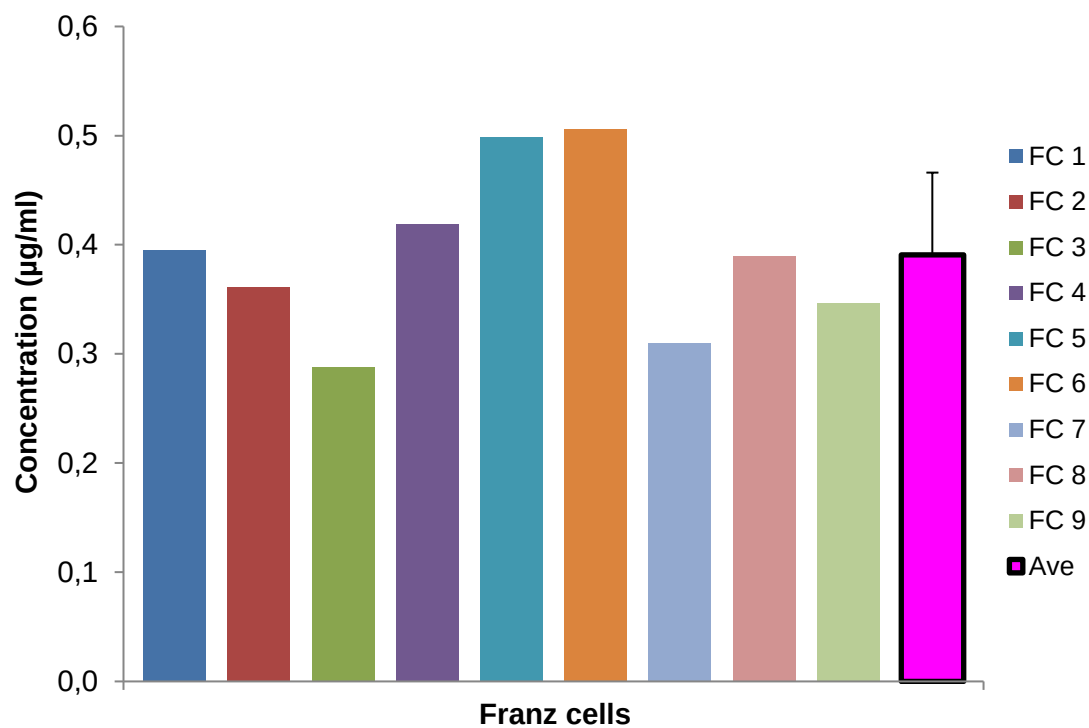


Figure D.44: Lovastatin concentration (µg/ml) from the **NEL1** present in the ED (n = 9)

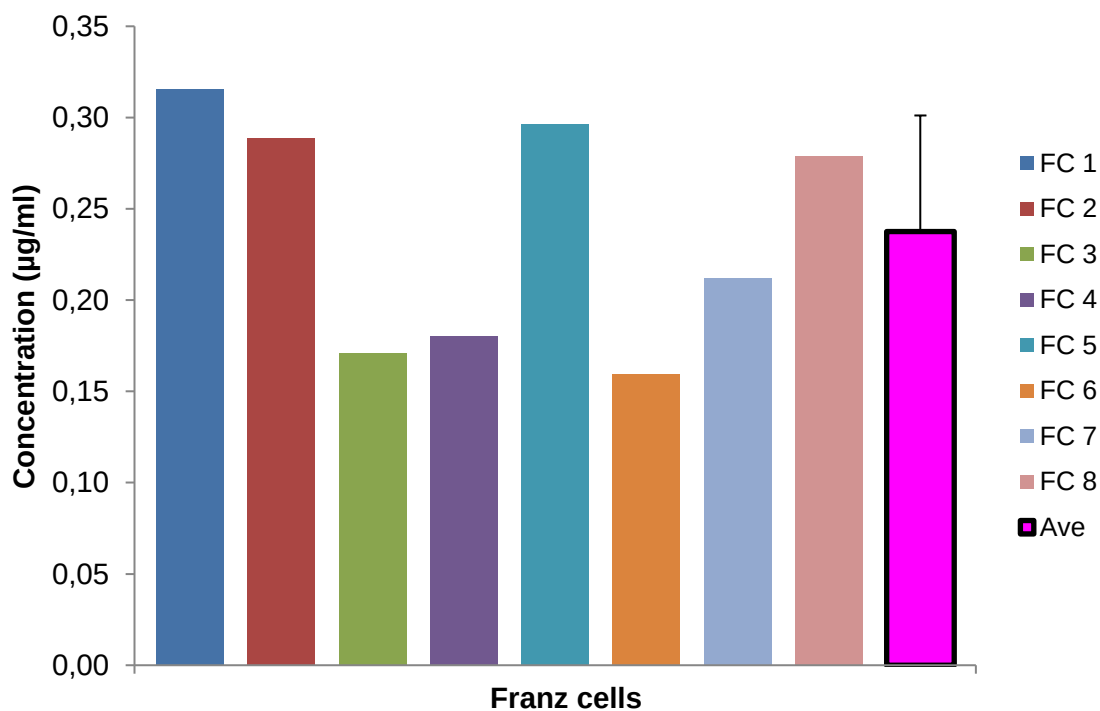


Figure D.45: Lovastatin concentration (µg/ml) from the **NEGL** present in the ED (n = 8)

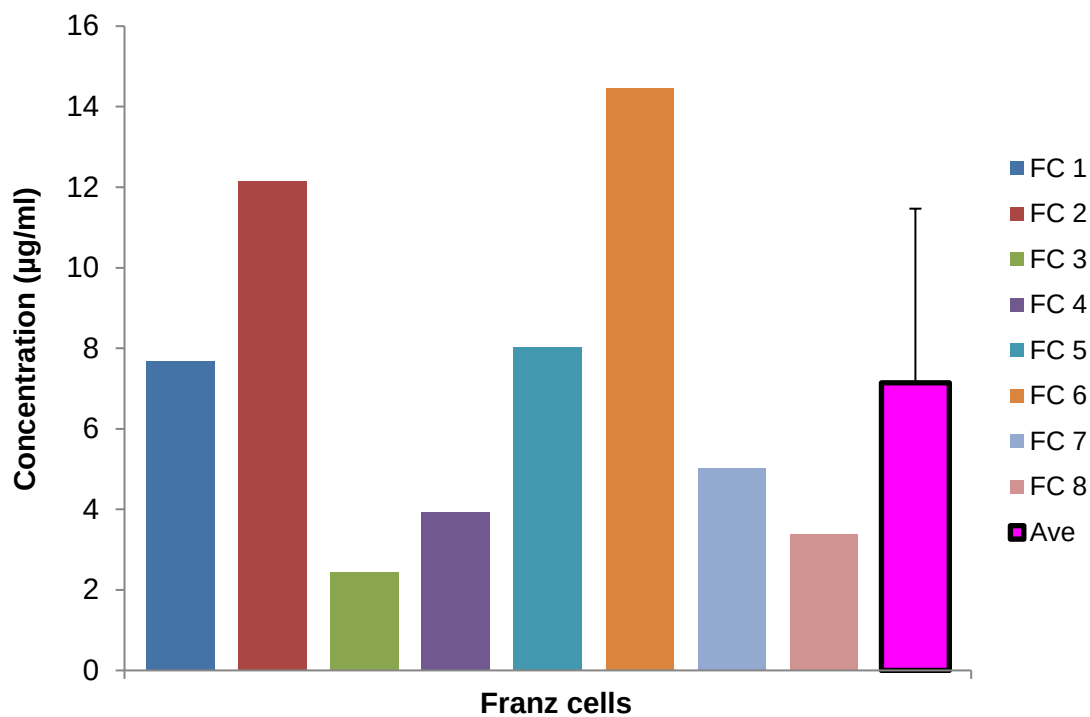


Figure D.46: Rosuvastatin concentration (µg/ml) from the **NER1** present in the ED (n = 8)

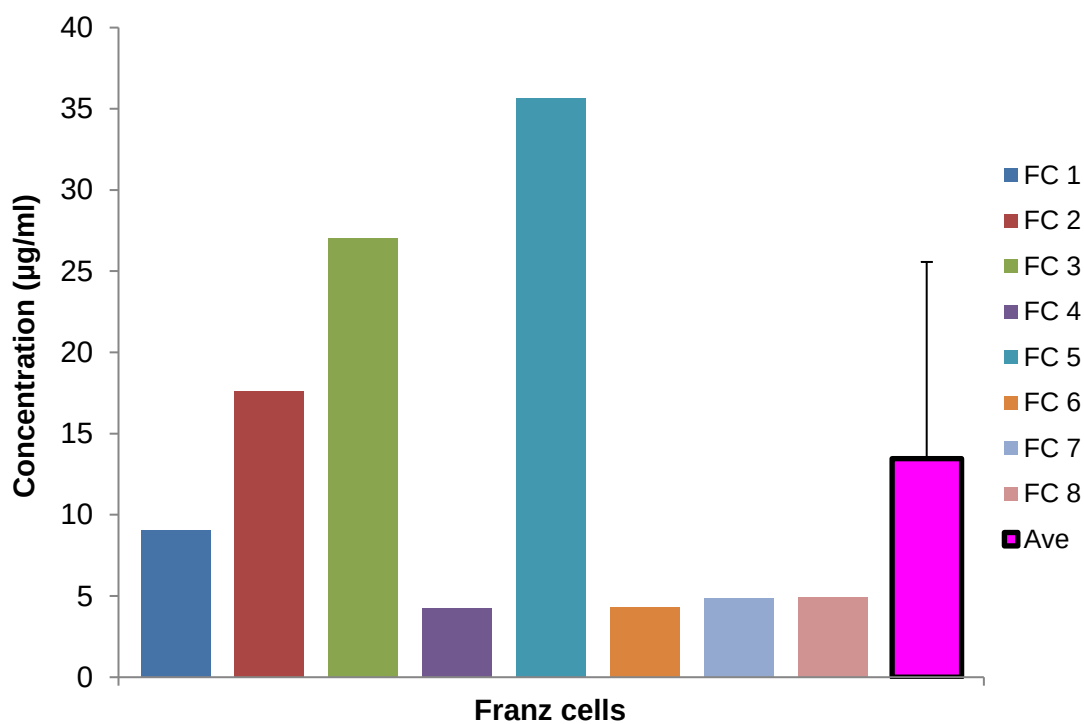


Figure D.47: Rosuvastatin concentration (µg/ml) from the **NEGR** present in the ED (n = 8)

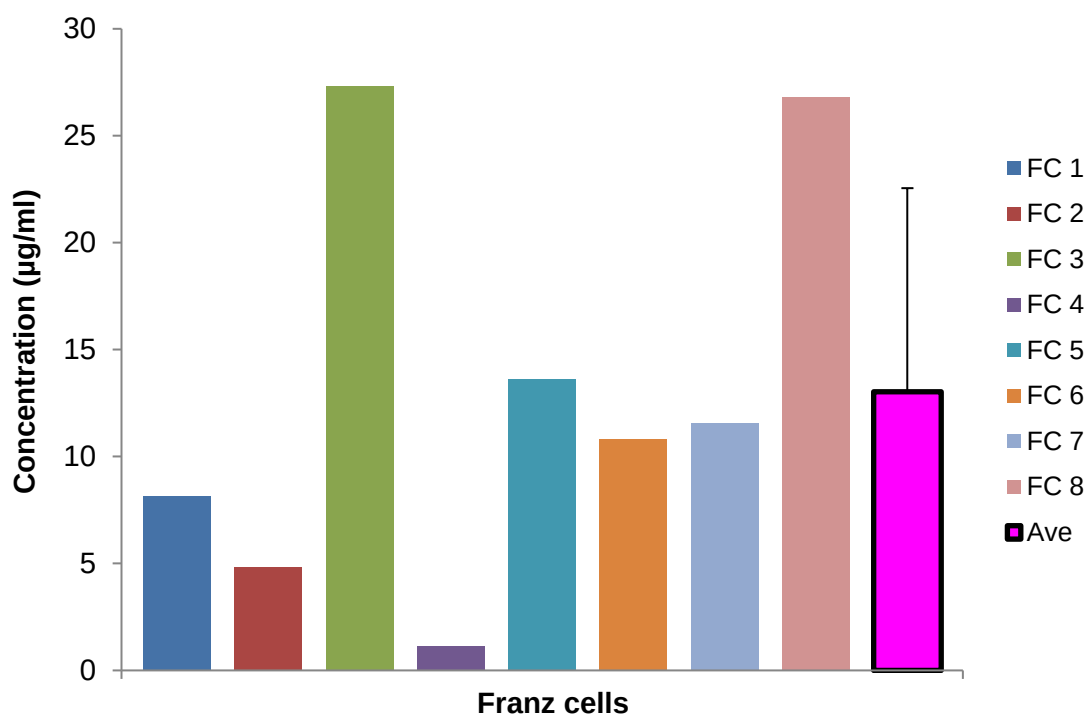


Figure D.48: Simvastatin concentration (µg/ml) from the **NES1** present in the ED (n = 8)

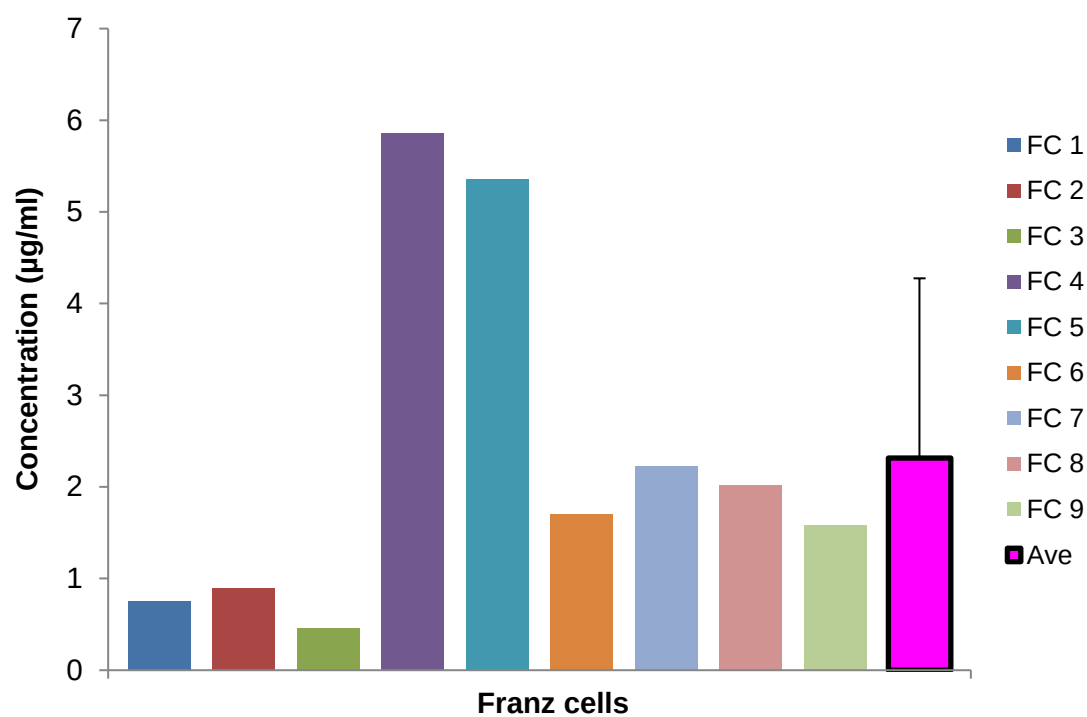


Figure D.49: Simvastatin concentration (µg/ml) from the **NEGS** present in the ED (n = 9)

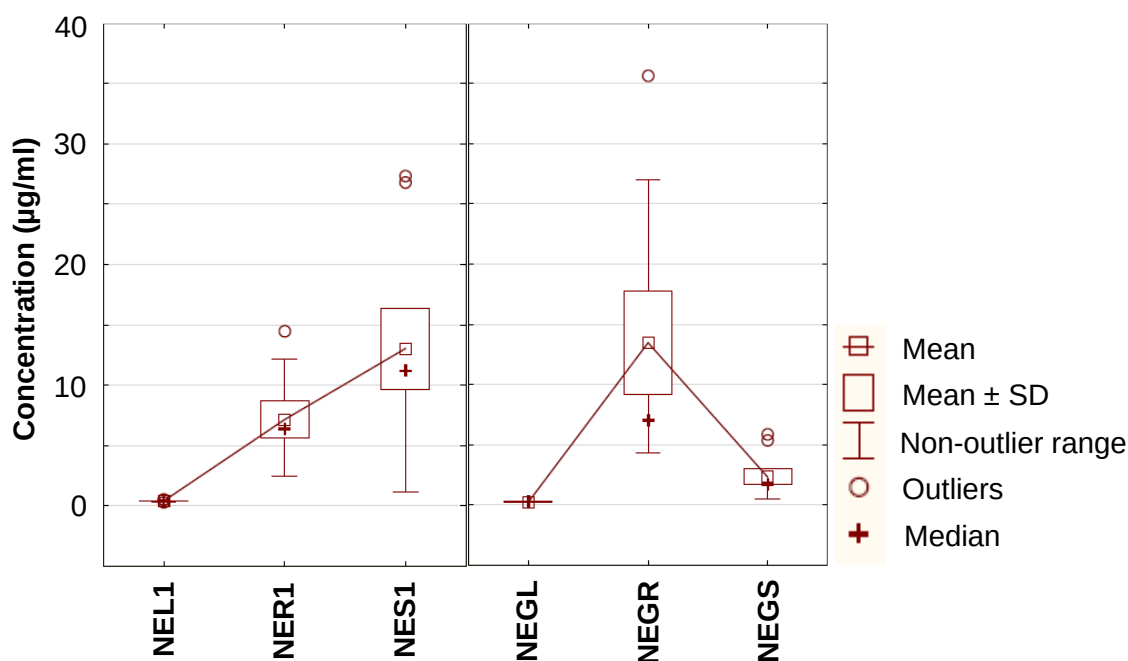


Figure D.50: Box-plots indicating the mean and median concentration (µg/ml) of the **(NE1)** and the **(NEG)** for each of the selected statins (excluding fluvastatin) present in the ED

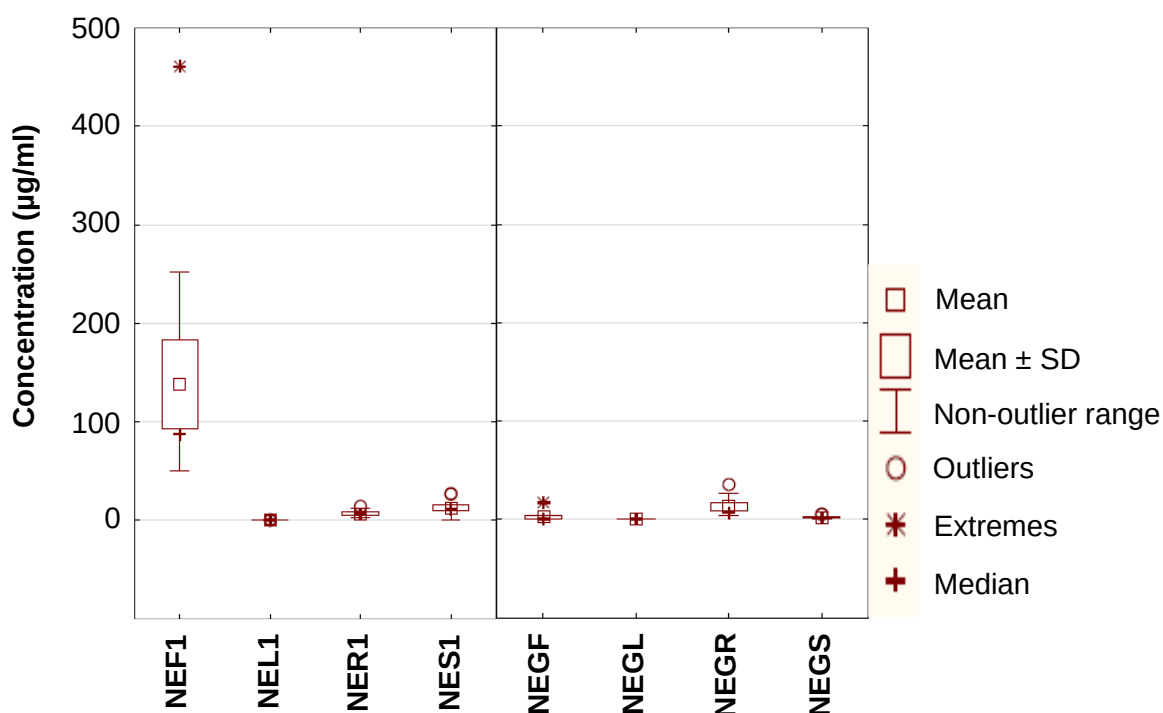


Figure D.51: Box-plots indicating the mean and median concentration (µg/ml) of the **(NE1)** and the **(NEG)** for each of the selected statins (including fluvastatin) present in the ED

Results were discussed by means of median concentrations, since average concentrations are influenced by outliers (Driscoll *et al.*, 2000:275). After the median concentrations were compared of the different **(NE1)** in the ED, it was found that **(NEF1)** had the highest median concentration value

(87.72 µg/ml), followed by **(NES1)** (11.19 µg/ml), **(NER1)** (6.36 µg/ml) and lastly, **(NEL1)** (0.39 µg/ml). A comparison was drawn between the **(NEG)** in the ED, and **(NEGR)** presented with the highest median concentrations (7.01 µg/ml), followed by **(NEGS)** (1.70 µg/ml), **(NEGF)** (0.53 µg/ml) and lastly, **(NEGL)** (0.24 µg/ml).

When comparing the **(NE1)** with the **(NEG)**, it was evident that the **(NE1)**, in general, obtained higher median statin concentrations than the **(NEG)** counterparts, except for **(NEGR)** that obtained a slightly higher median concentration for rosuvastatin than **(NER1)**. The **(NEG)** contained Carbopol® Ultrez 20 as a gelling agent; this gelling agent increases the particle size of the formulation, resulting in a lower median concentration for **(NEG)** (Shah *et al.*, 2012:1609). These results correlate with literature stating that the small droplet size of the nano-emulsions makes it easier for drug absorption and targeting (Sutradhar & Amin, 2013:97).

When the statins in the different formulas (**(NE1)** and **(NEG)**) were compared, it was expected that fluvastatin would obtain the highest median concentration in the ED, due to its more ideal physicochemical properties (log D (1.36) and aqueous solubility (0.53 mg/ml)) (Marais, 2018:200; Maree, 2018:232) compared to the other statins. This was observed for the **(NE1)**, but not for the **(NEG)**. The aforementioned might be due to the nano-emulsions being more hydrophilic than the nano-emulgels (Sharma & Sarangdevot, 2012:413), and also that nano-emulsions (being more hydrophilic) consequently targeted the more hydrophilic layers (Rangsimawong *et al.*, 2014:18). It was also anticipated that lovastatin would have the lowest permeation into the ED, due to its lipophilic log D value (4.50) and the hydrophilic environment of the ED (Rastogi & Singh, 2001:76). The aforementioned was observed for both the **(NE1)** and the **(NEG)**.

D.4 Statistical analysis

D.4.1 Statistical analysis of membrane release studies

After studies were performed, data analysis was done on results obtained. Firstly, membrane release studies were statistically analysed by means of an ANOVA, t-test for independent samples and Levene's test for homogeneity. The two-way ANOVA tests were performed on both the **(NE1)** and the **(NEG)**, and for both, the p-values were < 0.05. During this study, a p-value of < 0.001 was observed, indicating that the membrane release results were statistically significant. After the two-way ANOVA, a one-way ANOVA was performed for each of the statins formulated in the **(NE1)** and the **(NEG)** to obtain differences and compare them with one another.

Table D.5: Different p-values of one-way ANOVA for different formulations

Study	Comparison	p-Value
Membrane release	Statins for (NE1)	< 0.001
	Statins for (NEG)	< 0.001

Tukey's HSD test was performed for differences between the different statins, where averages between them were calculated. It can be observed from Tables D.6 and D.7 that it was suggested that simvastatin and lovastatin were homogenously grouped together, followed by rosuvastatin and fluvastatin, both of which had no similarities to the **(NE1)**. With the inspection of **(NEG)**, it was observed that no statin shared similar properties and was grouped individually.

Table D.6: Tukey's HSD-test performed for membrane release studies on the **(NE1)**

Nano-emulsions	Mean log flux	Statin order
(NEF1)	582.583	NES1^a
(NEL1)	10.502	NEL1^a
(NER1)	398.599	NER1^b
(NES1)	8.442	NEF1^c

Groups (a, b, c) as per homogeneity for each of the selected statins

Table D.7: Tukey's HSD-test performed for membrane release studies on the **(NEG)**

Nano-emulsions	Mean log flux	Statin order
(NEGF)	204.966	NEGL^a
(NEGL)	10.555	NEGS^b
(NEGR)	452.898	NEGF^c
(NEGS)	135.322	NEGR^d

Groups (a, b, c) as per homogeneity for each of the selected statins in nano-emulgel formulation

D.4.2 Statistical analysis of the *in vitro* skin diffusion studies

The statistical analysis was done for the *in vitro* skin diffusion studies by performing an ANOVA test by means of the two-way ANOVA for different formulas of **(NE1)** and **(NEG)**. The Kruskal-Wallis ANOVA (one-way ANOVA) was performed for the different ranks in the different formulations of each statin. Kruskal-Wallis method is the best method for data that are non-normal, and has an advantage in heterogeneity with the assumption it can meet the normality assumption on the one-way analysis with the ANOVA test (McDonald *et al.*, 2014:158).

Table D.8: p-Values obtained on different formulas for the *in vitro* skin diffusion studies

Study	Comparison	p-Value
<i>In vitro</i> skin diffusion studies	Statins for (NE1)	< 0.001
	Statins for (NEG)	< 0.001

Table D.9: Kruskal-Wallis ANOVA-test performed for the *in vitro* skin studies on the **(NE1)**

Nano-emulsions	Sum of ranks	Mean rank	Statin order
(NEF1)	270.000	30.000	NEL1
(NEL1)	73.000	8.111	NER1
(NER1)	80.000	10.000	NES1
(NES1)	172.000	21.500	NEF1

Table D.10: Kruskal-Wallis ANOVA-test performed for the *in vitro* skin studies on the **(NEG)**

Nano-emulsions	Sum of ranks	Mean rank	Statin order
(NEGF)	215.000	23.888	NEGL
(NEGL)	44.000	5.500	NEGS
(NEGR)	184.000	23.000	NEGR
(NEGS)	152.000	16.888	NEGF

From Table D.9, it can be observed that **(NEL1)** had the highest cumulative concentration, whereas **(NEF1)** had the lowest when the **(NE1)** were compared. According to Table D.10, it can be said that **(NEGL)** had the highest cumulative concentration in contrast to **(NEGF)** that had the lowest when the **(NEG)** were compared. It can be observed that the ranking of the **(NE1)** differed from that of the **(NEG)**, where the second and third positions of the statins were different from one another.

D.4.3 Statistical analysis of tape stripping

A one-way ANOVA was done on the interaction between the formula type, statin and layers of the skin and $p < 0.05$ values were obtained, which indicated statistical significance, this was done to determine what the effects of each of the four statins in different formulations were when compared to each other and the different layers of the skin during tape stripping.

Table D.11: The p-values for the different formulas in the one-way ANOVA and the accommodating skin layer during tape stripping

Combination	p-Value
(NE1) for SCE	< 0.001
(NE1) for ED	0.005
(NEG) for SCE	< 0.000
(NEG) for ED	<0.001

From Table D.11, it can be seen that both **(NE1)** and **(NEG)** in SCE and ED obtained p-values of statistical significance. After the one-way ANOVA was performed the Levene's test for homogeneity was performed on each statin to determine the difference between the four statins.

Table D.12: Unequal HSD-test performed on the **(NE1)** for SCE

(NE1)	Mean concentration
(NEF1)	1684.281
(NEL1)	62.518
(NER1)	75.107
(NES1)	67.341

It can be observed from Table D.12 that **(NEF1)** had the highest mean concentration and **(NEL1)** had the lowest value of mean concentration out of the four statins tested by the Unequal HSD-test on the SCE skin layers.

Table D.13: Levene's test performed on the **(NE1)** for ED

Study	Comparison	p-Value
ED	Statin for (NE1)	< 0.000167

Table D.14: Unequal HSD-test performed on the **(NE1)** for ED

(NE1)	Mean concentration
(NEL1)	0.391
(NER1)	7.141
(NES1)	13.031

Table D.13 shows the results for the Levene's test done on the ED for **(NE1)**. It can be observed that the p-value was < 0.05 and of statistical significance. Table D.14 displays the results for the **(NE1)** on ED performed by the Unequal HSD-test. This test showed no result for fluvastatin (since

no results were obtained) and also indicated that the mean concentration for statins tested showed differences between statins (the aforementioned test was completed after the ANOVA tests were performed). These results showed significant differences between statins.

Table D.15: Unequal HSD-test performed on the **(NEG)** for SCE

(NEG)	Mean concentration
(NEGF)	66.1969
(NEGL)	104.3036
(NEGR)	119.2681
(NEGS)	81.7639

It can be observed from Table D.15 that **(NEGR)** had the highest mean concentration and **(NEGF)** had the lowest value of mean concentration of the four statins tested on the **(NEG)** by means of the Unequal HSD-test on the SCE skin layers.

Table D.16: Levene's test performed on the **(NEG)** for ED

Study	Comparison	p-Value
ED	Statins for (NEG)	< 0.000025

Table D.17: Unequal HSD-test performed on the **(NEG)** for ED

(NEG)	Mean concentration
(NEGL)	0.238
(NEGR)	13.462
(NEGS)	2.315

Table D.16 displays the results for the Levene's test done on the ED for the **(NEG)**. It can be observed that the p-value was < 0.05 and of statistical significance. Table D.17 presents the results for **(NEG)** on ED performed by the Unequal HSD-test. This test showed no result for fluvastatin (as no results were obtained) and that the mean concentration for statins tested indicated differences (this test was done after the ANOVA tests were performed). These results showed significant differences between statins.

Table D.18: T-test for comparison of the skin layers (SCE vs ED) in terms of each of the four selected statins in the **(NE1)** and the **(NEG)**

Selected statin in the (NE1) and the (NEG)	p-Values SCE	p-Values ED
Fluvastatin	< 0.0000	0.0082
Lovastatin	< 0.0000	0.0004
Rosuvastatin	< 0.0000	0.1861
Simvastatin	0.0036	0.0047

Table D.18 indicates the p-values obtained from the t-test, which was performed on the ED layer of the skin. It indicated the p-values were < 0.001, except for rosuvastatin formulas which showed a p-value of > 0.05 and subsequently indicated the p-values of the rosuvastatin formulas were statistically insignificant. From the t-test performed on the SCE for the formulas of all four statins, it can be concluded that all the statins had a p-value of < 0.001 which indicated statistical significance, except for simvastatin, which showed statistical insignificance with a p-value of higher than 0.001.

D.5 Conclusion

During the membrane release studies, it was evident that the all APIs were released from the different formulas (**(NE1)** and **(NEG)**) respectively. It can be stated that **(NEF1)**, **(NEGF)**, **(NER1)** and **(NEGR)** presented with the highest median flux values for both the **(NE1)** and the **(NEG)**, where **(NEF1)** presented with the overall highest median flux value. **(NES1)**, **(NEL1)** and **(NEGL)** presented with the lowest median flux value.

For *in vitro* skin diffusion studies, **(NEF1)** and **(NEGR)** obtained the highest results for the **(NE1)** and the **(NEG)**, respectively; the same trend was observed during the SCE and the ED results. Fluvastatin has the most ideal physicochemical properties (log D of 1.36; aqueous solubility of 0.53 mg/ml) (Maree, 2018:232) when compared to the other statins (simvastatin (1.80, 0.24 mg/ml), rosuvastatin (- 0.20, 0.31 mg/ml) and lovastatin (4.50, 0.00 mg/ml), respectively) (Marais, 2018:245) for penetration through the layers of the skin (Naik *et al.*, 2000:319). Even though, fluvastatin did not excel in the **(NEG)** as well as in the **(NE1)**, it should be noted that the **(NEG)** improved delivery of fluvastatin through the SCE into the ED and finally into systemic circulation **(NEGF)** had the lowest concentration in the SCE and the second lowest concentration in the ED, but the second-best delivery into the systemic circulation after rosuvastatin of all the **(NEG)**). Although rosuvastatin did not exert ideal log D properties for formulation or penetration, it produced the highest results for the **(NEG)**. Since nano-emulgels enhance penetration of APIs and exert lipophilic properties, this could be the reason for the better penetration of rosuvastatin through the layers of the skin for deeper penetration (lipophilic and hydrophilic) (Chang *et al.*, 2013:41).

In general, lovastatin was the statin that achieved the lowest results of all the statins in the **(NE1)** and the **(NEG)** during the skin diffusion and tape stripping (SCE and ED) studies, except for the delivery of **(NEGL)** in the SCE (second best concentration). This may be ascribed to the poor physicochemical properties (log D of 4.50, aqueous solubility of 0.00 mg/ml) and therefore penetration into and through the layers of the skin were weak, especially to the hydrophilic ED and systemic circulation (hydrophilic) (Sharma & Sarangdevot, 2012:413).

In general, it can be stated that the **(NE1)** had the best results for skin diffusion studies, the **(NEG)** (if fluvastatin is excluded) obtained the best results in SCE and **(NE1)** obtained the best results in the ED (if rosuvastatin is not considered; although the difference in delivery between **(NEGR)** (7.01 µg/ml) and **(NER1)** (6.36 µg/ml) is not much). While the **(NE1)** generally performed better, the **(NEG)** was more ideal, as it released APIs in a more controlled manner (Kathe & Kathpalia, 2017:491).

Even if the statins were delivered in the SCE and ED, the target site (systemic circulation) was reached for each of the statins from their different formulas (**(NE1)** and **(NEG)**), thus the primary aim of the study was achieved.

The following conclusions were reached:

- * *In vitro* skin diffusion is an alternative route of administration of statins and results were obtained for each statin, confirming that it can be used.
- * When comparing the **(NE1)** and **(NEG)** formulas in transdermal delivery, **(NE1)** permeated better through skin and ED, whereas **(NEG)** had better results in membrane diffusion studies and SCE.
- * Fluvastatin obtained the best results from the **(NE1)** and rosuvastatin obtained the best results from the **(NEG)**.
- * Although the **(NE1)** had the highest results during the skin diffusion studies and ED, the **(NEG)** remained the preferable delivery system as it had a controlled release system.
- * It can be stated that the aim of this study was achieved for transdermal delivery, as both formulas (**(NE1)** and **(NEG)**) showed adequate concentrations in the receptor phase.

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Appendix E

CYTOTOXICITY STUDIES OF O/W NANO-EMULSIONS CONTAINING SELECTED STATINS IN GRAPESEED OIL

E.1 Introduction

The human skin consists of keratinocytes in the epidermis, which form a barrier against the environment (Zhang *et al.*, 2017:1). HaCaT cells were derived from human skin cells and utilised as an *in vitro* model (López-García *et al.*, 2014:44) in this study, as they are more applicable (Gao *et al.*, 2015:52). HaCaT cells used in this study exhibit a deoxyribonucleic acid (DNA) fingerprint copy, similar to that of human cells, which is unaffected by long-term cultivation or transformation (Pathakoti *et al.*, 2013:163). The effect of the various compounds on the HaCaT cell viability was assessed to determine the overall cytotoxicity of the statins.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay was used as an indicator of cell metabolic activity and potential cell viability (Grela *et al.*, 2018:303). MTT is a tetrazolium salt, that is converted from a yellow water-soluble product to a purple water-insoluble crystal-like structure (Young *et al.*, 2005:1051). Another toxicity measure performed was the NR-assay. The NR-dye is placed on the cells during the assay. Lysosomes in the cells absorb the red dye through active transport causing the red discolouration of the live cells, whereas dead cells cannot perform active transport and undergo no colour change (ATCC, 2011:1; Repetto *et al.*, 1993:503). NR (3-amino-7-dimethylamino-2-methylphenazine) generates an electrocatalytic effect and can be an indication of necrosis (cytotoxicity), since dead cells do not possess a mitochondrion (Fotakis & Timbrell, 2006:172; Singh *et al.*, 2019:196).

The purpose of this study was to determine the *in vitro* toxicity of each selected statin alone or as a formulation containing 2% (w/w) of each statin, 6% (w/w) Tween® 80 and Span® 60 formulated in 7.9% (w/w) grapeseed oil. The formulations were tested on the human HaCaT keratinocyte cells. Both the MTT- and NR-assays were utilised and results were directly compared.

E.2 Cell culture toxicity studies

E.2.1 The selection of an appropriate cell line

For the selection of an appropriate cell line, criteria such as species type, biosafety level restrictions, functional characteristics such as the expression of a specific gene, growth rate and plating efficiency should be kept in mind (Gibco®, 2016:18). For the purpose of this study, HaCaT cells were selected given that they consist of human keratinocytes with the characteristics of a proliferative epidermis

(López-García *et al.*, 2014:44). The HaCaT cells used for these experiments were between passages 24 and 31.

E.2.2 Non-assay experimental procedures

Procedures such as the general maintenance and seeding of the HaCaT cells into 96-well plates were performed prior to the cytotoxicity experiments. All the cell culturing, seeding and toxicity procedures were performed under the guidance of Dr C. Willers under aseptic conditions in the Mammalian Cell Culture Laboratory at the North-West University (NWU), Potchefstroom Campus (RSA).

E.2.2.1 Materials and reagents

The materials and reagents used for the *in vitro* procedures are presented in Table E.1.

Table E.1: Reagents used during the *in vitro* cytotoxicity studies

Reagents	Supplier	Catalogue number
Dulbecco's Modified Eagle Medium (DMEM) with 4 500 mg/l high glucose, 4.0 mM L-glutamine, sodium pyruvate	HyClone™	SH30243.01
MEM Non-essential Amino Acids (NEAA) (100X)	Sigma-Aldrich®	M7145
Penicillin/streptomycin (Pen/Strep) (10 000 U/ml)	Sigma-Aldrich™	P4333
L-glutamine (200 mM)	Lonza™	BE17-605E
Foetal Bovine Serum (FBS)	Gibco™	10270106
Trypan Blue Solution (0.4%)	HyClone™	SV30084.01
Phosphate buffered saline (1X)	HyClone™	SH30256.01
Methylthiazol tetrazolium (MTT)	Sigma-Aldrich®	M5655
Trypsin-Versene® (EDTA) (1X)	Lonza®	BE17-161E
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich®	D5879
Neutral Red solution (NRS; 0.33%)	Sigma-Aldrich®	N2889
96-Well Cell Culture Cluster plate	Corning Costar®	3596

The abovementioned reagents were only used under aseptic conditions in the laminar flow cabinets to avoid contamination and to ensure a sterile environment for the cells.

E.2.2.2 Data processing and statistical data analysis

Statistica® 13.3 (StatSoft, TIBCO® Software Inc., CA, U.S.A.) was used for the statistical data analysis of the toxicity screening results. Statistically significant differences ($p < 0.05$) were determined between each treatment group and the control groups. A Brown-Forsythe test was

performed to test the homogeneity of the data. Analysis of variance (ANOVA) was performed, as well as Tukey's Honest Significant Difference (HSD) and Kruskal-Wallis tests to indicate the significance between groups (Liu, 2015:2). IC_{50} values are based on a half-maximal inhibitory concentration (Touboul *et al.*, 2009:303), and the value can be defined as the concentration of a compound that inhibits the cell growth by 50% (Krippendorff *et al.*, 2019:914). Firstly, the %cell inhibition was calculated by subtracting the concentration of viable cells (calculated from Equation E.4) from a 100%. A regression analysis was then performed to determine 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, 2014:1606).

E.2.3 Concentrations tested

The normal concentrations of API used in the nano-emulsions and nano-emulgels were 2% (w/w). As less than 2% (w/w) of the API penetrated through the skin (as can be seen from the *in vitro* diffusion studies in Section D.3.4), low concentrations were used for the cytotoxicity studies. The nano-emulsions (from here on referred to as **(NE1)**) 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 dilution (with DMEM) range; the **NE1** without statins is termed the placebo. Each API (fluvastatin, lovastatin, rosuvastatin and simvastatin) was dissolved in 100% methanol (CH₃OH) and tested alone (referred to as **(AS)**) in a two-fold dilution (with DMEM) range from 0.00075% (7.5 µg/ml) to 0.01200% (120.0 µg/ml). The excipients used in the formulations namely Span® 60 and Tween® 80 have been tested in previous studies (Marais, 2018; Maree, 2018) and were therefore not included in this experiment. All the treatment groups were tested in triplicate and the experiments were repeated twice.

Table E.2: Representation of treatment groups and the different concentration ranges utilised

Treatment groups and concentration ranges	
Dispersions (NE1)	APIs (statins) alone (AS)
(NEF1) (fluvastatin)	(FS) fluvastatin
(NEL1) (lovastatin)	(LS) lovastatin
(NER1) (rosuvastatin)	(RS) rosuvastatin
(NES1) (simvastatin)	(SS) simvastatin
(NEP) (placebo)	
0.00250 – 0.04000%	0.00075 – 0.01200%

E.2.3.1 Treatment

After the preparation of the dispersions **(NE1)**, as discussed in Section B.8.1.1, each dispersion (0.2 ml) was diluted with 9.8 ml supplemented DMEM to obtain stock solutions. To prepare **(AS)** stock solutions of each statin (**(FS)**, **(LS)**, **(RS)** and **(SS)**), 400 mg of each was weighed and placed into a 100.0 ml volumetric flask and made up to volume with 100.0 ml CH₃OH. After the APIs were dissolved in CH₃OH, 0.3 ml of each solution was diluted with 9.7 ml DMEM and this served as the stock solutions for each API alone. The concentration of CH₃OH the cells were exposed to, never exceeded 3% (v/v). The dilution ranges of the various stock solutions are given in Table E.3.

Table E.3: Representation of the respective stock solution volumes added to the wells to obtain each concentration

Treatment groups and concentration ranges	
Dispersions (NE1)	APIs (statins) alone (AS)
0.00250% (12.5 µl)	0.00075% (12.5 µl)
0.00500% (25.0 µl)	0.00150% (25.0 µl)
0.01000% (50.0 µl)	0.00300% (50.0 µl)
0.02000% (100.0 µl)	0.00600% (100.0 µl)
0.04000% (200.0 µl)	0.01200% (200.0 µl)
0.00250 – 0.04000%	0.00075 – 0.01200%

The five different concentrations of **(NE1)** and **(AS)** (as seen in Table E.3) were administered to the HaCaT cells and the cells were exposed for a 12 h period in the incubator at 37 °C (5% carbon dioxide (CO₂), 95% humidity). The exposure period of 12 h corresponds with the *in vitro* skin diffusion studies. Cells were also exposed to a placebo of the **(NE1)** that contained no API (this served as the control group for **(NE1)**), those cells not exposed to any solvents are referred to as untreated control groups for both **(NE1)** and **(AS)**. The volumes (µl) applied to the wells were calculated in terms of the concentration of the APIs present in the nano-emulsions. An amount of DMEM was also added to each of the wells, respectively, to make up a final volume of 200.0 µl per well:

- * 12.5 µl of stock solution with 187.5 µl DMEM added.
- * 25.0 µl of stock solution with 175.0 µl DMEM added.
- * 50.0 µl of stock solution with 150.0 µl DMEM added.
- * 100.0 µl of stock solution with 100.0 µl DMEM added.
- * 200.0 µl of stock solution with no added DMEM

E.3 *In vitro* toxicity testing

E.3.1 Determination of cell viability

The HaCaT cells were maintained in high-glucose DMEM supplemented with 10% FBS, 1% NEAA, 2 mM L-glutamine and 1% Pen/Strep within cell culture flasks (75 cm²). This supplemented media is known as growth medium. The flasks were incubated at 37 °C with humidified air containing 5% CO₂.

It was important to determine the number of viable cells in a flask, seeing as the HaCaT cells were seeded at 15 000 viable cells/well. The method described by Potyrailo *et al.* (2019:97) was utilised for the determination of viable cells. A viable cell count is essential to evaluate the kinetics of cell growth (Candena-Herrera *et al.*, 2015:9). The flasks were removed from the incubator, thereafter the spent media in the flasks were removed. Each flask was then washed twice with 10 ml phosphate buffered saline to remove excess growth medium and dead cells. The phosphate buffered saline was removed and 3 ml Trypsin added directly onto the cell surface. The flasks were incubated for 9 min at 37 °C, 5% CO₂ in a humidified atmosphere, followed by the addition of 6 ml growth media to neutralise the trypsin. The cells were thoroughly washed from the flask and transferred into a 15 ml centrifuge tube and centrifuged for 5 min at 140 x *g* to obtain a cell pellet. The supernatant was discarded without disturbing the pellet and each pellet then re-suspended in 5 ml growth media to obtain a fresh cell suspension.

A haemocytometer was utilised to count the viable cells (illustrated in Figure E.1). A counting solution was prepared by mixing 25 µl Trypan Blue (0.4%), which provides better visualisation of viable and damaged cells (Cadena-Herrera *et al.*, 2015:11), 15 µl phosphate buffered saline and 10 µl cell suspension. A 10 µl aliquot of the counting solution was loaded in duplicate on the haemocytometer. The haemocytometer has nine large squares on each side, of which only the cells in the middle and corner squares were counted under the microscope. On each side only five squares were counted (a total of 10 squares) and the average per square calculated. The average was then multiplied by a dilution factor of 5×10^4 to obtain the number of cells per ml in the suspension (C_1 in Equation E.1). From this, the required dilution of the cell suspension for seeding was calculated with Equation E.1:

$$C_1V_1 = C_2V_2$$

Equation E.1

For both the MTT- and NR-assays, the HaCaT cells were seeded at a density of 15 000 cells per well of a 96-well plate. This density corresponds to 75 000 cells/ml seeded at a volume of 200 µl per well. After seeding, the plates were incubated at 37 °C, 5% CO₂, 95% humidity for 24 h to recover properly. After this recovery period, treatment of the cells with the various treatment groups commenced and this was considered as Time 0 h for both MTT- and NR-assays.

Table E.4: Cell concentration determination calculated for the number of plates used

MTT	Number of plates utilised	Amount of cells	Total amount of media (extra included)
1 st plates MTT (NEF1) (NEL1)	4	1.127 ml	30.273 ml
2 nd plates MTT	2	0.922 ml	30.478 ml
3 rd plates MTT	1	0.534 ml	17.666 ml

Table E.5: Cell concentration determination for NR

NR	Amount of plates utilised	Amount of cells	Total amount of media
1 st experiments (2 plates)	2	1.436 ml	29.964 ml

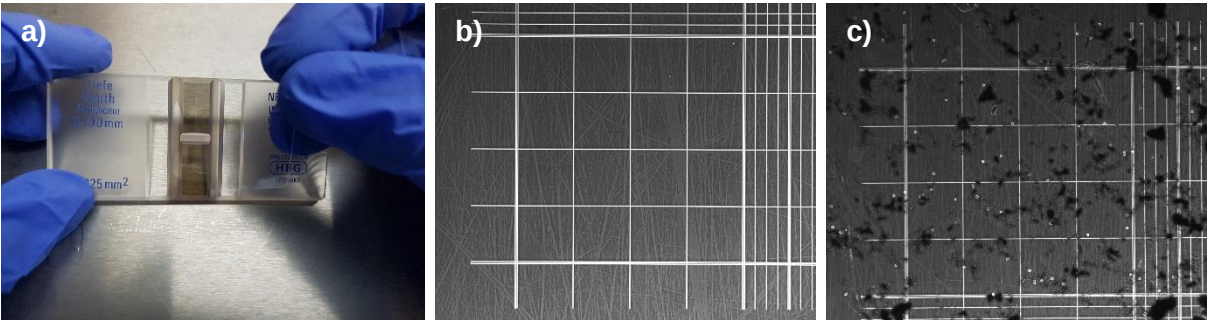


Figure E.1: a) Visual representation of a haemocytometer, b) one of the five grids of the haemocytometer used for counting and c) haemocytometer containing cells under the microscope



Figure E.2: A visual representation of seeding cells into a 96-well plate using a reservoir and multichannel pipette

E.3.2 MTT colorimetric assay

During the MTT-assay, the yellow water-soluble tetrazolium salt is metabolised by dehydrogenases to a violet-blue water-insoluble formazan product (Stockert *et al.*, 2012:785). DMSO is used for its significant ability to dissolve the insoluble formazan product (Twentyman & Luscombe, 1987:279). The MTT formazan is a direct equivalent to the number of living cells (Van Meerloo *et al.*, 2011:237). According to Fotakis and Timbrell (2006:172), cells that did not incorporate the purple colour of the formazan can be regarded as damaged cells, whereas cells that accumulate the purple colour are said to be healthy. Of a 96-well plate only 66 wells received 100 μ l MTT, in total seven plates were used. Amounts used were calculated as shown in Table E.6.

Table E.6: Calculated amount of MTT solutions needed for the treated 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 ml	1.6 ml	28.0 ml
3	198 wells	19.8 ml	1.2 ml	21.0 ml

Volumes of the assay media calculated in Table E.6 were pipetted into 50 ml centrifuge tubes. The MTT-solution must have a concentration of 0.5 mg/ml on the cells (Riss *et al.*, 2016:5), thus the amount of MTT added was weighed and calculated according to Equation E.2.

$$\text{MTT (mg)} = \text{Total volume (ml)} \times 0.5 \text{ mg/ml}$$

Equation E.2

The total amount of MTT used was calculated as 14 mg for the first four plates and 10.5 mg for the last three plates. For the first four plates, MTT was placed in the 50 ml centrifuge tube and 28 ml of DMEM (non-additive) was added, while 21 ml DMEM was added to the last three plates. The MTT-

solution was covered as it is light sensitive, and heated in a water bath at 37 °C until used for the experiments. The 96-well plate was removed from the incubator and the cells were inspected under a microscope. After inspection, the growth medium was extracted from the untreated control group and the dead cells were treated with 200 µl of 100% cold CH₃OH for more than 15 min. The remainder of wells with growth medium were rinsed with 150 µl phosphate buffered saline, followed by additional aspiration before MTT was added. MTT was added at 100 µl per well and incubated for 2 h in the CO₂ incubator (standard growth conditions). After incubation, the plates were removed and the cells inspected to determine if any formazan crystals formed. The MTT-solution was aspirated from the wells and 200 µl DMSO was added to each well. The plates were placed on a shaker to ensure adequate mixing for 15 to 30 min, the 96-well plates were then placed into a SpectraMax® Paradigm® Multimode Microplate reader (Molecular Devices, United States) and absorbance was measured at 560 nm and 630 nm.



Figure E.3: SpectraMax® Paradigm® Multimode Microplate reader (Molecular Devices, United States) used to measure absorbance



Figure E.4: Plate shaker adjusted to 300 rpm

Equation E.3 was used to determine the %viable cells as depicted in Fox *et al.* (2017:3).

$$\% \text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100 \quad \text{Equation E.3}$$

Modification was done to Equation E.3 to include the absorbance at both spectrum groups (560 nm and 630 nm). %Cell viability = $\frac{((\text{sample absorbance } 560 \text{ nm} - 630 \text{ nm}) - (\text{DMSO blank } 560 \text{ nm} - 630 \text{ nm}))}{((\text{untreated control } 560 \text{ nm} - 630 \text{ nm}) - (\text{DMSO blank } 560 \text{ nm} - 630 \text{ nm}))} \times 100$

Equation E.4 MTT colorimetric assay results and discussion

When MTT plates were visually inspected it was observed that the intensity of the purple colour increased with a lower concentration of formulation added, whereas higher concentrations had a lower intensity of purple colour in the wells.

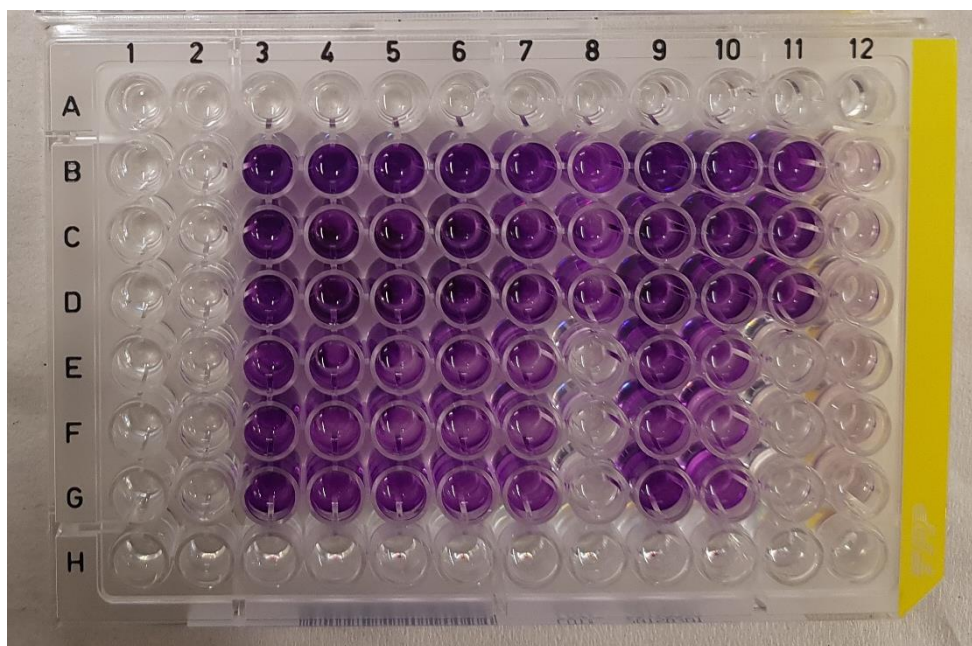


Figure E.5: Illustration of an MTT plate after the addition of DMSO (treatments were added from the lowest to the highest concentration)

Micrographs of the cells before and after MTT were obtained using a ZEISS Axio Vert.A.1 microscope and are shown in Figure E.6.

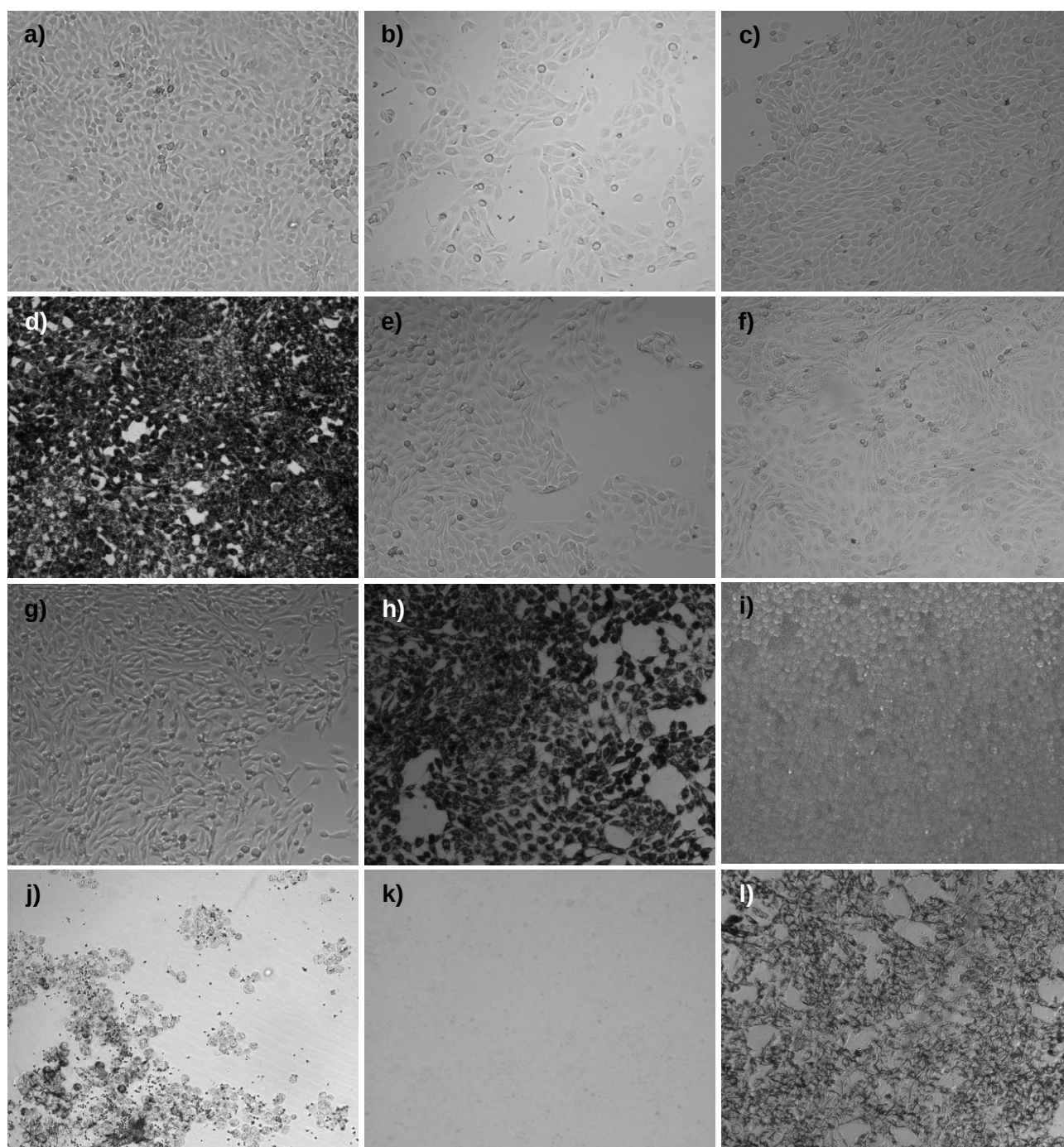


Figure E.6: a) Illustration of cells at 90% confluence before seeding took place, b) cells after seeding took place in each well, c) illustration of untreated cells before MTT assay, d) untreated cells after MTT-assay with crystals visible, e) dead cells before treatment with MTT, f) dead cells after treatment with MTT and no crystals visible, g) cells treated with high API concentrations before treatment with MTT, h) cells treated with high API concentrations after treatment with MTT, i) cells treated with high emulsion concentrations before MTT addition, j) cells treated with high emulsion concentrations after the addition of MTT, k) placebo treated cells before MTT addition and l) placebo treated cells after MTT addition

E.3.2.2 MTT-assay results on HaCaT cells

All cells were exposed to the various treatments over a 12 h period, the results of which are displayed in Tables E.7 and E.8, and Figures E.7 and E.8. The cell viability obtained for each treatment, dosed at five different concentrations are displayed as a percentage (%). The cells of **(NE1)** were also treated with the control group (placebo formulation (containing no API)) and an untreated control, while the cells of **(AS)** were treated with 5% CH₃OH and an untreated control group.

According to Lopez-Garcia *et al.* (2014:44), it can be said that a %cell viability lower than 40% is a measure of strong cytotoxicity, values of 40 to 60% can be seen as moderate cytotoxic, values of 60 – 80% can be seen as weak cytotoxic and lastly, values higher than 80% are non-toxic.

Table E.7: The %cell viability of HaCaT cells after treatment with the **(NE1)** as determined by the MTT-assay

%Cell viability						
Concentrations used for exposure	(NEF1)	(NEL1)	(NER1)	(NES1)	(NEP1) Control	Untreated control
0.0025% (0.025 mg/ml)	66.039 (± 38.303)	85.968 (± 33.848)	95.489 (± 9.881)	79.458 (± 13.875)	88.389 (± 4.909)	100.000
0.0050% (0.050 mg/ml)	65.988 (± 35.124)	79.892 (± 22.718)	59.095 (± 29.847)	41.103 (± 19.375)	87.225 (± 12.163)	
0.0100% (0.100 mg/ml)	34.795 (± 18.203) [▲]	60.127 (± 7.264)	53.536 (± 16.861)	33.425 (± 13.205) [▲]	84.938 (± 7.783) [▲]	
0.0200% (0.200 mg/ml)	17.606 (± 5.273)	25.654 (± 8.714)	35.091 (± 3.980)	6.792 (± 6.755) [▲]	64.414 (± 8.995)	
0.0400% (0.400 mg/ml)	0.893 (± 0.055)	10.769 (± 2.418)	20.752 (± 1.825)	0.192 (± 0.186)	32.989 (± 9.489)	

▲(NE1) Concentrations that differ significantly from the control group determined with Tukey's HSD test (p < 0.05)

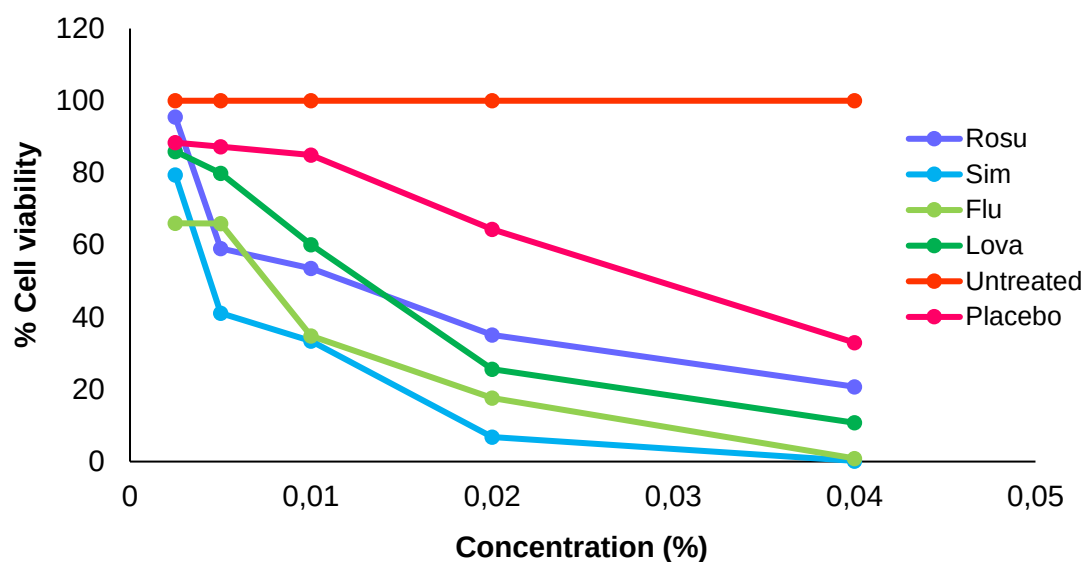


Figure E.7: The %cell viability after treatment with five concentrations of **(NEF1)**, **(NEL1)**, **(NER1)**, **(NES1)** and **(NEP1)**

Table E.7 and Figure E.7 clearly illustrates that a 12 h exposure to each of the nano-emulsions containing different statins showed a concentration-dependent effect on the HaCaT cells. Thus, a higher concentration of emulsion applied to the cells reduced the cell viability, whereas a lower concentration increased the cell viability. According to the guidelines stipulated by López-García *et al.* (2014:44), it can be stated that **(NEF1)** and **(NES1)** are strongly cytotoxic at concentrations of 0.100 mg/ml and higher with %cell viability values lower than 40%. In contrast, **(NEL1)** and **(NER1)** only showed %cell viability values lower than 40% at concentrations of 0.200 mg/ml and higher. **(NEP1)** showed a %cell viability of < 40% only at the highest concentration of 0.400 mg/ml. A non-cytotoxic value (> 80%) was obtained at the 0.025 mg/ml concentration for each **(NE1)** dispersion, except for **(NEF1)** and **(NES1)**. When comparing the different dispersions of **(NE1)** at concentration 0.025 – 0.100 mg/ml, it is observed that **(NEL1)** was the least cytotoxic dispersion with a %cell viability ranging from 60.127 – 85.968%.

Table E.8: The %cell viability of HaCaT cells after treatment with **(AS)** as determined by the MTT-assay

%Cell viability						
Concentrations used for exposure	(FS)	(LS)	(RS)	(SS)	5% CH ₃ OH	Untreated control
0.00075% (0.0075 mg/ml)	90.495 (± 6.044)	76.446 (± 4.170) [▲]	84.440 (± 1.015) [▲]	94.482 (± 1.488)	112.695 (± 3.356)	100.000
0.00150% (0.0150 mg/ml)	69.262 (± 2.948)	60.234 (± 13.862) [▲]	84.814 (± 2.421)	97.071 (± 3.329)		
0.00300% (0.0300 mg/ml)	71.302 (± 12.391)	52.605 (± 6.086) [▲]	82.186 (± 0.526)	76.856 (± 8.014)		
0.00600% (0.0600 mg/ml)	33.588 (± 8.603) [▲]	44.919 (± 8.133) [▲]	80.213 (± 9.929)	63.743 (± 3.686)		
0.01200% (0.1200 mg/ml)	-0.725 (± 0.075) [▲]	0.333 (± 0.219)	84.594 (± 1.232) [▲]	7.039 (± 5.797) [▲]		

▲(AS) Concentrations that differ significantly from the control group determined with Tukey's HSD-test ($p < 0.05$)

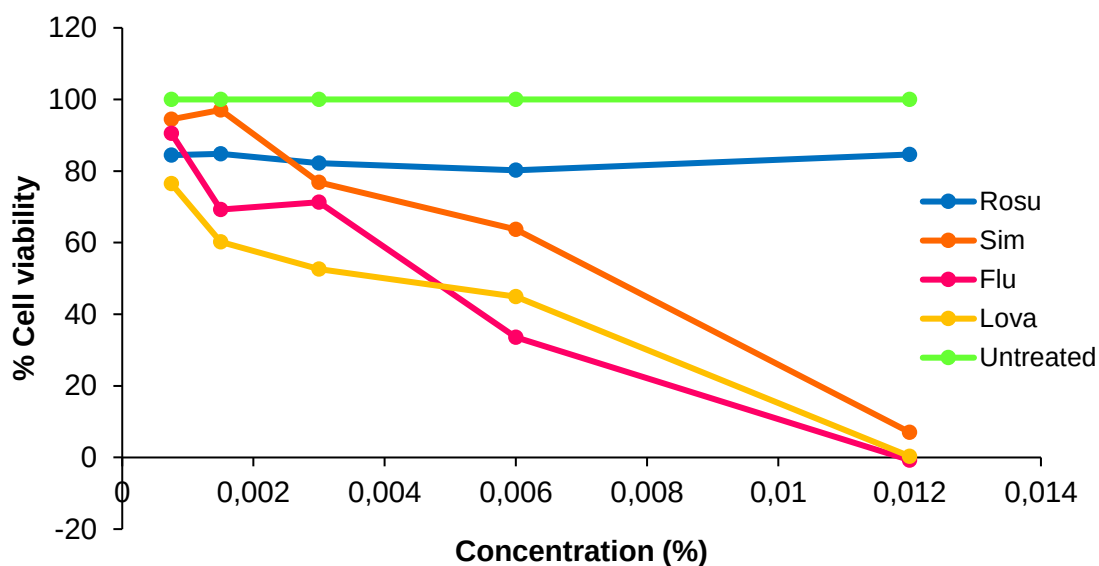


Figure E.8: The %cell viability after treatment with five concentrations of **(FS)**, **(LS)**, **(RS)** and **(SS)**

It is clear from Table E.8 and Figure E.8 that 12 h exposure showed a concentration-dependent effect on the HaCaT cells. The %cell viability is given for the MTT-testing on HaCaT cells where the APIs were tested alone on the cells. For each of the statins used, it can be seen that a higher concentration applied to the cells reduced cell viability, as with **(NE1)**, the highest incorporated concentration did not incorporate the purple formazan colour, whilst lower concentrations did (Saravanan *et al.*, 2003:214). **(FS)** and **(LS)** became weak cytotoxic at a concentration of

0.0150 mg/ml, whereas **(SS)** were weak cytotoxic at a concentration of 0.030 mg/ml. All the APIs (except **(RS)**) became either weak or strong cytotoxic as the concentration of the API increased. When comparing the different **(AS)**, it can be stated that **(RS)** was the least cytotoxic at concentrations of 0.0075 – 0.1200 mg/ml, with a %cell viability that ranged from 84.440 – 84.594% and was therefore non-toxic (> 80%).

E.3.2.2.1 Determination of IC₅₀ values for MTT-assay

After the %cell viability (toxicity) was determined, the IC₅₀ value for each **(NE1)** and **(AS)** was determined.

Table E.9: IC₅₀ values of the **(NE1)** obtained from the MTT-assay

(NE1)	IC₅₀ (mg/ml)
(NEF1)	0.082
(NEL1)	0.167
(NER1)	0.173
(NES1)	0.052
(NEP1)	0.300

Table E.10: IC₅₀ values of the **(AS)** obtained from the MTT-assay

(AS)	IC₅₀ (mg/ml)
(FS)	0.050
(LS)	0.041
(RS)	13.735
(SS)	0.069

From the results obtained, it was evident that the IC₅₀ value of **(NES1)** was the lowest (0.052 mg/ml), confirming that it has the highest toxicity of all the emulsions tested. When comparing the other **(NE1)** with each other, the nano-emulsion with the lowest toxicity was **(NER1)** (0.173 mg/ml) followed by **(NEL1)** (0.167 mg/ml) and then **(NEF1)** (0.082 mg/ml). **(NEP1)** had the highest IC₅₀ value of 0.300 mg/ml confirming that it had the least cytotoxic effects, which may be due to the placebo not containing any API. According to Jiang *et al.* (2014:1564), the IC₅₀ value of fluvastatin is 0.922 μ M (399.59 mg/ml) over an exposure time of 36 h. When the fluvastatin value from HaCaT cells was determined, the value obtained was much higher than suggested by Jiang *et al.* (2014:1564), since the study was only done for 12 h on the HaCaT cell line. From Table E.10, it can be observed that **(LS)** had the lowest IC₅₀ value (0.041 mg/ml) and thus presented with the highest toxicity in compared to the other **(AS)**. **(FS)** presented with an IC₅₀ value of 0.050 mg/ml and was the second most toxic of all the **(AS)**. **(SS)** had an IC₅₀ value of (0.069 mg/ml) and **(RS)** had the highest IC₅₀

value of (13.735 mg/ml), thus, **(RS)** was the least toxic statin. This corresponds to previous findings by Marais (2018) and Maree (2018). The IC₅₀ values of **(NE1)** and **(AS)** differ and can be attributed to the effect of Tween® 80, Span® 60 and grapeseed oil on the HaCaT cells.

E.3.3 Neutral red colorimetric assay

Neutral red (NR), also known as toluylene red, is used as a stain that is absorbed by the cells via diffusion. NR is a cationic stain and is trapped inside the lysosomes of the cells (Miller *et al.*, 2015:904). The NR-dye is only absorbed by the lysosomes of healthy living cells (Mamaca *et al.*, 2005:195), and thus living cells will show a red discolouration, while dead cells will have no discolouration.

The NRS was prepared a day before the assay initiation and left to incubate overnight at 37 °C. The solution was first filtered through a 0.45 µm syringe filter before adding to the cells. The required volume of NRS was prepared as portrayed in Table E.11.

Table E.11: Amount of NRS calculated for the intended number of plates used

Number of plates	Wells used in plates	Volume needed for plates (ml)	Extra volume (ml)	Total volume (NRS) needed (ml)
3	198	19.8	0.2	20

The NR stock (0.33%) should be diluted to 10% of the culture medium volume needed. To obtain the 20 ml of NRS, the volume of NR stock added can be calculated with Equation E.5.

$$\text{Amount NRS} = \text{Total amount} \times 10\%$$

Equation E.5

From Equation E.5, it can be determined that the total amount of NRS (20 ml) contains 2 ml NR stock and 18 ml DMEM (non-additive).

Three plates were taken out of the incubator after the 12 h exposure. Spent media from the dead cell control wells were aspirated and treated with 200 µl of 100% CH₃OH. Spent media from the wells of the treated groups were aspirated and washed with 150 µl phosphate buffered saline. All the cells were then treated with 100 µl of NRS and incubated for 2 h at 37 °C, 5% CO₂, 95% humidity. After 2 h of incubation, the plates were removed and NRS aspirated. The cells were then fixed with 100 µl fixative (0.1% CaCl₂ in 0.5% formaldehyde) followed by the addition of 200 µl of solubilisation solution (1% acetic acid in 50% ethanol). The plates were placed on a mechanical shaker for 15 min at room temperature to ensure proper mixing. Thereafter, the absorbance was measured using a SpectraMax® Paradigm® Multimode Microplate reader (Molecular Devices, United States) at 540 nm and 690 nm. The %cell viability was determined by means of Equations E.3 and E.4.

E.3.3.1 Neutral red colorimetric assay results and discussion

Cytotoxic effects of the treatments will be classified according to the specific guidelines set out by López-García *et al.* (2014:44).

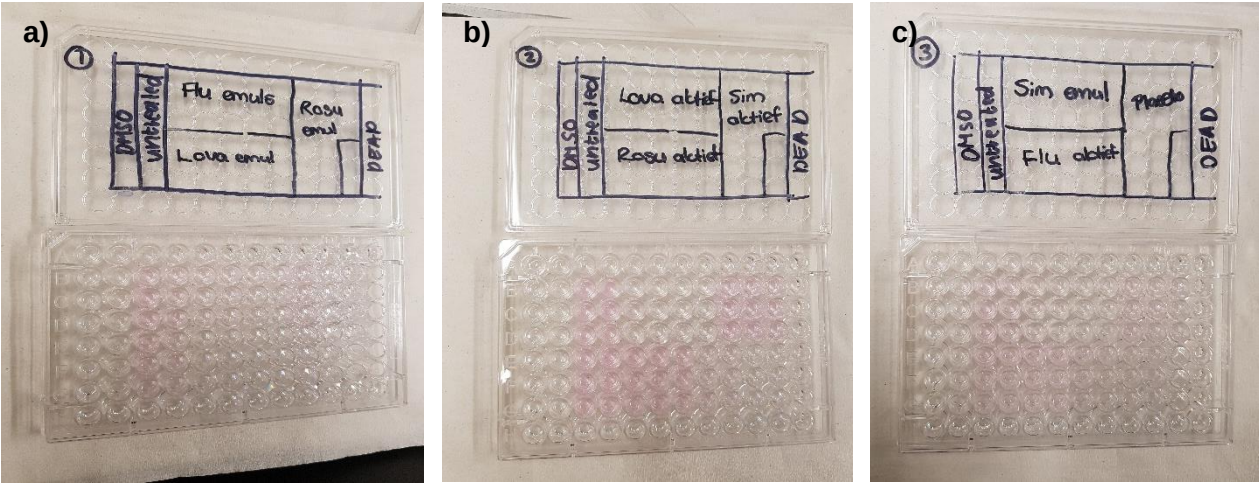


Figure E.9: Illustration of the three 96-well plates used in this experiment after the NR-assay for a) (NEF1), (NEL1) and (NER1), b) (LS), (RS) and (SS), c) (NES1), (NEP1) and (FS)

E.3.3.2 NR-assay results on HaCaT cells

Concentrations used for dose of NR on the HaCaT cells are displayed in Table E.2. Results are displayed in Tables E.12 and E.13, as well as Figures E.10 and E.11.

Table E.12: The %cell viability of HaCaT cells after the treatment with the **(NE1)** as determined by the NR-assay

%Cell viability						
Concentrations used for exposure	(NEF1)	(NEL1)	(NER1)	(NES1)	(NEP1) Control	Untreated Control
0.0025% (0.025 mg/ml)	81.451 (± 6.248) [▲]	76.678 (± 9.543) [▲]	109.671 (± 10.433) [▲]	80.031 (± 9.386) [▲]	101.745 (± 9.337) [▲]	100.000
0.0050% (0.050 mg/ml)	45.269 (± 6.232) [▲]	9.107 (± 6.075) [▲]	25.336 (± 6.203) [▲]	30.527 (± 6.227) [▲]	52.743 (± 4.746) [▲]	
0.0100% (0.100 mg/ml)	41.011 (± 1.760)	15.599 (± 2.299) [▲]	25.145 (± 6.161)	22.293 (± 0.657)	41.442 (± 4.573) [▲]	
0.0200% (0.200 mg/ml)	23.695 (± 11.335)	11.570 (± 3.313) [▲]	31.159 (± 2.291) [▲]	2.449 (± 9.628) [▲]	26.304 (± 4.270) [▲]	
0.0400% (0.400 mg/ml)	-4.926 (± 3.016) [▲]	2.806 (± 3.276)	18.749 (± 14.485) [▲]	-1.504 (± 3.121) [▲]	18.053 (± 1.527) [▲]	

▲(NE1) Concentrations that differ significantly from the control group determined with Tukey's HSD test ($p < 0.05$)

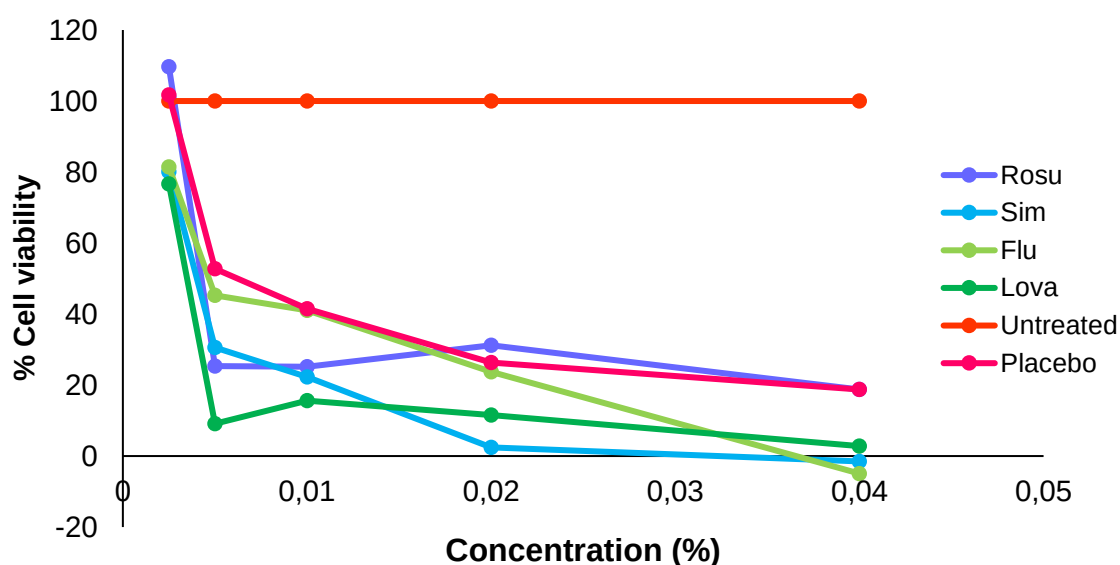


Figure E.10: The %cell viability after treatment with five concentrations of **(NEF1)**, **(NEL1)**, **(NER1)**, **(NES1)** and **(NEP1)**

Results were compared to parameters set out by López-García *et al.* (2014:44) when treated with NR and the results discussed accordingly. From Table E.12, it can be observed that **(NEF1)** at a concentration of 0.025 mg/ml has a non-toxic concentration (81.451%) as the concentration of exposure increases, the cytotoxic effects on the cells increased to strong cytotoxic. **(NEL1)** had a weak cytotoxic effect at a concentration of 0.025 mg/ml and toxicity increased to strong cytotoxic effects with higher concentrations. At a concentration of 0.025 mg/ml, a non-cytotoxic value was

obtained for **(NER1)**, **(NES1)** and **(NEP1)** as the %cell viability was greater than 80%. With higher concentrations of exposure (0.050 - 0.400 mg/ml), the cytotoxicity increased for **(NER1)**, **(NES1)** and **(NEP1)** to strong cytotoxic.

Table E.13: The %cell viability of HaCaT cells after treatment with **(AS)** as determined by the NR-assay

%Cell viability						
Concentrations used for exposure	(FS)	(LS)	(RS)	(SS)	5% CH ₃ OH	Untreated control
0.00075% (0.0075 mg/ml)	95.565 (± 8.533)	101.077 (± 5.596)	107.163 (± 10.675)	98.733 (± 9.749)	75.612 (± 6.365)	100.000
0.00150% (0.0150 mg/ml)	94.349 (± 5.756)	40.239 (± 15.677)	99.613 (± 8.827)	87.578 (± 5.656)		
0.00300% (0.0300 mg/ml)	85.748 (± 1.949)	-0.399 (± 1.150)	83.389 (± 9.906)	75.414 (± 4.966)		
0.00600% (0.0600 mg/ml)	59.815 (± 9.253)	-1.226 (± 0.493)	76.54852 (± 2.287)	40.450 (± 3.972)		
0.01200% (0.1200 mg/ml)	45.114 (± 4.824)	-0.047 (± 0.602)	37.754 (± 11.118)	1.403 (± 1.069)		

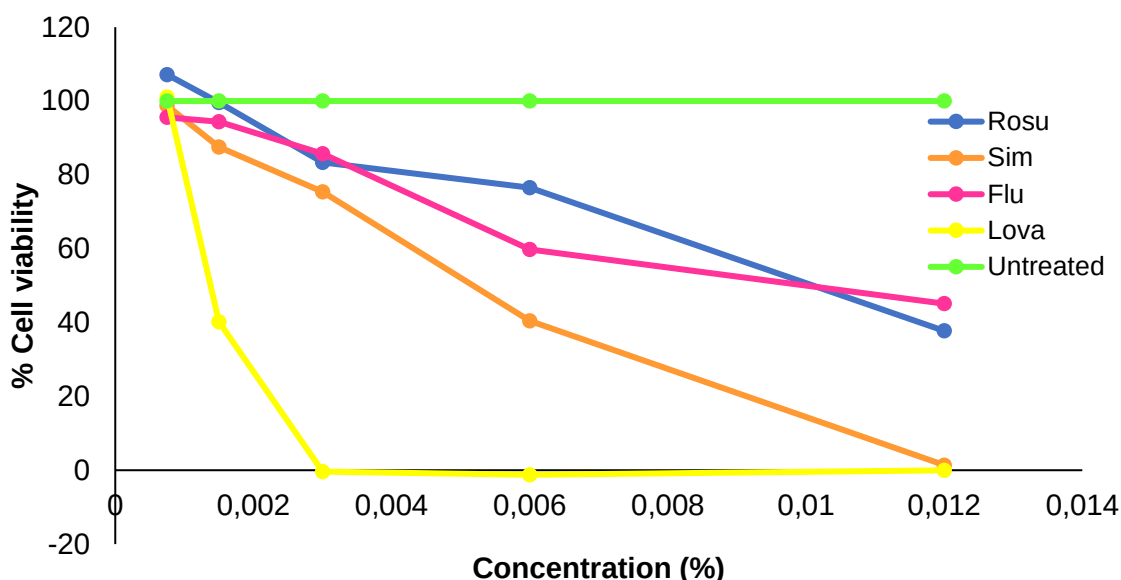


Figure E.11: The %cell viability after treatment with five concentrations of **(FS)**, **(LS)**, **(RS)** and **(SS)**

From Table E.13 and Figure E.11 the concentrations and results of the **(AS)** can be observed. The %cell viability for **(FS)**, **(LS)**, **(RS)** and **(SS)** have a non-toxic effect at a concentration of 0.0075 mg/ml as each of the selected statins presented with values > 80%. **(LS)**, **(RS)** and **(SS)** have strong cytotoxicity (< 40%) at a concentration of 0.01200 mg/ml, while **(LS)** already presented with strong cytotoxic effects at a concentration of 0.0300 mg/ml, where the %cell viability was already

at -0.399%. There were no significant differences between the data for **(AS)** determined by the ANOVA, thus no other tests were performed.

E.3.3.2.1 Determination of IC₅₀ values for NR-assay

As stated in Section E.3.2.2.1, the IC₅₀ value is used for the determination of the concentration of a drug that leads to a 50% inhibition in cells (Koba *et al.*, 2009:31). Representation of results obtained from NR-assay after 12 h exposure is displayed in Tables E.14 and E.15. The method used for the determination of IC₅₀ values is described in Section E.3.2.2.1.

Table E.14: IC₅₀ values of the **(NE1)** obtained from the NR-assay

(NE1)	IC₅₀ (mg/ml)
(NEF1)	0.0884
(NEL1)	-0.0811
(NER1)	0.0946
(NES1)	0.0118
(NEP1)	0.1440

Table E.15: IC₅₀ values of the **(AS)** obtained from the NR-assay

(AS)	IC₅₀ (mg/ml)
(FS)	0.101
(LS)	0.011
(RS)	0.099
(SS)	0.059

From results obtained, it is evident that **(NEP1)** was the least cytotoxic (0.1440 mg/ml) with the highest IC₅₀ value, this may be due to the placebo not containing any API. When comparing the other **(NE1)**, it is evident that **(NEL1)** had the lowest IC₅₀ value (-0.0811 mg/ml), confirming it has the highest toxicity of all the nano-emulsions tested. The **(NE1)** with the lowest toxicity was **(NER1)** (0.0946 mg/ml), followed by **(NEF1)** (0.0884 mg/ml) and then **(NES1)** (0.0118 mg/ml). This data correlates with the IC₅₀ values of the MTT results, where **(NER1)** was also the lowest cytotoxic with the highest IC₅₀ value.

In Table E.15 a difference in values can be observed as **(FS)** has the highest IC₅₀ value (0.101 mg/ml) and the lowest cytotoxicity effect, while **(LS)** has the lowest IC₅₀ value (0.011 mg/ml) and the highest cytotoxicity of the selected **(AS)**. The data correlates with the MTT-assay data for the **(AS)** in terms of **(LS)** that, in both cases, had the lowest IC₅₀ value but highest cytotoxic effects and therefore it can be concluded that **(LS)** is the most cytotoxic API of all the **(AS)**. According to

Jiang *et al.* (2014:1564), the study done on the effect of statins on tumour cells were tested and the IC₅₀ was obtained in the following order - fluvastatin < simvastatin < lovastatin < rosuvastatin - which confirms that fluvastatin exerts the lowest cytotoxicity on cells and lovastatin is amongst one of the statins with the highest cytotoxicity.

E.4 Conclusion

The MTT- and NR-assays were performed to determine the cytotoxicity effects of the different **(NE1)** and the different **(AS)** on HaCaT cells. When results of both MTT and NR were compared, it can be concluded that for **(NE1)** the most cytotoxic dispersion is **(NEL)** with the NR-assay, and **(NES)** for the MTT-assay; for the **(AS)** the most cytotoxic statin was **(LS)** for both MTT and NR. The least cytotoxic dispersion of the **(NE1)** was found to be **(NER1)** for both MTT and NR, and the least cytotoxic statin of the **(AS)** alone was **(RS)** at a concentration of 0.0075%. These findings are in accordance with those of Marais (2018) and Maree (2018).

When IC₅₀ values were compared between MTT and NR for the **(NE1)**, **(NES1)** (0.052 mg/ml) had the lowest IC₅₀ value for MTT and **(NEL1)** (-0.0811 mg/ml) had the lowest IC₅₀ value for NR, which makes them the most toxic towards cells. When a comparison was drawn between the IC₅₀ values of the **(AS)** for both MTT and NR it was found that with both assays, **(LS)** showed the highest toxicity towards cells with the lowest IC₅₀ value of 0.041 mg/ml and 0.011 mg/ml, respectively. The **(NE1)** that showed the highest IC₅₀ value with the lowest cytotoxicity towards cells was **(NER1)** for both MTT and NR. The **(NEP1)** formulation showed little toxicity towards cells with a high IC₅₀ value in both assays. The **(AS)** tested in the MTT-assay showed that **(RS)** had the highest value (13.735 mg/ml) and **(FS)** was the least cytotoxic for NR. In studies of the effects of linalool and simvastatin, in combination or alone on the human liver cancer cell line (HepG2) and adenocarcinomic human alveolar basal epithelial cells (A549), cells over 48 h were evaluated and it was found that those incubated with simvastatin, exhibited a growth inhibition from 10 to 40 µM (16 700 mg/ml) on both cell lines; these results were obtained for both MTT and NR. After 48 h of exposure to simvastatin, MTT results obtained were 18.2 ± 1.1 µM (7 618 mg/ml) and the NR results were 23.0 ± 1.2 µM (9 627.8 mg/ml) (Kladniew *et al.*, 2014:61). Treatment of cells with lovastatin for 16 h resulted in an IC₅₀ value of 3.1 µM (1 253.95 mg/ml) for treatment on human erythroleukemia cell line with spontaneous and induced globin expression (HEL) cells (Lawler *et al.*, 2001:1644). A study done on rosuvastatin on thyroid cells (B-CPAP) over a period of 48 and 72 h intervals exhibited results of 12.9 µM (6 211.35 mg/ml) and 4.6 µM (2 214.9 mg/ml), respectively (Zeybek *et al.*, 2011:108). Effects of fluvastatin and rosuvastatin were tested on tumour cell lines (Human epithelial type 2 cells (Hep-2) and human epithelial cells (HeLa) cells). IC₅₀ values were calculated for each of the selected statins. Fluvastatin had an IC₅₀ value of 2.43 ± 0.56 µg/ml (Hep-2) and 5.02 ± 1.52 µg/ml (HeLa). Rosuvastatin showed poor or no growth inhibition to activity of cells (> 20 µg/ml) (Lara & Espinoza, 2011:190).

Finally, it can be concluded that the concentration that caused the statins to be cytotoxic on the HaCaT cells, were much higher than the concentration that was 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 at a minimum. Further tests can be performed *in vivo* to determine cytotoxicity more accurately.

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Appendix 7

AUTHOR GUIDELINES: Die Pharmazie

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:

Reviews

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

Original articles

Publications from all fields mentioned above.

Short communications

Brief publications about the fields mentioned above (see Preparation of manuscripts)

Book reviews

Conditions

1. For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be determined and classified in a

suitable way, problems must be formulated in view of the data, hypotheses should be suggested and/or the author should give possible explanations for any inconsistencies.

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

2. Authors are requested to submit all manuscripts online.

Paper copy submissions are no longer acceptable.

Articles are considered for publication depending on their value and pharmaceutical relevance and with the understanding that they have not been published previously and are submitted exclusively to the journal *Die Pharmazie*.

3. All manuscripts are subject to experts review. Additional corrections may be done by the editors.
4. A PDF-file of the article is delivered free of charge after the paper has been published in the journal. Please note that, by copyright reasons, this is for personal use of the authors only and must not be made available, e.g. by posting on a freely accessible website.
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Preparation of manuscripts

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

1. Below the title, the surname(s) of the author(s) with initials should be given without academic and professional degrees. The full address of the author for correspondence should appear below author names. Details on the institution where the work was done are requested and should be given above the title.
2. Each manuscript should start with an abstract, containing the most essential results of the study. Extensive review papers and articles for continuous education should be preceded by an outline of topics.

Papers should be subdivided into chapters and subchapters according to the decimal system (e.g. 2.1.3.).

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

Introduction: This should indicate the question under investigation which is generally based on a brief interpretation of the literature considering the current state of knowledge in the subfield and explaining the necessary theoretical foundations.

Investigations and results or synthesis of compounds: Methods should only be described generally (see "Experimental"), referring to previous or analogous studies. The presentation of results should be precise, with necessary formulas (numbered in sequence with Arabic numerals), diagrams, tables and figures added separately (together with the legend) to the manuscripts. Numerical values of results should generally be presented either in tables or curves (please mark statistical limits).

Discussion (unless covered by 2. as Investigations, results and discussion): It should not repeat results already given, but should state the conclusions drawn from the results or provide a theoretical debate and comparison with literature citations.

Experimental: This part describes briefly the detailed experimental conditions. Unless directions taken from literature have been modified, it suffices to refer to the original source. In the case of well known inorganic or organic compounds chemical formulae or common abbreviations may be used (e.g. NaCl, H₂SO₄, CH₃OH, C₆H₆: Ac, Eth, Me, Phe, DMSO) under "Experimental". In other parts of the paper this is not desirable.

Results of elemental analyses can be omitted if it is stated that all the results were in an acceptable error range.

4. Short communications are published as rapidly as possible. The length of a manuscript is limited to 100 lines (including short summary; subdivisions are not required; the "Experimental" - if there is one - should be marked), up to 15 citations of literature and a maximum of 2 supplementary materials (schemes, figures, tables) are allowed.
5. Only the surnames of authors are given in the text. When there are more than two authors, only the name of the first one is used, followed by et al.
6. References in the text have to be cited by author and year, if there are three or more authors, use et al. (Miller 1997; Miller and Smith 2000; Miller et al. 2001). If the year is the same for several references identify these with a, b, c etc (Smith 1998a; Smith 1998b etc.) both in the text and in the reference list. At the end of the paper, references are listed in alphabetical order under the first authors' surname. If there are several references to items with the same first author, arrange these chronologically regardless the alphabetical order of the co-authors ("alphabetic-

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

Quotations have to follow the following style:

Journal articles:

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

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

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

Only if each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

7. For the identification of pharmaceutical substances, the International Nonproprietary Names (INN) proposed or recommended by the WHO should be used. Registered Trade Marks (usually indicated with R; in an article this sign should only be used when it is first mentioned or used in the summary), trivial names and chemical nomenclature can be added.
8. Nomenclature and spelling should conform to the directions given by IUPAC and IUB.
9. Units of measurement are determined by the directions of the International Units System SI as symbols; M instead of mol/l or mol * l⁻¹ is allowed
10. Botanical names (species, genus) should be marked in italics.
11. The following abbreviations should be used consequently (except in the title and all subtitles). All other abbreviations have to be explained in the manuscript at first usage, if aforementioned directions are not applicable. Abs. = absolute; anh. = anhydrous; b.p.; b.r. = boiling point, -range; calcd. = calculated; CC = column chromatography; conc. = concentrated; dec. = decomposition, eq. = equation; Fig. = figure; GC = gas chromatography, - chromatogram, HPLC = high performance liquid chromatography, - chromatogram; i.m. = intramuscular; i.p. = intraperitoneal; IR = infrared; i.v. = intravenous; m.p.; m.r. = melting point, -range; MS = mass spectrometry, mass spectrum; NMR = nuclear magnetic resonance spectrum; PC = paper chromatography, - chromatogram, % = per cent, percentage, p.o. = peroral; s.c. = subcutaneous; TLC = thin layer chromatography, - chromatogram; UV = ultraviolet.

12. Footnotes must be numbered consecutively and are to be added separately to the manuscript. They are printed following the "Experimental".
13. Dedications (e.g., on the occasion of the 60th or higher birthday) should be inserted between author(s) and summary.
14. Additions to legends of table should be marked by *, **, *** or a,b,c,d etc.
15. Figures have to be of sufficient quality for reproduction process. Even after size reduction the figures' key has to be easy to read. Manuscripts containing figures of insufficient quality cannot be accepted.

Introduction

Scope

JPharmSci focuses on two major questions of importance to pharmaceutical scientists: (i) What are the physical and biological barriers that limit the access of drugs to their therapeutic targets?; and (ii) How can drugs, excipients, traditional formulations, novel drug delivery systems and drug products be designed to maximize therapeutic efficacy? Answers to these questions have in the past and will in the future be forthcoming from research in a variety of scientific disciplines including but not limited to the following: physical pharmacy; pharmaceutics; pharmaceutical technology; drug delivery; pharmaceutical engineering; materials science; nanotechnology; animal, human, cellular and molecular biopharmaceutics; animal and human pharmacokinetics, pharmacodynamics and pharmacogenomics; drug metabolism and transport; biotechnology; medical chemistry, including drug design and prodrug strategies; biophysical chemistry; analytical and bioanalytical chemistry; physical organic, organic, and computational chemistry; molecular modeling; immunology; biochemistry; and cell and molecular biology. The scientific content of manuscripts submitted to *JPharmSci* should fit into one of the following subject categories:

Drug Discovery-Development Interface

Manuscripts in this scientific category should include descriptions of quantitative and mechanistic research in pharmaceutics, biopharmaceutics, pharmacokinetics, pharmacodynamics and drug metabolism and transport that are normally conducted during the discovery of organic chemistry-based and biotechnology-based hits, leads and potential drug candidates. Research results of particular interest to the readers of *JPharmSci* would include those that afford valuable, new information about how a molecule's *in vitro* and *in vivo* behavior are influenced by its molecular and physico-chemical properties, traditional formulations and novel delivery systems used in lead optimization studies. This scientific category would also encompass manuscripts that describe: (i) new and novel analytical methodologies and that would facilitate and/or more accurately and completely characterize the physico-chemical and biological properties of hits, leads and potential drug candidates; and (ii) new and novel formulations strategies and drug delivery systems, including those built on bio- and nanotechnologies, that would enhance the delivery of these molecules to their pharmacological targets in animal models.

Pharmaceutical Biotechnology

Manuscripts in this scientific category should include descriptions of quantitative and mechanistic research in pharmaceuticals, drug delivery and pharmaceutical technology that are normally conducted during the preclinical and clinical drug development of biotechnology-based drug candidates and drugs (e.g. peptides, proteins, antibodies, vaccines, DNA, RNA). Research results of particular interest to the readers of *JPharmSci* would include those that afford valuable, new information about how a molecule's *in vitro* and *in vivo* behavior is influenced by its molecular and physico-chemical properties, traditional formulations and novel drug delivery systems used in preclinical and clinical studies and the manufacturing processes that give rise to the final drug product. This scientific category would also encompass manuscripts that describe: (i) new and novel analytical methodologies that would facilitate and/or more accurately and completely characterize the physico-chemical and biological properties of biotechnology-based drug candidates and drugs; and (ii) new and novel formulations strategies and drug delivery systems, including those built on bio-and nanotechnologies, that would enhance the delivery of these types of molecules to their pharmacological targets in animals and humans.

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Manuscripts in this scientific category should include descriptions of quantitative and mechanistic research in pharmaceuticals, drug delivery and pharmaceutical technology that are normally conducted during the preclinical and clinical development of organic chemistry-based drugs based drugs or drug candidates. Research results of particular interest to the readers of *JPharmSci* would include those that afford valuable, new information about how the *in vitro* and *in vivo* behavior of a drug molecule or formulation excipient is influenced by its molecular and physico-chemical properties, traditional formulations and novel drug delivery systems used in pre-clinical and clinical studies and the manufacturing processes that give rise to the final drug product. This scientific category also encompasses manuscripts that describe: (i) new and novel analytical methodologies that facilitate and/or more accurately and completely characterize physico-chemical and biological properties of biotechnology-based drugs and drug candidates; (ii) new and novel pro-drug strategies and formulation strategies and drug delivery systems, including those built on bio- and nanotechnologies, that enhance the delivery of these types of molecules to their pharmacological targets in animals and humans; and (iii) new and novel developments in manufacturing of drugs and drug delivery systems, including continuous manufacturing and the Quality by Design concept.

Pharmaceutical Nanotechnology

Manuscripts in this scientific category should describe quantitative and mechanistic experimental or theoretical research in nanoscale-based pharmaceuticals or diagnostics in which the innovation resides specifically in the nanoscale aspects of the work. Manuscripts reporting advances in

pharmaceutical nanotechnology that are being disclosed for the first time would be of particular interest. Suitable topics in this category include advances in the fabrication of nanoscale materials with demonstrably new or significant functionality potential for pharmaceutical applications. Additional topics include improved quantitative methods of characterization of nanoscale pharmaceutical materials and mechanistic studies that contribute to an improved understanding of functionality of nanoscale-based technologies with clear therapeutic implications. The manuscript's conclusions should be supported by relevant *in vitro* and/or *in vivo* experimental data and appropriate statistical analysis. Notable exceptions to the requirement for appropriate physical and biological characterization are: (i) comprehensive and complete theoretical or computational studies; and (ii) meta-analyses of historical data or reviews of the existing literature.

Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Manuscripts in this scientific category should encompass quantitative and mechanistic research normally conducted during the preclinical and clinical drug development of organic chemistry-based or biotechnology-based drug candidates or drugs that affords valuable, new information (e.g. drug-drug interactions) about the molecule's *in vitro* metabolism and/or *in vitro* absorption, distribution, metabolism and excretion (ADME) and how these properties relate to the molecule's *in vivo* pharmacological and toxicological properties. This scientific category would also encompass manuscripts that describe new and novel analytical methodologies that would facilitate and/or more accurately and completely characterize the pharmacokinetics, pharmacodynamic and drug metabolism and transport properties of these types of drug candidates and drugs in animals and humans.

Global Health

Manuscripts in this scientific category should encompass descriptions of quantitative and mechanistic research in pharmaceuticals, biopharmaceuticals, pharmacokinetics, pharmacodynamics, and metabolism and transport properties that are normally conducted during the discovery of organic chemistry-based and biotechnology-based hits, leads and potential drug candidates and the preclinical and clinical development of drug candidates and drugs targeting diseases common in developing countries. Research results of particular interest to the readers of *JPharmSci* would include those that afford valuable, new information about how a molecule's *in vitro* and *in vivo* behavior are influenced by its molecular and physico-chemical properties, traditional formulations, novel drug delivery systems and manufacturing processes. The scientific category would also encompass manuscripts that describe new and novel analytical methodologies that would facilitate and/or more accurately and completely characterize the pharmaceuticals, pharmacokinetics, pharmacodynamics, drug metabolism, drug delivery and manufacturing properties of these types of drug candidates and drugs in animals and humans.

Types of article

Types of article

The Editor-in-Chief and one Editor, as well as members of the Journal's Editorial Advisory Board and independent experts, will review most manuscripts submitted to *JPharmSci*. However, the Editor-in-Chief and the Editors reserve the right to reject a manuscript without conducting an in-depth review if they feel that the manuscript is "out of scope" or it does not meet the minimal acceptance criteria for publication in *JPharmSci*.

Rapid Communications are preliminary accounts of significant and original experimental and/or theoretical results that fit within the scope of *JPharmSci*. The results must be of sufficient significance, originality and general interest to justify accelerated publication. Authors are asked to write their manuscripts in a clear and concise manner and to include only data crucial to arriving at their final conclusions. Preferably manuscripts should not exceed 2,000 words of text and a total of 4 figures and/or tables. Extra experimental and/or theoretical data in the form of figures and tables should be deposited under Supporting Information.

Research Articles are comprehensive accounts of significant and original experimental and/or theoretical results that fit within the scope of *JPharmSci*. Authors are asked to write their manuscripts in a clear and concise manner and to include only data crucial to arriving at their final conclusions. Preferably manuscripts should not exceed 5,500 words of text and a total of 8 figures and/or tables. Extra experimental and/or theoretical data in the form of figures and tables should be deposited under Supporting Information.

Notes differ from Rapid Communications in that they are final reports and from Research Articles in that they are limited in scope. Authors are asked to write their manuscripts in a clear and concise manner and to include only data crucial to arriving at their final conclusions. Preferably manuscripts should not exceed 2,000 words of text and a total of 4 figures and/or tables. Extra experimental and/or theoretical data in the form of figures and tables should be deposited under Supporting Information.

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