

Influence of acid-base imbalance and hyperlipidaemia on statin-induced myotoxicity

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Abstract

Disturbances in the acid-base balance, such as acidosis and alkalosis, alone or in the presence of postprandial or pathological hyperlipidaemia can alter the pharmacological and toxicological outcomes of statin therapy. Both acid-base imbalance and hyperlipidaemia are quite common among statin users. Statins are commonly prescribed for elderly patients who have multiple co-morbidities such as diabetes mellitus, cardiovascular and renal diseases. These conditions are risk factors for the development of metabolic acidosis. In addition, patients with abnormal plasma lipoproteins levels are usually treated with statins. There is also a general consensus by clinicians to recommend such patients to use unsaturated fat and fatty acids such as olive oil for prevention of cardiovascular and atherosclerotic diseases. The use of such oils is associated with transient but significant elevation in plasma triglyceride-rich lipoproteins (TRL), mainly chylomicrons.

The effect of disturbances in acid-base balance on the inter-conversion of simvastatin and pravastatin between lactone and hydroxy acid forms have been investigated in physiological buffers, human plasma, and cell culture medium over pH ranging from 6.8–7.8. The effects of such inter-conversion on cellular uptake and myotoxicity of statins were assessed *in vitro* using C2C12 skeletal muscle cells under conditions relevant to acidosis, alkalosis, and physiological pH.

Results indicate that the conversion of the lactone forms of simvastatin and pravastatin to the corresponding hydroxy acid is strongly pH-dependent. At physiological and alkaline pH, substantial proportions of simvastatin lactone (~87% and 99%, respectively) and pravastatin lactone (~98% and 99%,

respectively) were converted to the active hydroxy acid forms following 24 hours of incubation at 37°C. At acidic pH, conversion occurs to a lower extent, resulting in greater proportion of statin remaining in the more lipophilic lactone form. However, pH alteration did not influence the conversion of the hydroxy acid forms of simvastatin and pravastatin to the corresponding lactones. Furthermore, acidosis has been shown to hinder the metabolism of the lactone form of statins by inhibiting hepatic microsomal enzyme activities. Lipophilic simvastatin lactone was found to be more cytotoxic to undifferentiated and differentiated skeletal muscle cells compared to the more hydrophilic simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid. Enhanced cytotoxicity of statins was observed under acidic conditions and is attributed to increased cellular uptake of the more lipophilic lactone or unionised hydroxy acid form.

Statins association with plasma lipoproteins was examined using an *in silico* model, artificial chylomicrons-like lipid particles, rat and human lipoprotein fractions under conditions of physiological and altered pH levels. The effect of statins association with plasma lipoproteins on cellular uptake and myotoxicity of these drugs was also assessed at different pH levels using C2C12 cells that overexpress lipoprotein lipase (LPL).

Lipophilic simvastatin displayed considerable association with plasma lipoproteins. The association was more significant with the non-polar lipoprotein fractions (TRL and Low-density lipoprotein [LDL]). This association contributed to increased cellular uptake of statins by C2C12 cells through LPL-mediated process, resulting in a higher intracellular concentration of statins in hyperlipidaemic conditions. These high intracellular

concentrations of statins induced significantly higher cytotoxicity in hyperlipidaemic environment comparing to normolipidaemic conditions. Furthermore, a combination of low pH environment (representing acidosis) with hyperlipidaemia enhanced the association of lipophilic statins with plasma lipoproteins and increased cellular uptake and myotoxicity of these drugs.

These studies suggest that comorbidities such as hyperlipidaemia, especially when coincident with acidosis, can enhance the statin-associated muscle toxicity, and therefore require extra caution and close monitoring by prescribing clinicians. Hydrophilic rather than lipophilic statins could be a preferable choice in this patient population.

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List of Abbreviations

ACC/AHA	American College of Cardiology/American Heart Association
ACD	Advanced Chemistry Development
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CK	Creatine kinase
C_{max}	Maximum plasma concentration
Ct	Cycle threshold
DDT	4,4-Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphates
ESI	Electrospray ionization
FDA	Food and Drug Administration
F-PP	Farnesyl pyrophosphate
FRB	Freely rotatable bonds
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
G-PP	Geranyl pyrophosphate
GC-MS	Chromatography-mass spectrometry
H-acceptors	Hydrogen-bond acceptors
HDL	High-density lipoprotein
H-donors	Hydrogen-bond donors
HEPES	4-2-hydroxyethyl -1-piperazineethanesulfonic acid
hLPL	Human lipoprotein lipase
HMG-CoA	Hydroxymethyl glutaryl-Co enzyme-A
HPLC	High-Performance Liquid Chromatography
Hprt	Hypoxanthine phosphoribosyltransferase
HQC	High concentration quality control
IC_{50}	Half-maximal inhibitory concentration
IL-6	Interleukin-6
IS	Internal standard
KBr	Potassium bromide
LC3	Light chain 3
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein-cholesterol
LLOQ	Lower limit of quantification
Log$D_{7.4}$	Distribution coefficient at pH 7.4
Log*P*	Partition coefficient
LOV-A	Lovastatin hydroxy acid
LOV-L	Lovastatin lactone
LPDP	Lipoprotein-deficient plasma
LPL	Lipoprotein lipase
LQC	Low concentration quality control
MAFbx	Muscle atrophy F-box
MCT4	Monocarboxylate transporter 4
MHC	Myosin heavy chain
mLPL	Mouse lipoprotein lipase

MQC	Medium concentration quality control
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
Mrp	Multi-resistant protein
MS	Mass spectrometry
MSCV	Murine stem cell virus
MTBE	Methyl *tert*-butyl ether
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
Murf	Muscle ring finger 1
m/z	Mass to charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NP-40	Nonidet P-40
Oatp	Organic anion transporting polypeptide
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Photodiode Array Detector
PDK4	Pyruvate dehydrogenase kinase isozyme 4
PLS	Partial least squares regression
PRIMO	Prediction of Muscular Risk in Observational conditions
PSA	Polar surface area
PVA	Lovastatin hydroxy acid
PVL	Pravastatin lactone
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RD	Rhabdomyosarcoma
RE	Relative error
RIPA	Radioimmunoprecipitation assay
Rps12	Ribosomal protein S12
RSD	Relative standard deviation
SD	Standard deviation
SE	Standard error
SVA	Simvastatin hydroxy acid
SVL	Simvastatin lactone
SV-LDL	Simvastatin-loaded low-density lipoprotein
SV-Sol	Simvastatin solution
SV-TRL	Simvastatin-loaded triglyceride-rich lipoproteins
Tbp	TATA box-binding protein
THF	Tetrahydrofuran
TNF-α	Tumour necrosis factor alpha
TRL	Triglyceride-rich lipoproteins
UDP	Uridine diphosphate
ULN	Upper limit of normal
UV	Ultraviolet
VLDL	Very low-density lipoprotein

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Chapter One

1 Introduction

1.1 Statins

Statins, also called hydroxymethyl glutaryl-CoA (HMG-CoA) reductase inhibitors, are lipid-lowering drugs widely used for prevention and treatment of cardiovascular and coronary heart diseases.[1] These drugs act primarily by inhibiting the rate-limiting step of cholesterol biosynthesis in the liver which is catalysed by the enzyme 3-hydroxy-3-methylglutary-CoA reductase. Reduction of intracellular cholesterol concentrations induces the expression of low-density lipoprotein receptors on the hepatocyte surface cells. This, in turn, increases the clearance of low-density lipoprotein-cholesterol (LDL-C) from the circulation and reduces the circulating LDL-C concentrations. Furthermore, the depletion of intracellular cholesterol pools inhibits very low-density lipoprotein (VLDL) synthesis and secretion by the hepatocytes.[2, 3] (Figure 1.1).

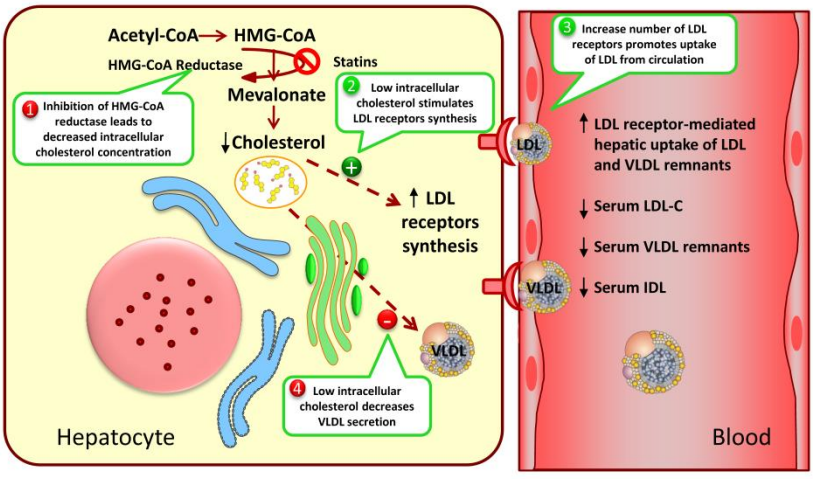


Figure 1.1: Mechanism of action of statins.[4]

Beside cholesterol-lowering effects, statins have a number of clinical benefits called pleiotropic effects.[5] These pleiotropic effects include immune-modulatory actions, improvement of endothelial function, stabilisation of atherosclerotic plaques, and inhibition of vascular inflammation, smooth muscle cells proliferation, thrombogenesis and LDL-C oxidation.[5, 6]

Mevastatin, formerly called compactin, was the first statin discovered by Akira Endo and his colleagues in 1970s from the mould *Penicillium citrinum*.[7] Although it didn't reach the market, Mevastatin has laid the first stone for the development of a number of analogues that showed therapeutic applications. Currently, 7 statins are available for clinical use (Figure 1.2). These include lovastatin which was approved by the Food and Drug Administration (FDA) in 1987, followed by simvastatin, 1988; pravastatin, 1991; fluvastatin, 1994; atorvastatin, 1997; rosuvastatin, 2003; and pitavastatin, 2009.[8] Another agent, called cerivastatin, was approved by the FDA and launched into the market in January 1998. However, it was subsequently withdrawn from the market in August 2001 because of adverse event reports of rhabdomyolysis.[9] Between 1998 and 2001, forty cases of cerivastatin-induced myotoxicity were reported to be fatal.[8] Lovastatin is a natural statin isolated from the mould *Aspergillus terreus*. Simvastatin and pravastatin are semisynthetic derivatives of lovastatin and compactin, respectively while the remaining statins (fluvastatin, atorvastatin, rosuvastatin and pitavastatin) are completely synthetic compounds.[10, 11]

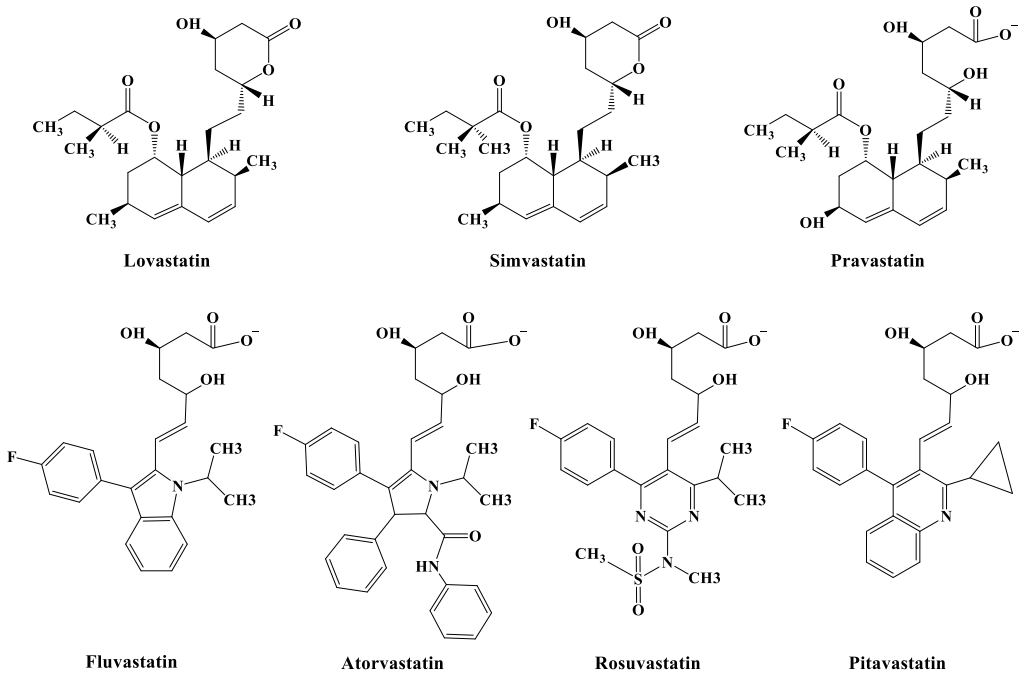


Figure 1.2: Chemical structures of statins.[12]

Lovastatin and simvastatin are administered as prodrugs in the lactone forms while other statins are given in the active hydroxy acid forms.

Although statins share a common mechanism of action, they differ in terms of their chemical structures, pharmacokinetic profiles, and lipid-modifying efficacy. The chemical structures of statins govern their water solubility, and this in turn influences their absorption, distribution, metabolism and excretion.[10] Pravastatin hydroxy acid, the most hydrophilic statins, has poor oral absorption, protein binding, and bioavailability compared to the more lipophilic simvastatin lactone (Table 1.1).

Table 1.1: Comparative pharmacokinetics of statins.

Pharmacokinetic property	Simvastatin	Pravastatin
Equipotent dose (mg)	10	20
Lipophilicity	lipophilic	hydrophilic
Fraction absorbed (%)	60–80	34
Cmax (ng/mL)	10–34	45–55
Tmax (h)	1.3–2.4	0.9–1.6
Bioavailability (%)	<5	18
Protein binding (%)	94–98	43–55
Hepatic extraction ratio	≥80	45
Metabolism	CYP3A4	Isomerisation and sulfation
Metabolites	active	inactive
Clearance (L/hr/kg)	0.45	0.81
Elimination half-life (h)	2-3	1.3-2.8
Urinary excretion (%)	13	20
Fecal excretion (%)	58	71

Adapted from[10, 13-15]

Statins are generally well tolerated. Common adverse events include dyspepsia, flatulence, constipation, generalised gastrointestinal discomfort, elevated transaminase levels, myalgia, headache, sleep disorders and central nervous system disturbances.[14, 16] Muscle complaints constitute the major adverse effects that limit the clinical use of statins.[17] The severity of these adverse effects could range from mild muscle pain and weakness without creatine kinase (CK) elevation to more pronounced and potentially fatal breakdown of the muscle tissue (rhabdomyolysis).[18]

1.2 Definition and Incidence of Statin-Induced Myotoxicity

Statins are among the most commonly prescribed medicines worldwide. In the recent years, significant increase in the number of statin prescriptions has been reported by the British Heart Foundation with more than seven million people currently taking either prescribed or over the counter statins in the UK.[19] Recent data from the National Centre for Health Statistics reveals that 27.9% of American men and women of 40 years and older are taking statins.[20]

According to the American College of Cardiology/American Heart Association (ACC/AHA) guidelines[21, 22] for prediction of cardiovascular risk factors, more than one billion people worldwide are now estimated to use statins.[23]

The terminology used to describe statin-related muscle side effects is inconsistent and confusing. The majority of randomised controlled trials used the term myopathy to reflect an increase in CK below 10 times the upper limit of normal (ULN) with or without muscle symptoms. In an attempt to address these inconsistencies, the American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute adapted the definitions presented in Table 1.2.[24]

Table 1.2: Definitions of muscle-related side effects of statins according to the American College of Cardiology/American Heart Association/National Heart, Lung, and Blood Institute consensus.[24]

Condition	Definition
Myopathy	A general term referring to any disease of muscle
Myalgia	Muscle ache or weakness without CK elevation
Myositis	Muscle symptoms with moderate CK elevation
Rhabdomyolysis	Muscle symptoms with marked CK elevation (typically > 10 times the upper limit of normal), and creatinine elevation (usually with brown urine and urinary myoglobin)

CK, creatine kinase

It has been reported that in clinical practice, as many as 5%–10% of patients receiving statins develop some sort of muscle-related side effects.[25-27] These side effects have been underestimated in randomised controlled trials, probably because of the exclusion of high-risk patients, such as patients with a history of muscular symptoms or CK elevations, and the omission of mild symptoms in patient interviews and clinical trial adverse event reports.[26] The most common muscle-related adverse event associated with statin administration is myalgia with an incidence of 190 per 100,000 patient-years.[28]

According to the US National Lipid Association Statin Safety Assessment Task Force,[29] a meta-analysis of 21 clinical trials found that myopathy, defined as muscle symptoms and CK levels greater than 10-folds ULN, occurs in 5 per 100,000 patient-years. Rhabdomyolysis, defined as CK levels more than 10,000 IU/L or CK levels more than 10-times the ULN, plus elevation in serum creatinine or requirement for hydration therapy, occurs in 1.6 per 100,000 patient-years.[30-33]

The incidence of statin-induced myotoxicity is different across members of the statin class. The incidence of rhabdomyolysis from simvastatin, atorvastatin, and lovastatin are about four times higher than fluvastatin or pravastatin.[34-36] Moreover, the PRIMO (Prediction of Muscular Risk in Observational conditions) study has suggested a lower risk of muscle-related side effects from fluvastatin (5.1%) compared with pravastatin (10.9%), atorvastatin (14.9%), and simvastatin (18.2%).[25]

1.3 Pathophysiological Mechanisms of Statin-Induced Myotoxicity

Many hypotheses have been suggested to explain the mechanisms of statin-induced myotoxicity. One theory states that blocking cholesterol synthesis by HMG-CoA reductase inhibitors reduces the cholesterol content of skeletal muscle cell membranes, making them unstable.[37] The membrane lipids are in dynamic equilibrium with plasma lipids. Low plasma cholesterol levels are usually associated with reduced intracellular cholesterol and may result in decreased membrane lipid contents. This, in turn, can cause physical alteration of membrane fluidity and inhibition of cell proliferation.[16] This change in membrane fluidity can affect different ionic channels such as sodium,

potassium, and chloride channels resulting in altered muscle membrane excitability.[32, 38]

There is also a postulation that statin-induced myotoxicity is not a result of decreased cholesterol synthesis, but rather a reduction in the availability of the isoprenoid co-metabolites farnesyl pyrophosphate (F-PP) and geranyl pyrophosphate (G-PP)[39] (Figure 1.3). Depletion of these metabolites leads to a reduction in the prenylation of small guanosine triphosphate-binding proteins, such as Rac, Rho, and Ras, which is thought to result in cell apoptosis.[12, 40] Replacement of depleted isoprenoid metabolites (F-PP and G-PP) has been found to ameliorate the adverse effects of statins in rat skeletal muscle cells in a concentration-dependent manner. In contrast, inhibition of cholesterol synthesis by squalene synthase inhibitors does not induce myotoxicity.[41]

HMG-CoA reductase catalyses the synthesis of mevalonate, an important precursor not only of cholesterol, but also of ubiquinone (coenzyme Q), dolichol, and isopentenyl adenosine. All these products are involved in cell replication, and dolichol is necessary for glycoprotein synthesis. A deficiency in these products may adversely affect myocyte membranes, predisposing the cell to myotoxic consequences.[16] Ubiquinone protects against free radicals-induced oxidative stress, participates in electron transport during oxidative phosphorylation, regulates mitochondrial permeability transition pores, activates mitochondrial uncoupling proteins, and regulates the physicochemical properties of membranes.[37, 42-44] Several studies have reported that statins reduce blood and tissue levels of ubiquinone in animals as well as in humans.[45-47] A study of preclinical safety evaluation of cerivastatin in dogs reported a marked reduction in ubiquinone concentrations in the serum and

skeletal muscle tissue. This reduction was inhibited completely by concurrent administration of mevalonic acid.[48]

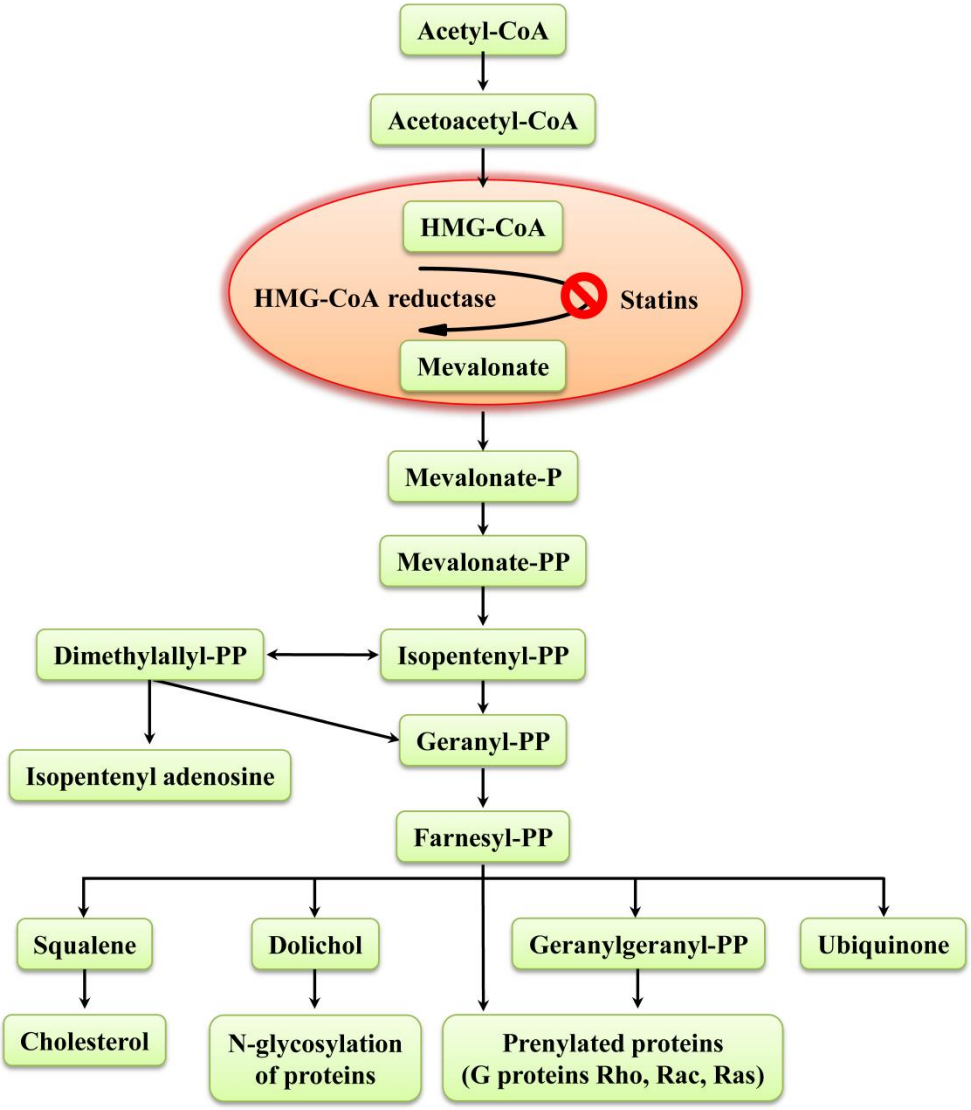


Figure 1.3: Schematic diagram of cholesterol biosynthesis.[40]
(-P, phosphate; -PP, pyrophosphate).

In addition to the previously mentioned mechanisms, statins have been reported to reduce mitochondrial ATP production and induce mitochondrial dysfunction by inhibiting mitochondrial complex I and III activities.[38, 49] Statins can induce sustained increase in cytosolic Ca^{2+} levels by interfering with intracellular storage in mitochondria and the sarcoplasmic

reticulum.[50] Altered Ca^{2+} homoeostasis in muscle cells could lead to muscle dysfunction.[51]

Another proposed mechanism of statin-induced myotoxicity relies on the finding that statin use can lead to secondary carnitine deficiency that is manifested clinically as myalgia and/or myositis. Carnitine plays a role in the transfer of long-chain acyl-CoA across the inner mitochondrial membrane, activation of fatty acid β-oxidation, and prevention of accumulation of potentially toxic levels of mitochondrial and cytosolic acyl-CoA.[52] Statins trigger myotoxicity in people with pre-existing asymptomatic carnitine palmitoyl transferase deficiency and in patients who have abnormalities in muscle carnitine content, reduced activities of carnitine palmitoyl transferase or respiratory chain enzymes.[53]

There is some evidence indicating that statins can lead to over-accumulation of lactic acid in skeletal muscle by inhibiting lactic acid efflux from myocytes, resulting in intracellular acidification and apoptosis.[54] Lactic acid efflux is mediated by the monocarboxylate transporter 4 (MCT4) isoform.[55, 56] These transporters are responsible not only for the efflux of lactic acid but also play a role in the transport of compounds that have monocarboxylate structures within their molecules (including statins). A number of statins have been shown to significantly inhibit MCT4 and thereby induce damage to the muscle cells.[54, 57]

It has been shown that α-cyano-4-hydroxycinnamic acid, a lactate efflux inhibitor, increases cerivastatin-induced growth inhibition of cultured human rhabdomyosarcoma (RD) cells.[58] On the other hand, citrate and bicarbonate suppress cerivastatin-, simvastatin-, and atorvastatin-induced reduction of cell

viability, caspase activation, and morphological change in RD cells. Moreover, citrate prevents cerivastatin-induced elevations in CK levels in a concentration-dependent manner. Accordingly, these alkalinizing agents can suppress statin-induced apoptosis and can be used to reduce the chance of rhabdomyolysis.[58, 59]

Alterations of protein synthesis and protein degradation have also been implicated in statin-induced myotoxicity.[60] Statins have been shown to induce the expression of atrogenes-1, a key gene encoding ubiquitin protein ligase that is involved in muscle atrophy resulting in an increase in the rate of muscle proteolysis.[61-63] Recently, simvastatin has been shown to suppress protein synthesis in C2C12 cell line with simultaneous reduction in the expression of eukaryotic initiation factor 2B (a regulated and rate-controlling guanine nucleotide exchange factor known to affect global rates of protein synthesis).[60]

1.4 Risk Factors of Statin-Induced Myotoxicity

The likelihood of developing myotoxicity from statin use appears to be greater in the presence of certain risk factors. These risk factors can be classified broadly into environmental and genetic risk factors. Most of the environmental risk factors are modifiable. However, genetic risk factors appear to be unavoidable.[64] Environmental risk factors can further be divided into either drug-related or patient-related risk factors. Table 1.3 summarises the risk factors of statin-induced myotoxicity. The next sections focus on the risk factors that were considered in the framework of our project. More detailed descriptions of myotoxicity-related risk factors are provided in our review article (Taha *et al*., 2014).[64]

Table 1.3: Risk factors of statin-induced myotoxicity[31, 64, 65]

Environmental risk factors	
Drug-related	
High-dose statin therapy	
Using lipophilic statin	
Using the lactone form of statin	lovastatin and simvastatin
Drug interactions	Fibrate (especially gemfibrozil), itraconazole, fluconazole, ketoconazole, danazol, erythromycin, clarithromycin, ciclosporin, diltiazem, verapamil, amiodarone, tacrolimus, and ritonavir
Patient-related	
Advanced age	
Comorbidities	pre-existing muscle disorders (acquired or inherited), multisystem diseases such as hepatic or renal failure, diabetes, hypothyroidism, major surgical operation
Dietary habits	High-fat meals, grapefruit juice, and alcohol consumption
Excessive extraneous exercise	
Genetic risk factors	
Female gender	
Small body mass index	
Asian race	especially Japanese, Chinese and Malaysian descent
Genetic variability in drug transporters	
Genetic variability in drug metabolising enzymes (e.g. polymorphism of SLCO1B1 gene which encode for OATP1B1 transporter).	

1.4.1 Lipophilic versus hydrophilic statins

Lipophilicity varies between statins. Cerivastatin, simvastatin, atorvastatin, fluvastatin and lovastatin exhibit lipophilic properties, in contrast to the hydrophilicity of other statin molecules (pravastatin and rosuvastatin).[6, 66] Lipophilic statins were found to be superior to hydrophilic statins in protecting against cardiovascular events but at the same time have more side effects.[67-69] It has been postulated that the risk of myotoxicity is much greater with lipophilic statins because of their ability to penetrate skeletal muscle cells and alter membrane structure.[70] Lipophilic statins tend to accumulate inside the

cells more than hydrophilic statins. Such accumulation appears to be correlated with cytotoxicity.[71]

In vitro data obtained from neonatal rat skeletal muscle cells indicate that pravastatin is less myotoxic than simvastatin. While both statins showed comparable inhibition of hepatic cholesterol synthesis, pravastatin was 85 times less potent in inhibiting cholesterol synthesis in rat skeletal muscle cells. Furthermore, pravastatin was 100-200 times less myotoxic.[72] The lower myotoxicity of pravastatin appears to be correlated with the hydrophilicity of the drug molecule.[65] Pravastatin is about 300 times less lipophilic than simvastatin at physiological pH conditions.[73]

Gadbut *et al*., 1995 compared the effect of lipophilic and hydrophilic statins by examining the viability of C2C12 cells after treatment with different concentrations of simvastatin, lovastatin, and pravastatin for 24 hours. They found that simvastatin and lovastatin decrease cell viability by approximately 50% at low concentrations of 1 and 5 μM, respectively, and by 90% at 10 and 15 μM respectively. On the other hand, pravastatin at a concentration of 300 μM produced less than 50% reduction in cell viability.[74]

1.4.2 Lactone versus hydroxy acid forms

Statins are available on the market either as lactones (simvastatin and lovastatin) or hydroxy acid forms (atorvastatin, fluvastatin, pravastatin, and rosuvastatin).[75, 76] *In vivo*, the lactone forms are inactive (prodrug) while the hydroxy acid forms are the active forms that lower plasma cholesterol levels. The lactone form of statins can be absorbed from the gastrointestinal tract and converted to the active drug form by esterases in the intestinal wall, liver, and

plasma.[75, 77] On the other hand, the hydroxy acid form of statins can be converted to the corresponding lactone forms mostly by glucuronidation by UDP-glucuronosyltransferase enzymes and to a lesser extent by β-oxidation of the dihydroxy heptanoic acid side chain both *in vitro* and *in vivo*.[78-83]

Both lactone and hydroxy acid forms were detected in the systemic circulation of human and animals after oral administration of lovastatin,[84] atorvastatin,[85] simvastatin[86, 87] and cerivastatin[88] suggesting the presence of inter-conversion between both forms.

A marked difference in solubility exists between the lactone and hydroxy acid forms of statin. The lactone forms of statins are generally more lipophilic than the corresponding hydroxy acid forms which have poor lipid solubility.[89] Such difference in statin lipophilicity makes the lactone forms more potent in inducing myotoxicity compared to the corresponding acid forms with a 14 fold for atorvastatin lactone, 23 fold for pravastatin lactone, 26 fold fluvastatin and 37 fold of simvastatin lactone.[76]

1.4.3 Roles of comorbidities in statin-induced myotoxicity

The incidence of statin-induced myotoxicity is higher among patients with pre-existing muscle disorders which could be either hereditary (e.g. carnitine palmitoyl transferase II deficiency, McArdle's disease and myoadenylate deaminase deficiency) or acquired (e,g. post-poliomyelitis syndrome).[65]

Conditions that are known to increase plasma and tissue concentration of statins are expected to enhance the risk of statin-induced myotoxicity. Patients with multi-system disease are more likely to be at higher risk of developing myopathy, especially those with hepatic or renal failure, diabetic mellitus,

hypertension, hypothyroidism, serious infections, drug abusers and those undergoing major surgical operations.[24, 90-92] Exercise in combination with statin treatment has been shown to induce greater muscle damage as assessed by CK levels.[93] In patients with comorbidities, the prevalence of statin-related myopathy rates increases from 0.75% with monotherapy to 2.12% with combination therapy.[94]

1.4.3.1 Acid-base imbalance and statin-induced myotoxicity

The pH of blood is normally maintained within a very narrower range of 7.35 - 7.45.[95] Acidosis or alkalosis usually develop when there is a mild disturbance in blood pH outside this range.[96] Deviations of systemic pH in either direction can have profound impacts on many aspects of drug actions.[97] It is well established that blood pH alteration has a significant influence on the pharmacokinetics and tissue distribution of a number of drugs. In fact, pH differences across both sides of cell membranes govern the transfer of many weak acidic and basic drugs across the cell membranes by affecting the lipophilic/ hydrophilic proportions of the drug.[98] Alteration of plasma pH has also been reported to affect plasma protein binding of some drugs such as amitriptyline, fentanyl, and lidocaine resulting in clinically significant consequences.[99, 100]

A number of lactone-containing drugs have been reported to undergo pH-dependent inter-conversion into open ring forms resulting in alteration in the physicochemical properties and drug efficacy and safety. The list of these drugs include statins,[87, 101] camptothecin anticancer drugs (such as camptothecin, topotecan, irinotecan and SN-38)[102, 103] and eplerenone (a new

antihypertensive drug).[104, 105] It has been reported that the lactone form of statins could be easily converted to the hydroxy acid form in water and plasma, and the conversion would be enhanced in alkaline solutions.[77] At physiological pH and higher, the lactone form is unstable and favours hydrolysis to the hydroxy acid form.[77, 101, 106]

An *in vitro* study evaluated the relationship between pravastatin and rosuvastatin cytotoxicity and medium pH alteration using L6 rat skeletal muscle and RD cell lines. This study showed that, at acidic pH, rosuvastatin induces morphological changes with significantly higher cytotoxicity, reduction of cell viability and caspase activation than at neutral pH.[107] Although this study did not determine the concentrations of the hydroxy acids and lactone forms, the higher cytotoxicity was accompanied by increased rosuvastatin accumulation under acidic conditions, probably due to enhanced rosuvastatin lipophilicity by the acidic medium.

A number of *in vitro* and *in vivo* studies have suggested protective effects of bicarbonate and citrate against statin-induced muscle damage without exploring the molecular mechanisms by which these compounds produce their actions.[59, 108] In one study, bicarbonate has been suggested to suppress statin-induced caspase activation and apoptosis in RD cell line by inhibiting intracellular acidification provoked by cerivastatin.[59]

1.4.3.2 Hyperlipidaemia and statin-induced myotoxicity

Hyperlipidaemia is defined as an elevation in plasma cholesterol, triglyceride levels or both. This elevation is always accompanied by an increase in plasma lipoprotein levels since fats are not soluble in the aqueous

environment of the blood and require special carriers in order to be transported in the circulation.[109, 110] Pathological hyperlipidaemia is a chronic condition associated with elevated levels of VLDL and LDL. It could be either primary due to genetic abnormality in lipoprotein receptors, apoproteins or lipoprotein lipase (LPL) or secondary to other underlying disorders.[111-113] Postprandial hyperlipidaemia, on the other hand, is often induced by consumption of fat-containing meals and is characterised by a transient elevation in triglyceride-rich lipoproteins (TRL) in plasma, mostly chylomicrons.[114]

Lipoproteins are heterogeneous macromolecular aggregates composed of a core of non-polar lipids (triacylglycerides and cholesteryl esters) surrounded by a monolayer of amphipathic lipids (phospholipids and unesterified cholesterol) in which apoproteins are embedded (Figure 1.4).[115, 116] These lipoproteins are classified according to their density into chylomicrons which have the lowest density followed by VLDL, LDL and high-density lipoprotein (HDL).[109, 110]

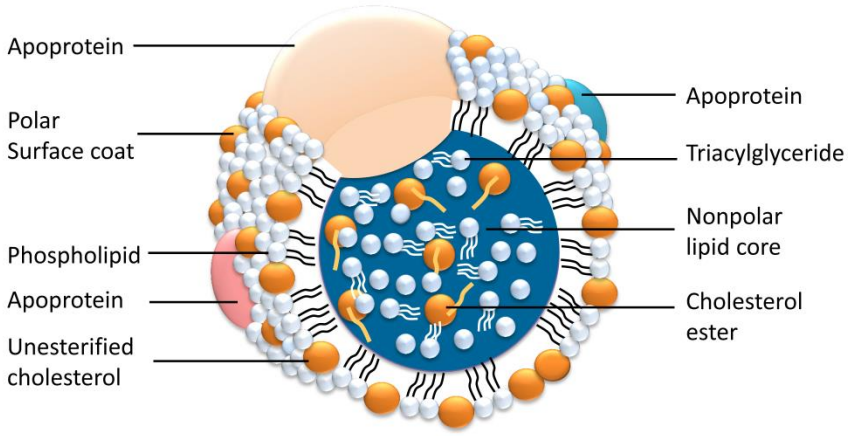


Figure 1.4: Structure of lipoprotein particles.[117]

The principle function of plasma lipoprotein is the transport of endogenous and exogenous lipids around the body.[118] Lipophilic drugs have

the tendency to associate with plasma lipoproteins. These lipoproteins can serve as potential carriers that deliver lipophilic drug molecules to various tissues via the lipoprotein receptors present on the surface of different cells.[119] Alteration in plasma lipoprotein levels and/or compositions in response to postprandial or pathological hyperlipidaemia can influence the extent of drug association with plasma lipoproteins [116, 120, 121] This would affect not only the pharmacokinetics and tissue distribution of lipophilic drugs but also the pharmacological activities and potential toxicities of these drugs.[117, 122]

There is growing evidence to indicate that binding of some lipophilic drugs such as amphotericin and ciclosporin to plasma lipoproteins enhances the toxicity of these drugs.[123-126] Furthermore, elevation of plasma lipoprotein levels has been reported to prolong the serum half-lives of a number of lipophilic drugs that demonstrated considerable associations with these lipoproteins.[127] It is conceivable that lipophilic statins would associate with plasma lipoproteins and that such association could influence their efficacy and toxicity profiles since these drugs are often administered to hyperlipidaemic patients.

Association of lipophilic drugs with plasma lipoprotein may occur either within the non-polar core of lipoprotein particles or on the outer, more polar surface. The apoproteins present on the outer surface of the lipoprotein particle help in recognising the lipoprotein receptors existing on the cell membrane of various tissues. Therefore, unlike drug binding with plasma proteins which often restrict drug from entering the tissues, the association with plasma lipoproteins can facilitate receptor-mediated uptake of drug by the tissues.[117]

1.5 Project Hypotheses

1.5.1 Effect of disturbance in acid-base balance on statin-induced myotoxicity

Disturbance in acid-base balance is expected to affect the inter-conversion of statins between the lactone and hydroxy acid forms. Since each form has different lipophilicity, it is possible that such pH-dependent inter-conversion would affect the distribution and tissue uptake of statins into skeletal muscle cells resulting in altered therapeutic and toxic profiles of statins (Figure 1.5).

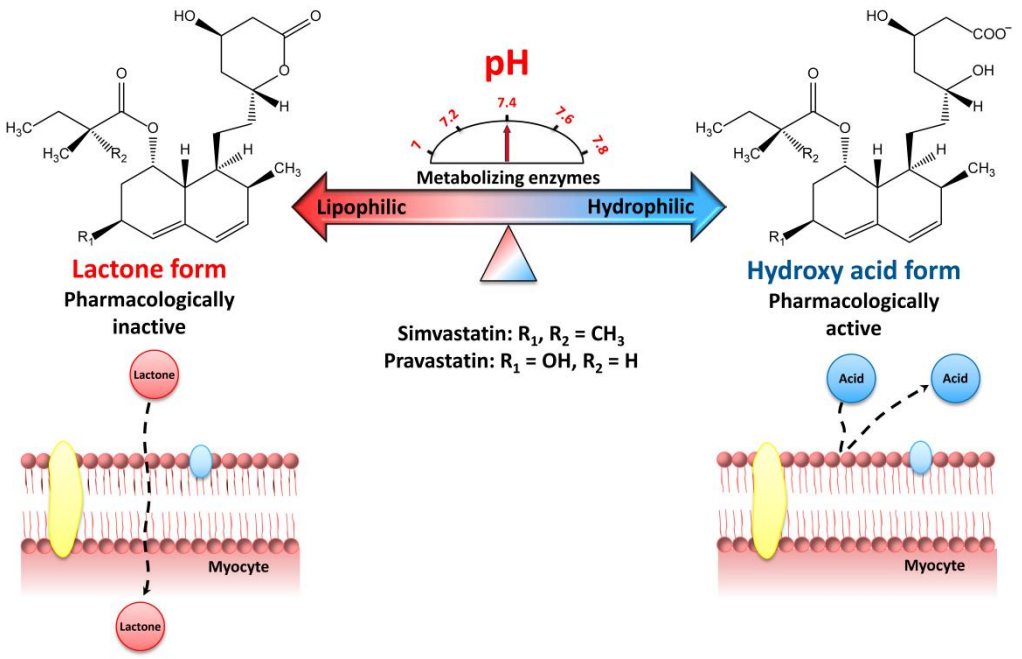


Figure 1.5: Schematic diagram for the possible mechanisms of statin inter-conversion between lactone and hydroxy acid forms and the potential effect on membrane permeability. The inter-conversion between the two forms is mediated by pH and enzyme-dependent processes. The higher lipophilicity of statins in the lactone form can potentially facilitate their penetration into muscle cells and consequently induce high local drug concentrations within skeletal muscle tissues.

1.5.2 Effects of postprandial or pathological hyperlipidaemia on development and potentiation of statin-induced myotoxicity

Administration of lipophilic statins to patients with pathological hyperlipidaemia (persisting despite the statin treatment) or postprandial hyperlipidaemia could potentially contribute to enhanced muscle toxicity. Transient elevation in TRL, mainly chylomicrons, occurs following the consumption of a high-fat meal.[128] Similarly, pathological hyperlipidaemia is associated with elevated levels of some lipoproteins (VLDL and LDL). These lipoproteins are the major carriers of lipids in the systemic circulation, and can also serve as potential carriers for lipophilic statins.[129, 130] It is conceivable that lipophilic statins can associate with plasma lipoproteins in the general circulation, and this, in turn, can facilitate their delivery to the peripheral tissues. Lipoprotein lipase present at the surface of the capillary endothelium of cardiac and skeletal muscle and adipose tissue mediates the hydrolysis of triglycerides within the TRL to fatty acids and monoacylglycerol. Released free fatty acids are then transported across cell membranes to the tissues of LPL synthesis and secretion.[131] It is reasonable to assume that this process could be accompanied by an increase in the uptake of lipoprotein-associated lipophilic statins by the skeletal muscle and consequently contribute to increased muscle toxicity. In addition, LPL also mediates the internalisation of LDL into cells (as reported in several studies[132-134]), which could be an additional mechanism of enhanced intracellular accumulation and toxicity of statins in hyperlipidaemic state. Therefore, the administration of lipophilic statins to patients with persisting pathological or postprandial hyperlipidaemia could potentially contribute to enhanced muscle toxicity. (Figure 1.6).

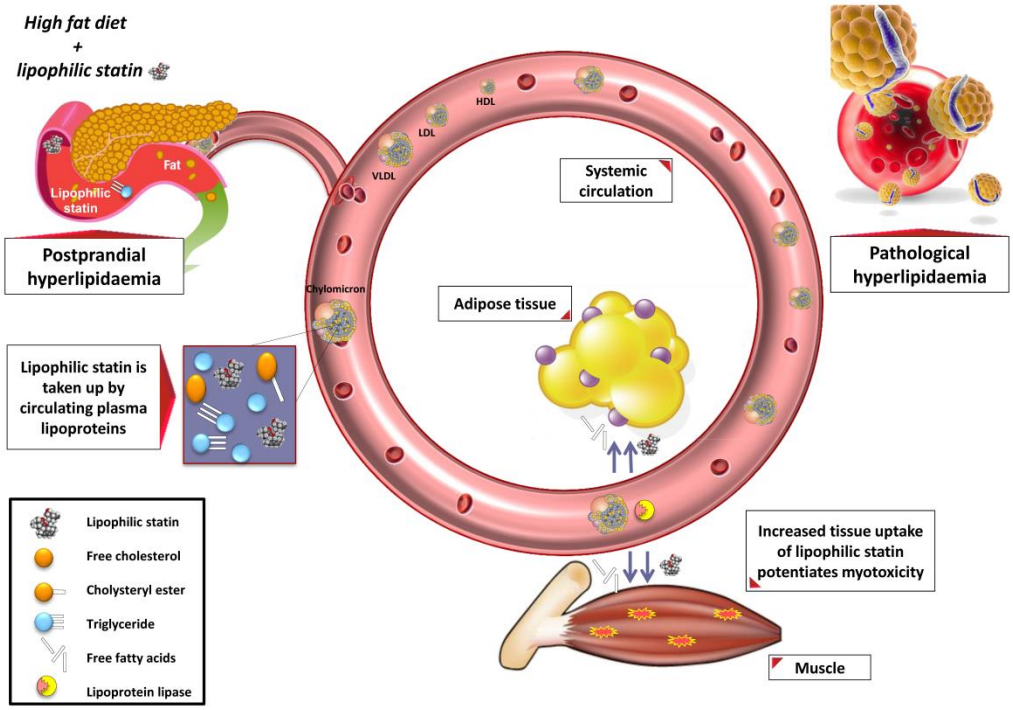


Figure 1.6: Schematic diagram for the possible role of elevated plasma lipoprotein levels in tissue distribution and myotoxic potential of lipophilic statins.

1.5.3 Combined effects of acid-base imbalance and hyperlipidaemia on statin-induced myotoxicity

The coincidence of hyperlipidaemia with acid-base imbalance can impose even higher risk to statin-induced myotoxicity and could potentially affect the pharmacological and toxicological outcomes of statin therapy. Acid-base imbalance can alter the lipophilicity of statins by inter-converting statins between hydroxy acid and lactone forms. Higher lipophilicity of statins might occur as a result of hindering the chemical conversion of the lipophilic lactone form at acidic pH. This would enhance the association of statins with plasma lipoproteins and consequently increase their penetration into muscle cells resulting in high local drug concentrations within skeletal muscle tissues.

1.6 Project Aim

The overall aim of this project is to elucidate the effects of acid-base imbalance, postprandial or persisting pathological hyperlipidaemia, as well as the combined effects of acid-base imbalance and hyperlipidaemia on the development and severity of statin-induced muscle toxicity.

Simvastatin was selected as a model lipophilic statin in these studies due to its high lipophilicity, wide clinical use and high incidence of reported simvastatin-associated muscle toxicity.[20, 135] Pravastatin was selected as a model hydrophilic statin with expected lower myotoxicity.

The objectives include assessment of the effect of disturbances in acid-base balance on the inter-conversion of statins between the lactone and hydroxy acid forms, determination of statin uptake by muscle cells under conditions relevant to physiological pH, acidosis and alkalosis and evaluation of the role of statin inter-conversion in skeletal muscle toxicity.

The study also involves prediction of statins association with chylomicrons using *in silico* model, assessment of statin association with artificial chylomicron-like particles rat and human lipoproteins at different pH levels. The influence of statin association with plasma lipoproteins on the cellular uptake and cytotoxicity were evaluated using skeletal muscle cells that express different levels of LPL under conditions of altered pH levels.

Chapter Two

2 Materials and Methods

2.1 Materials

Simvastatin lactone (SVL, 99.3%) and pravastatin hydroxy acid sodium (PVA, 99.4%) were purchased from Kemprotec Ltd (Lancashire, UK). Simvastatin hydroxy acid ammonium salt (SVA, 98.0%) and pravastatin lactone (PVL, 98%) were obtained from Toronto Research Chemicals Inc (Toronto, Canada), lovastatin lactone (LOV-L, 97.0%) from Cayman (Leicestershire, UK) and 4,4-dichlorodiphenyltrichloroethane (DDT, 98.0%) from Sigma-Aldrich (Dorset, UK). Lovastatin hydroxy acid sodium (LOV-A, 98.0%), griseofulvin (97.0%), methyl *tert*-butyl ether (MTBE), thiazolyl blue tetrazolium bromide (MTT) and Bradford reagents were purchased from Alfa-Aesar (Lancashire, UK). Dulbecco's Modified Eagle Medium (42430025-DMEM, High glucose, HEPES without sodium pyruvate), pooled human liver microsomes, and SuperScript™ IV reverse transcriptase were purchased from Invitrogen-Life Technologies (Paisley, UK). Total RNA isolation kit (NucleoSpin RNA II) was supplied by Macherey-Nagel, GmbH & Co. KG (Düren, Germany). GoTaq® qPCR Master Mix was obtained from Promega (Southampton, UK). Random hexamers (50 µM), deoxynucleotide triphosphates (dNTP) mix (10 mM each) and Pierce™ lactate dehydrogenase (LDH) cytotoxicity assay kit were purchased from Thermo Fisher Scientific (Loughborough, UK). Magnesium chloride ($MgCl_2$), monobasic potassium phosphate (KH_2PO_4), dibasic potassium phosphate (K_2HPO_4) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (Gillingham, UK). Pooled hyperlipidaemic human plasma was

obtained from Biological Specialty Corporation (Pennsylvania, USA). LPL activity fluorometric assay kit and total cholesterol and cholesteryl ester colorimetric assay kit were purchased from T/A Source BioScience Ltd (Wiltshire, UK). Microsep™ advance centrifugal device with Omega™ membrane was obtained from VWR International Ltd (Leicestershire, UK). Hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium bromide (KBr), mono and dibasic sodium phosphate, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), Intralipid® 20% emulsion, triglyceride reagent, free glycerol reagent and glycerol standard were obtained from Sigma-Aldrich (Dorset, UK). Peanut oil, acetonitrile (ACN), ammonium acetate, and HPLC grade water were purchased from Fisher Scientific (Leicestershire, UK). C2C12 cells transfected with mouse lipoprotein lipase (mLPL), human lipoprotein lipase (hLPL) and empty vectors were kindly donated by Professor Robert H. Eckel at School of Medicine, University of Colorado. All reagents were of HPLC grade and were used without further purification.

2.2 Development and Validation of HPLC Methods

2.2.1 Preparation of standard and quality control samples

Stock solutions of analytes (simvastatin hydroxy acid, simvastatin lactone, pravastatin hydroxy acid and pravastatin lactone) were prepared in DMSO at a concentration of 1 mmol/L. DDT and griseofulvin were used as internal standards for simvastatin and pravastatin, respectively. Stock solutions of internal standards (IS) were prepared in acetonitrile at a concentration of 100 µg/mL. Working solutions of the analytes were prepared immediately before preparation of calibration curves and quality control samples by diluting stock

solutions in ACN. Nine-point calibration curves were prepared by spiking 10 µL of working solutions to 90 µL of the matrix (phosphate buffer saline [PBS], human plasma, and DMEM culture medium). Quality control samples were prepared at four levels for lower limit of quantification (LLOQ), low (LQC), medium (MQC), and high concentration quality control (HQC). Ammonium acetate buffer (100 mmol/L) was prepared using double de-ionized distilled water (resistivity = 18.2 MΩ-cm). The pH of the buffer was adjusted to 4.5 using glacial acetic acid and the buffer was filtered with through a 0.45-µm Millipore nylon membrane filter.

2.2.2 Sample preparation

Samples for HPLC analysis were prepared by protein precipitation followed by liquid-liquid extraction. A 100 µL of human plasma or DMEM samples were pipetted into 1.5 mL Eppendorf tubes and 50 µL of ammonium acetate buffer (100 mmol/L, pH 4.5) along with 10 µL of internal standard solution (DDT or griseofulvin 100 µg/mL for simvastatin and pravastatin containing samples, respectively) was added and samples were vortex mixed for 30 seconds. Proteins were precipitated by the addition of 300 µL of chilled acetonitrile and samples were vortex mixed for 1 minute and then centrifuged at 900*g* for 10 minutes at 4°C. After centrifugation, the acetonitrile layer was transferred into new glass test tubes and 3 mL of methyl *tert*-butyl ether were added to each sample, vortex mixed for 10 minutes and centrifuged at 1,615*g* for 10 minutes at 4°C. Finally, the upper organic layers were separated using glass Pasteur pipette, transferred into new glass tubes and the contents of the tubes were evaporated to dryness using Techne® sample concentrator (Bibby

Scientific Ltd, UK). The dry residues were reconstituted with 100 µL of the mobile phase, vortex mixed and placed into appropriate HPLC vials. Samples from PBS were extracted in the same way with one exception that the protein precipitation step was skipped.

2.2.3 HPLC conditions

HPLC analysis was performed using Waters® Alliance 2965 separation module equipped with Waters® 996 Photodiode Array Detector (PDA) and integrated autosampler. System control and data processing were performed using Empower software. Chromatographic separation was achieved by ACE® Excel Super C18 column (100 X 3mm, 3 µm) under isocratic conditions with a mobile phase consisting of acetonitrile: 5 mmol/L ammonium acetate buffer, pH 4.5 (73:27 and 55:45, v/v for simvastatin and pravastatin samples, respectively). The flow rate was set at 0.4 mL/min for simvastatin and 0.3 mL/min for pravastatin samples. Samples temperature was kept at 4°C and the column temperature was set at 40°C. Chromatographic separation was monitored by PDA detector at 238 nm with an injection volume of 20 µL.

2.2.4 HPLC-UV methods validation

Validation of the analytical methods was carried out by determining the intra- and inter-day accuracy and precision. Six replicates of statin-containing samples (PBS, human plasma, and DMEM) were analysed at four quality control levels (lower limit of quantification, low, medium and high concentration quality control [QC]). Precision was expressed as relative standard deviation (RSD%), whereas accuracy was described as relative error

(RE%) and was determined by comparing the calculated concentration obtained using calibration curves to the theoretical concentrations. Intra- and inter-day precisions and accuracies were calculated by analysing QC samples on the same day and on 6 different days over a period of one month, respectively.[136-138] The acceptance criteria for intra- and inter-day precisions and accuracies were set at 15% for the high, medium and low concentration QC samples and at 20% for lower limits of quantification.[136-138]

Extraction recoveries of the lactone and hydroxy acid forms of statins were also determined at high, medium, and low QC levels by comparing the peak area ratios of the analytes spiked after extraction to those spiked before extraction.[139-141] Six replicates were evaluated at each QC level.

2.3 Development and Validation of LC-MS/MS Methods

An LC-MS/MS analysis was used to determine the concentrations of statin hydroxy acid and lactone forms recovered from microsomal stability samples, plasma lipoprotein fractions and cellular uptake studies by undifferentiated and differentiated C2C12 cells in response to medium pH alteration, hyperlipidaemia or both.

2.3.1 Sample preparation

100 µL of cell suspension or microsomal stability samples were transferred into 1.5 mL Eppendorf tubes and 50 µL of ammonium acetate buffer (100 mmol/L, pH 4.5) along with 10 µL of IS solutions (lovastatin hydroxy acid and lovastatin lactone, 2.5 µg/mL) were added and samples were vortex mixed for 30 seconds. 300 µL of ice-cold ACN was added to each

sample to precipitate proteins and cell lysis was performed using Retsch MM-301 mixer mill. Samples were centrifuged at 15,000 *g* for 10 minutes at 4 °C to remove the debris. Following centrifugation, the acetonitrile layer was transferred into new glass test tubes and liquid-liquid extraction was performed in the same way as described for HPLC-UV method using methyl tert-butyl ether (Section 2.2.2).

Samples from lipoprotein fractions were prepared in the same way as for microsomal stability and cell suspension samples except 300 µL of THF was used instead of ACN.

2.3.2 LC-MS/MS Conditions

LC-MS/MS system consisted of Quattro Ultima triple quadrupole mass spectrometer (Micromass, UK) interfaced via an electrospray ionization (ESI) probe with Agilent (1100 Series, Agilent Technologies) HPLC system. The HPLC system consisted of a binary pump, online degasser, temperature-controlled autosampler and column compartment. Chromatographic separation was achieved by ACE® Excel Super C18 column (100 X 3 mm, 3 µm) with a mobile phase consisting of acetonitrile: 5 mmol/L ammonium acetate buffer, pH 4.5. An isocratic condition consisted of ACN: ammonium acetate (80:20, v/v) was used for simvastatin separation. Pravastatin was separated using gradient flow as follows: 65% ACN over the first four minutes, increased to 75% ACN over a period of one minute and kept at 75% ACN for another two minutes, and then the flow was returned to 65% ACN in the last minute to prepare for the next run. The flow rate was set at 0.3 mL/min. Samples temperature was kept at 4°C and the column temperature was set at 40°C.

Quantification was performed using multiple-reaction monitoring (MRM) scan. The mass spectrometric system was operated in the negative ionization mode for quantification of the hydroxy acid forms and positive mode for determination of the lactone forms. Instrument control and data acquisition were performed by Masslynx software packages (version 4.1). Data processing and analysis were performed using Quanlynx software. Nitrogen was used for nebulization and as a dissolution gas, while argon was used as a collision gas. Source temperature and desolvation temperature were setup at 125 and 350 °C, respectively. The flow rate of the cone gas and desolvation gas were set at 150 and 565 L/hr, respectively. Capillary voltage, cone voltage, and collision energy were optimized individually for each compound as indicated in Table 2.1 by direct infusion with mobile phase using injection pump.

Table 2.1: Optimized LC-MS/MS parameters used for analysis of statins.

Compound	Precursor type	Precursor ion *m/z*	Product ion *m/z*	Capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)
SVL	**$[M+Na]^+$**	**441**	**199.17**	**4.00**	**35**	**23**
SVA	**$[M-H]^-$**	**435.49**	**319.43**	**3.20**	**45**	**14**
PVL	**$[M+Na]^+$**	**429**	**183.22**	**3.5**	**35**	**18**
PVA	**$[M-H]^-$**	**423.23**	**321.37**	**3.2**	**35**	**16**
LOV-L	**$[M+Na]^+$**	**427.15**	**325.36**	**4.00**	**35**	**23**
LOV-A	**$[M-H]^-$**	**421.08**	**319.54**	**3.20**	**45**	**14**

SVL, simvastatin lactone; SVA, simvastatin acid; PVL, pravastatin lactone; PVA, pravastatin acid; LOV-L, lovastatin lactone; LOV-A, Lovastatin acid; *m/z*, mass to charge ratio.

2.3.3 LC-MS/MS methods validation

Validation of the LC-MS/MS methods was carried out by determining the intra- and inter-day accuracy and precision of six replicates of lipoprotein and cell lysate samples. Assay variability was examined at the four QC levels (LLOQ, LQC, MQC, and HQC) as described previously (Section 2.2.4).

2.4 Inter-conversion of Statins in Phosphate Buffer Saline, Human Plasma, and DMEM Culture Medium

2.4.1 pH modification of PBS, human plasma, and DMEM culture medium

The pH of PBS was adjusted to the target values (6.8-7.8) by adding an appropriate volume of either 1.0 N HCl or NaOH followed by pH measurement using Mettler Toledo T50 pH titrator.

Sodium phosphate buffer was used to adjust the pH of human plasma as previously described with some modification.[142] The pH adjustment was done in three steps. In the first step, a fixed volume of human plasma samples was adjusted to the target pH (6.8-7.8) by adding appropriate volumes of either mono or dibasic sodium phosphate solution. After that, series of sodium phosphate buffer solutions of predefined pH were prepared at a concentration of 1 mol/L by mixing appropriate volumes of mono and dibasic sodium phosphates solution. Finally, one volume of phosphate buffer solutions (of defined pH) was mixed with nine volumes of plasma samples, the pH of the resulting mixture was measured by Mettler Toledo T50 pH titrator and minor adjustments were made as appropriate.

The pH of DMEM culture medium was adjusted to the target values (6.8-7.8) by adding an appropriate volume of either 1.0 N HCl or NaOH to a medium containing 20 mmol/L HEPES buffer. The pH was measured at incubation temperature (37°C) by Mettler Toledo T50 pH titrator. Before the experiments, the medium was kept in an incubator for 24 hours under cell culture conditions (37°C and 5% CO_2) to allow the desired pH ranges to equilibrate. After equilibration, minor adjustment in pH was occasionally

required to reach the desired final pH.[143] Maintaining medium under a constant CO_2 environment of 5% stabilizes the pH through CO_2-HCO_3^- equilibrium.[144]

2.4.2 Inter-conversion study

The inter-conversion of the lactone and hydroxy acid forms of simvastatin and pravastatin was investigated by incubating tested compounds with PBS, human plasma, and DMEM culture medium of defined pH values at 37°C for a predetermined period of time. Four different concentrations were examined for each statin (12.5, 25, 50 and 100 µmol/L for simvastatin lactone and hydroxy acid and 25, 50, 100 and 200 µmol/L for pravastatin lactone and hydroxy acid). PBS and human plasma samples were incubated in a C25 classic incubator shaker (New Brunswick Scientific, USA). For DMEM samples, CO_2 supply was maintained at 5% throughout the experiment in a tissue culture incubator. The pH of different matrices was adjusted to simulate physiological pH, acidosis, and alkalosis as described previously (Section 2.4.1). After incubation, the inter-conversion reaction was stopped at predetermined time points by adding 50 µL of ice-cold ammonium acetate buffer (100 mmol/L, pH 4.5) to 100 µL of tested samples.[87] Six replicates were tested at each pH level and the concentrations of parent and corresponding forms were measured by fully validated HPLC analytical methods as described previously (Section 2.2).

2.5 Metabolic Microsomal Stability

Microsomal metabolic stability assay was conducted using human liver microsomes. The reaction mixture consisted of $MgCl_2$, human liver

microsomes, NADPH and simvastatin lactone at final concentrations of 10 mmol/L, 0.5 mg protein/mL, 1 mmol/L and 1 µmol/L, respectively, in 84.7 mmol/L potassium phosphate buffer. The buffer was prepared at three different pH levels of 6.8, 7.4 and 7.8 (simulating acidosis, physiological pH and alkalosis, respectively). The reaction mixture was pre-incubated at 37°C in a water bath for 3 minutes prior to the assay. The reaction was initiated by addition of NADPH to the mixture. Samples of 100 µL volume were withdrawn from the reaction mixture at 0.25, 5, 10, 20, 30 and 40 minutes, and transferred into new tubes containing 1 mL of acetonitrile to terminate the reaction. Vortex-mixing of 30 seconds was applied to each sample and then the samples were subjected to sample preparation for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using the method described in the previous section (2.3). Half-life ($t_{1/2}$) of simvastatin lactone was calculated using the following equation:

$$t_{1/2} = -\frac{0.693}{k} \qquad \textit{(Eq. 1)}$$

Where *k* is the slope of the plot of natural log percentage of simvastatin lactone versus incubation time. The experiment was performed in triplicates.

2.6 Cellular Uptake of Statins in Response to Altered Medium pH

2.6.1 C2C12 growth and differentiation

C2C12 are mouse myoblast cells capable of spontaneous differentiation into myotubes under low serum conditions. These cells were originally derived from satellite cells of the thigh muscle of C3H mice after a crush injury.[145, 146] C2C12 mouse myoblast cells were cultured in a humidified environment of 5% CO_2 at 37°C. Cells were maintained sub-confluent (70-80%) by growing in

DMEM medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin antibiotic mixture. Myogenic differentiation was induced by growing the cells in differentiation medium containing 2% horse serum. The cells were cultured over a period of 4 to 6 days to allow complete differentiation and the medium was replaced every 24 hours.[147]

2.6.2 Characterisation of myogenic differentiation of C2C12

To verify the differentiation of C2C12, the expression of 2 markers of myogenic differentiation (myogenin and myosin heavy chain [MHC]) were assessed by real-time reverse transcriptase quantitative polymerase chain reaction (qPCR) at different stages of cells differentiation. C2C12 was cultured in a 6-well plate at a density of 2 x 10^5/ mL. The cells were allowed to attach to the well surface by incubation at 37°C and 5% CO_2. Differentiation was induced on 80% confluence by switching the cells to differentiation medium. On differentiation, the medium was aspirated from the wells and the cells were harvested. Total RNA was extracted from undifferentiated and differentiated cells at different differentiation stages (day 1, 3, 5, 7 and 9) using NucleoSpin RNA II extraction kit (the manufacturer's protocol was followed). The extracted RNA was reverse transcribed into cDNA using random hexamer primers, dNTP mix (10 mM each) and SuperScript™ VI (reverse transcriptase purified from *Escherichia. coli*), and the resultant cDNA was used as a template for PCR amplification.

The expression of differentiation marker genes (myogenin and MHC) and 4 reference genes (TATA box-binding protein [Tbp], glyceraldehyde-3-phosphate dehydrogenase [Gapdh], hypoxanthine phosphoribosyl transferase

[Hprt] and ribosomal protein S12 [Rps12]) was assessed by real-time qPCR using GoTaq® qPCR Master Mix (Promega, UK) and previously published primer sets.[148] The primers used for the qPCR were synthesized by Sigma-Aldrich Biotechnology, UK (sequences are listed in Table 2.2).

Table 2.2: Primers used for gene expression quantification of differentiation markers.

Gene	Direction	Primer sequence (5´-3´)	Melting temperature (°C)	Amplicon length (bp)*
Myogenin	Forward	CAGGAGATCATTTGCTCG	55.2	122
	Reverse	GGGCATGGTTTCGTCTGG	62.5	
MHC	Forward	AGGGAGCTTGAAAACGAGGT	60.2	260
	Reverse	GCTTCCTCCAGCTCGTGCTG	65.9	
Tbp	Forward	TGCACAGGAGCCAAGAGTGAA	64.4	132
	Reverse	CACATCACAGCTCCCCACCA	65.6	
Gapdh	Forward	ATGTTTGTGATGGGTGTGAA	57.2	151
	Reverse	ATGCCAAAGTTGTCATGGAT	57.9	
Hprt	Forward	ATGGACTGATTATGGACAGGACTG	61.5	124
	Reverse	TCCAGCAGGTCAGCAAAGAAC	62.8	
Rps12	Forward	AAGGCATAGCTGCTGGAGGTGTAA	65.3	156
	Reverse	AGTTGGATGCGAGCACACACAGAT	67.4	

*bp= base pairs.

DNA amplification was carried out using Rotor-Gene® Q, (Qiagen thermal cycler). The PCR thermocycling program consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at the optimal annealing temperature of 55°C and 30 seconds at 72°C for extension. To confirm the amplification specificity, PCR products were subjected to melting curve analysis. A standard curve was constructed for each sample and was derived from 10-fold serial dilution of cDNA template.

The levels of gene expressions in each sample over the days of differentiation were calculated relative to their expression in undifferentiated cells. Data were normalized relative to the expression of Tbp gene (a reference gene with minimal fluctuation in expression over the periods of cell differentiation). All samples were run in triplicate and the mean value was used

for subsequent analysis. The ΔΔ cycle threshold (Ct) value for each gene was determined by calculating the difference between the Ct value of the target gene and the Ct value of the reference gene. The normalized level of the gene expression in each sample was calculated using the formula $2^{-\Delta\Delta Ct}$, and the results were expressed as fold changes in gene expression from the baseline level observed with undifferentiated C2C12 myoblast cells. Three reference genes (Gapdh, Hprt, and Rps12) were used for validation of gene expression. The size and the integrity of the amplicon in each sample were assessed by running the qPCR products on 1.2% agarose gel after staining with SYBR® safe (Invitrogen-Life Technologies, UK) and the bands were visualized under UV light.

2.6.3 Uptake study

C2C12 cells were cultured in 10 cm dishes at a density of 2×10^5/ mL. Cells were allowed to attach for 24 hours or to differentiate for 4 days by switching to differentiation medium containing 2% horse serum. Before starting the uptake study, the medium was removed and replaced with a fresh medium of modified pH (relevant to physiological pH, acidosis, and alkalosis) and the cells were allowed to equilibrate for 10 minutes. The uptake study was initiated by adding simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone or pravastatin hydroxy acid to the medium at a concentration of 1 µmol/L. Cells were incubated for a predetermined period of time at 37°C under CO_2 environment of 5%. Statins uptake was terminated by suctioning off the DMEM containing the tested drug, and the cells were washed twice with ice-cold PBS (pH 7.4). Cells were harvested by trypsinisation and resuspended in

200 µL PBS (pH 7.4). Five replicates were tested at each pH level and the intracellular concentration of both the lactone and the corresponding hydroxy acid forms of simvastatin and pravastatin were determined in 100 µL of cell suspension using fully validated LC-MS/MS assays. Cellular protein content was determined in the remaining cell suspension after pelleting and lysing the cells with radioimmunoprecipitation assay (RIPA) lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 8.0], 0.5% Nonidet P-40 [NP-40], supplemented with proteinase inhibitor and phosphatase inhibitor) as described in the next Section 2.6.4.

2.6.4 Determination of cellular protein concentration

Protein concentration from cell lysate samples was determined using Bradford assay.[149] This colorimetric assay is based on the formation of a blue coloured complex as a result of protein interaction with the dye coomassie brilliant blue G-250. The complex has a maximum absorbance 595 nm and the intensity of the complex's colour is proportional to the amount of protein present in the sample.

The assay was performed according to the manufacturer's instructions. Briefly, cell lysate samples were first diluted by 4-6 folds using PBS to achieve protein concentrations within the assay limit. Then 5 µL of unknown protein sample, standard protein solution, and lysis buffer was added to 200 µL of Bradford reagent in separate wells of a 96 well plate. The plate was briefly shaken for 30 seconds and the samples were incubated at room temperature for 30 minutes protected from light. Absorbance was measured at 595 nm using EnVision® Multilabel Plate Reader (PerkinElmer, USA). A standardised curve

was constructed using bovine serum albumin (BSA) over concentrations ranging from 0-1.4 mg/mL by plotting the net absorbance versus the protein concentration from each standard sample. Finally, protein concentrations of the unknown samples were determined by comparing the net absorbance values at 595 nm against the standard curve.

2.7 Expression of Cellular Transporters

The mRNA expression levels of organic anionic transporting polypeptide Oatp1a4 and Oatp2b1 (uptake transporters) and multiresistant protein Mrp1, Mrp4 and Mrp5 (efflux transporters) were assessed using reverse transcriptase-PCR analysis. C2C12 cells were cultured in 10 cm dishes as described previously (Section 2.6.3). Both undifferentiated and differentiated cells were maintained in their corresponding mediums of modified pH (relevant to the physiological pH, acidosis, and alkalosis) for 6 hours. RNA extraction, cDNA synthesis, and gene amplifications were performed as described early (Section 2.6.2). The primer sequences of the transporter and reference genes are listed in Table 2.3.

Three different sets of primers were attempted to amplify Oatp1a4 and Oatp2b1 influx transporters. The levels of gene expressions in undifferentiated and differentiated cell samples maintained at different pH levels were calculated relative to their expression in undifferentiated cells maintained at physiological pH. Transporters genes were normalized to Gapdh whereas each reference gene was normalized relative to the other three reference genes. Results were expressed as fold changes in gene expression relative to the

baseline level observed with undifferentiated C2C12 maintained at physiological pH.

Table 2.3: Primers used for gene expression quantification of cellular transporters.

Gene	Direction	Primer sequence (5´-3´)	Melting temperature (°C)	Amplicon length (bp) *
Mrp1	Forward	GTTATGGAAGCCTGTGCCCT	60.03	296
	Reverse	ATGACATCCACTTGGGGCAG	60.03	
Mrp4	Forward	CCTGGTGAGTTGTTAGCCGT	59.97	326
	Reverse	AGGTTCACCCGAGCTTTCTG	59.96	
Mrp5	Forward	CTTCCTGGGATCAGCGGTTT	60.04	244
	Reverse	GCTCTGAAAGTACCCGGCTT	60.04	
Oatp1a4	Forward	AACTCCCATCATGCCCTTGG	60.03	246
	Reverse	CCTGCACAGACCAAAAAGCC	59.97	
Oatp2b1	Forward	GCCAGAAGGAGGCATCAACT	60.03	233
	Reverse	TTAAAGGCTCGTGCTGGGAG	60.04	
Oatp1a4	Forward	CTCACTTGGCTGTGATTCTTGT	58.86	254
	Reverse	TCTTCTGATTGTCTCCAAATGTCT	57.88	
Oatp2b1	Forward	TCATCCTGAGAGGTGTGAAGAAAG	60.02	203
	Reverse	TATGAATCGGTTTCGGAGCAGG	60.48	
Oatp1a4	Forward	CCACGTCTGTAGTTGGGCTT	59.97	291
	Reverse	TCACACACTCTGTTGGGTCT	58.50	
Oatp2b1	Forward	TGCCAGAAGGAGGCATCAAC	60.32	346
	Reverse	ACCAGCAAGAAGATGGGGTG	59.96	
Gapdh	Forward	TGTGAACGGATTTGGCCGTA	59.96	159
	Reverse	ACTGTGCCGTTGAATTTGCC	59.97	
Hprt	Forward	AGTCCCAGCGTCGTGATTAG	59.54	170
	Reverse	TGATGGCCTCCCATCTCCTT	60.33	
Rps12	Forward	ACAAGAGGTGCTGAAGACCG	59.97	275
	Reverse	CTACGCAACTGCAACCAACC	60.04	
Tbp	Forward	CTCAGTTACAGGTGGCAGCA	59.96	118
	Reverse	GCACAGAGCAAGCAACTCAC	60.04	

* bp= base pairs.

2.8 *In Vitro* Cytotoxicity of Statins at Different Medium pH Levels

The effect of medium pH alteration on the cytotoxicity of statins was evaluated by MTT and LDH cytotoxicity assays. Both undifferentiated and differentiated C2C12 cells were used in these studies.

2.8.1 MTT assay

MTT assay is based on the capacity of the mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble tetrazolium salt into water-insoluble dark-blue/ purple formazan product. The amount of formazan product is directly proportional to the number of viable cells.[150] For MTT

assay, cells were seeded at a density of 4,000 cells/ well in 96-well plates and allowed to attach for 24 hours or to differentiate for 4 days. Before starting treatment, the medium was replaced by a fresh one with modified pH (6.8, 7.4 and 7.8). C2C12 cells were treated with different concentrations of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone or pravastatin hydroxy acid prepared in DMSO for 72 hours. The final level of DMSO in culture medium was determined by DMSO tolerance study and was found to be 0.25% (v/v) with no obvious cytotoxicity (100% viability compared to untreated cells). Constant volume of DMSO was maintained for all samples and DMSO alone without statins has been used as a control. Positive control samples with 100% cell death were obtained by treating cells with 1% Triton X-100. Four hours before the end of the incubation period, 10 µL of 5 mg/mL MTT solution in PBS was added to each well, and the plates were incubated at 37°C for the remaining time. Finally, the medium was removed and the formazan crystals formed were dissolved in 100 µL DMSO. Absorbance was measured at 570 nm using EnVision® Multilabel Plate Reader (PerkinElmer, USA). Results were expressed as percentage of the control and the half-maximal inhibitory concentration (IC_{50}) was calculated, when applicable, using Prism 6 (GraphPad Software Inc.).

2.8.2 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in different cell types. The enzyme is released into the cell culture medium when there is damage to the plasma membrane. LDH activity in the culture medium is used as an indicator of cell membrane integrity.[151] The enzyme activity can be

quantified by an enzymatic reaction that involves the conversion of lactate to pyruvate via the released LDH. This conversion is coupled with the reduction of NAD^+ to NADH. Diaphorase then utilities NADH to reduce a tetrazolium salt to a red formazan product. The level of formazan product generated is proportional to the amount of LDH released into the medium (Figure 2.1).

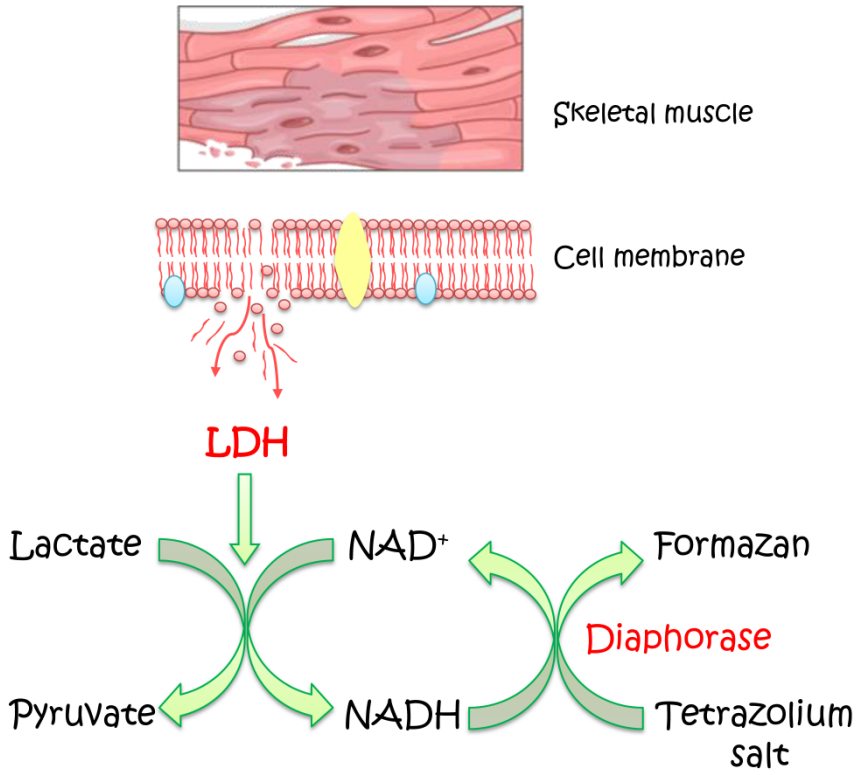


Figure 2.1: Schematic diagram of LDH cytotoxicity assay principle. Abbreviations: LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide.

For LDH assay, cells were cultured and treated as described above and the assay was performed according to manufacturer's instructions. Briefly, 50 µL of cell culture medium was transferred a flat-bottom 96-well plate and mixed with 50 µL of the reaction medium (containing a mixture of NAD^+, LDH Diaphorase, iodotetrazolium chloride, and sodium lactate in the assay buffer). The plate was then incubated at room temperature for 30 minutes

protected from light. At the end, 50 µL of stop solution was added to each sample well and samples were mixed gently by tapping. The formation of red formazan product was monitored at 490 nm with a reference wavelength of 680 nm. Positive control samples with 100% LDH release were obtained by fully lysing the cells using Triton X-100 (final concentration of 1%) in order to determine the maximum amount of LDH present in the cells. All assays were performed in triplicate and data were expressed as the ratio of the amount of LDH released, per treatment, to the maximum amount of LDH released from the control cells.

2.9 *In Silico* Prediction of Statins Association with Chylomicrons

A partial least squares regression (PLS) model[152] was applied to predict the degree of association of simvastatin hydroxy acid, simvastatin lactone, pravastatin hydroxy acid and pravastatin lactone with plasma-derived chylomicrons. This model relies on a number of physicochemical parameters including Log$D_{7.4}$ (distribution coefficient at pH 7.4), Log$P$-Log$D_{7.4}$ (mathematical difference between partition coefficient and distribution coefficient at pH 7.4), polar surface area, number of H-bond donors and acceptors, number of freely rotatable bond, density, and molar volume (Table 6.1).

For calculation of the degree of statins association with plasma-derived chylomicrons, the above-mentioned parameters for each individual compound were derived from ACD/I-Lab (Advanced Chemistry Development Inc.) and the following equations were applied to calculate the degree of association:

Total LOGIT value = ∑ (physicochemical parameters x regression coefficients) *(Eq. 2)*

Degree of association (Y) =Total LOGIT value + Model constant *(Eq. 3)*

$X=10^{Y}$ *(Eq. 4)*

Predicted association with chylomicrons (%) $= \frac{X}{1+X}$ *x 100* *(Eq. 5)*

2.10 Association of Statins with Artificial Lipid Particles, Rat, and Human Chylomicrons

2.10.1 Preparation of artificial lipid particles

Artificial triglyceride-phospholipid particles (Intralipid®) were used to provide a reasonably close estimate for the degree of simvastatin association with natural chylomicrons.[152] The uptake of simvastatin lactone by protein-free artificial emulsion was evaluated as previously described[153] with an important change that all incubation experiments were carried out at 3 pH levels corresponding to physiological pH (pH 7.4), acidosis (pH 6.8), and alkalosis (pH 7.8). Association was also studied with Intralipid® emulsion of pH 4.5. At this pH, simvastatin is expected to be entirely preserved in the lipophilic lactone form. Intralipid® emulsion was diluted to a triglyceride concentration of 100 mg/dL by PBS before performing the uptake study.

2.10.2 Preparation of rat chylomicrons

Male Sprague-Dawley rats (weighing 275-300 g, n=4) were used in this experiment. The study was performed in accordance with the standards of animal care and ethics described in Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

Animals were acclimatized for 6 days in Bio Support Unit at the University of Nottingham with a 12 hours light/dark cycle, a temperature of 21 ± 2^{o}C, and relative humidity of 55 ± 15%. In order to obtain plasma with high TRL levels for production of chylomicrons emulsion, animals were fasted overnight with free access to drinking water. Next day, 0.5 mL of peanut oil were administered to all animals by oral gavage 3 hours before, followed by two additional doses of 0.3 mL peanut oil 2 and 1 hour before blood sampling. Animals were anaesthetized by isoflurane 5% and terminal blood samples (10-12 mL) were collected from the caudal vena cava. Chylomicrons were separated from 4 mL volumes of plasma by density gradient ultracentrifugation as described previously.[152, 153]

Standard solutions with densities of 1.006, 1.019 and 1.063 g/mL were prepared by dissolving appropriate amounts of potassium bromide (KBr) in PBS. 4 mL of plasma were adjusted to a density of 1.1 g/mL by adding 0.57 g of KBr and the density gradient was built in 12 mL polyallomer tubes (Beckman Coulter) by placing 4 mL of plasma (density of 1.1 g/mL) at the bottom of the tube, then 3 mL of 1.063 g/mL solution was carefully layered on top of the plasma, followed by 2.5 mL of 1.019 g/mL solution and 2 mL of 1.006 g/mL. The tubes were loaded on a Sorvall® TH-641 Swinging Bucket Rotor and centrifuged in Sorvall® Discovery 100 SE Ultracentrifuge at 274,044g and 15°C for 35 minutes. Following centrifugation, the top 1 mL white milky layer, representing the chylomicron fraction, was collected using a Pasteur pipette. Chylomicron fractions from several tubes were combined and triglyceride content was measured using a triglyceride colorimetric kit.

Chylomicrons emulsion was diluted with PBS to a triglyceride level of 100 mg/dL and kept at 4°C pending uptake experiments (< 24 hours).

2.10.3 Preparation of human chylomicrons

Chylomicron fractions were obtained from human plasma following induction of transient postprandial hypertriglyceridemia. The study was approved by Faculty of Medicine and Health Sciences Research Ethics Committee, Queens Medical Centre, Nottingham University Hospitals (BT12102015 CBS SoP). Three healthy male volunteers (25-35 years old) were recruited for this study after obtaining informed consents. Participants were instructed not to use any over the counter medication for one week prior to the study. Following 12 hours of overnight fasting, participants had long-chain triglyceride-rich breakfast (full English breakfast or olive oil-fried eggs). Three-four hours following the meal (expected time of peak plasma chylomicron levels)[154, 155] blood samples (30 mL) were collected and plasma was separated by centrifugation (800 *g*, 10 min, 15°C). Chylomicrons were separated from human plasma by density gradient ultracentrifugation as described early (Section 2.10.2).

2.10.4 Association study

The association of statins with Intralipid®, rat and human chylomicron emulsions was evaluated as previously described with some modification.[152, 153] All incubation experiments were carried out at normal physiological pH of 7.4 in addition to pH levels that correspond to acidosis (pH 6.8) and alkalosis (pH 7.8).[156] These pH values, which represent the relevant extremities of the

pathophysiological pH range, were used to identify the trend in statins association with plasma lipoproteins in response to plasma pH alteration. The selection of this pH range was based on extreme but clinically relevant disturbances in acid-base balance that are encountered in intensive care units among patients with long-term diuretics use,[157] prolonged vomiting,[158] diabetic ketoacidosis,[159, 160] lactic acidosis,[161, 162] renal tubular acidosis,[163] hypovolemic shock,[164, 165] and ethylene glycol intoxication.[166-168] In most of these cases, blood pH of less than 7 or greater than 7.65 was reported.

Stock solutions of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid (350 µmol/L) were prepared in propylene glycol and an appropriate volume of each solution was added to a volume of 1 mL of diluted Intralipid®, rat or human chylomicron emulsions to achieve a final statin concentration of 1.75 µmol/L. Samples were incubated at 37°C for 1 hour with constant mixing and the statin-loaded chylomicron particles were separated by density gradient ultracentrifugation (Sorvall Discovery 100 SE Ultracentrifuge; TH-641 Rotor, 35 min, 274,044g, 15°C) as described previously.[153, 169, 170] The top 1 mL, white milky layer resembling the chylomicron fraction was collected using Pasteur pipette and kept at -80°C until analysis. Control samples were also run for all statins using standard PBS solution with a density of 1.006 g/mL instead of artificial or natural chylomicron emulsions in order to check the diffusion of free drug molecules through the density gradient under the conditions of the experiment.[153] The concentrations of statins recovered in Intralipid® and chylomicron fractions were determined by LC-MS/MS analysis as described previously (Section 2.3).

2.11 Effect of Hyperlipidaemia Alone or in Combination with Acidosis/ Alkalosis on Simvastatin Association with Plasma Lipoproteins

Both normolipidaemic and hyperlipidaemic plasma were used for the i*n vitro* evaluation of simvastatin association with plasma lipoproteins as described previously.[129, 171] The pH of plasma samples was adjusted to achieve physiological pH of 7.4, a pH of 6.8 corresponding to acidosis and a pH of 7.8 corresponding to alkalosis as described previously.[156]

An appropriate volume of simvastatin lactone stock solution was added to 3 ml of normolipidaemic or hyperlipidaemic human plasma of defined pH to get final simvastatin concentration of 2 µg/mL. Samples were incubated at 37°C for 1 hour with constant mixing. Following incubation, the density of plasma samples was adjusted to 1.25 g/mL by addition of 0.34g KBr per mL plasma. Standard solutions of different densities (1.21, 1.063 and 1.009 g/mL) were prepared by dissolving appropriate amounts of KBr in PBS. Once KBr had dissolved into the plasma, the density gradient was built in 12 ml polyallomer tubes (Beckman Coulter) by placing 3 mL of plasma (density of 1.25 g/mL) at the bottom of the tube, then 2.8 mL of the highest density solutions were carefully layered on top of the plasma followed by 2.8 ml of 1.063 g/mL and 1.006 g/mL solutions, respectively. The tubes were loaded on a Sorvall® TH-641 Swinging Bucket Rotor and centrifuged in Sorvall® Discovery 100 SE Ultracentrifuge at 274,044g and 15°C for 18 hours. Following centrifugation, four distinctive layers were spotted and identified. Those layers representing, from the top to the bottom, TRL, LDL, HDL and the lipoprotein-deficient plasma fraction (LPDP) which consist primarily of albumin and α_1-acid glycoprotein. The layers were collected using Pasteur

pipettes, transferred to new Eppendorf tubes and the volumes were measured and recorded. The concentrations of simvastatin lactone and hydroxy acid forms recovered in individual lipoprotein fractions were determined by LC-MS/MS analysis as described early (Section 2.3).[156] The total triglyceride and cholesterol levels of hyperlipidaemic plasma, normolipidaemic plasma, and individual lipoprotein fractions were determined by enzymatic methods using commercially available kits. Normolipidaemic plasma showed triglyceride levels of 63.0 ± 2.9 mg/dL and cholesterol level of 134.7 ± 8.9 mg/dL, whereas hyperlipidaemic plasma had total triglyceride and cholesterol levels of 448.0 ± 12.1 and 348.5 ± 15.5 mg/dL**,** respectively.

2.12 Determination of Triglyceride Concentration

Triglyceride concentration was determined in hyperlipidaemic and normolipidaemic plasma samples, individual lipoprotein fractions, Intralipid®, rat, and human chylomicron emulsions using Triglyceride Determination Kit from Sigma-Aldrich. The assay involves enzymatic hydrolysis of triglycerides by LPL to glycerol and free fatty acids. Glycerol then converted by coupled enzymatic reactions to quinoneimine dye that has a maximum absorbance at 540 nm.[172] The increase in absorbance at 540 nm is directly proportional to triglyceride concentration of the sample.

Triglyceride assay was performed in 96-well plates using a slightly modified protocol from manufacturer's instructions. Briefly, 200 µL of free glycerol reagent was pipetted into separate wells and 2.5 µL of unknown sample, glycerol standard or water (blank) were then added. The plate was mixed gently, incubated at 37°C for 5 minutes and the initial absorbance (Ab1)

was measured at 540 nm using EnVision® Multilabel Plate Reader (PerkinElmer, USA). After that, 50 µL of triglyceride reagent was added to each well and the plate was mixed, incubated for another 5 minutes at 37°C and the final absorbance (Ab2) was measured again. Finally, triglyceride concentration was determined using the following equation:

$$Triglyceride\ concentration = \frac{((Ab2\ Sample - (Ab1\ Sample\ X\ F))}{((Ab2\ Standard - (Ab1\ Blank\ X\ F))}\ X\ Concentration\ of\ standard \quad (Eq\ 6)$$

Where F = 202.5/252.5 = 0.80

2.13 Determination of Cholesterol Concentration

Cholesterol concentration was determined in individual lipoprotein fractions, hyperlipidaemic and normolipidaemic plasma samples using Total Cholesterol and Cholesteryl Ester Colorimetric Assay Kit II. The assay is based on enzymatic hydrolysis of cholesteryl esters present in the samples to free cholesterol by cholesterol esterase. Oxidation of free cholesterol by cholesterol dehydrogenase is then used to activate a colorimetric probe resulting in a strong absorbance at 450 nm.

Samples were first diluted with an appropriate volume of cholesterol assay buffer to achieve cholesterol concentration within the assay limit. Then 10 µL of sample and cholesterol standard was transferred into separate wells in 96 well plate and the volume was adjusted to 50 µL using the assay buffer. To hydrolyze cholesteryl ester present in the samples, 2µL of esterase enzyme was added to each well and the plate was mixed and incubated at 37°C for 30 minutes. After that, 48 µL of the reaction mixture was added to each well and

the plate was mixed, incubated for another 30 minutes at 37°C and the absorbance was measured at 450 nm using EnVision® Multilabel Plate Reader (PerkinElmer, USA). A standard curve was constructed using serial dilutions of cholesterol standard and was used to determine cholesterol concentration.

2.14 Effect of Hyperlipidaemia Alone or in Combination with Acidosis/Alkalosis on Cellular Uptake of Lipophilic Statin

2.14.1 Cell culture and differentiation

C2C12 cells that overexpress mouse (mLPL) or human (hLPL) lipoprotein lipase were used in these studies. Cells transfected with empty vector were used as a control. mLPL and hLPL were cloned into murine stem cell virus (MSCV) mammalian expression vector. To produce retrovirus for stable overexpression of hLPL and mLPL, 6 µg of each plasmid (mLPL, hLPL, and the empty MSCV vector) was combined with a transformation mixture containing 3 µg pCL-Eco packaging plasmid, 2 µg VSVG pseudotyping plasmid, and 33 µl Lipofectamine 2000 (Invitrogen) in 1 ml Opti-MEM (Invitrogen). The transformation cocktails were added to Phoenix 293T retroviral packaging cells grown to ~85% confluence. Virus-containing supernatant was then harvested from the packaging cells 48 hours following transfection. C2C12 cells at P2 were then grown to 30% confluence and incubated with the virus-containing supernatant along with 10 µg/ml polybrene. The cells were incubated with the virus for 24 hours followed by a medium change and a 24-hour recovery period. C2C12 cells were cultured and differentiated as described early (Section 2.6.1).

2.14.2 Uptake study

The effect of hyperlipidaemia and acidosis/alkalosis on cellular uptake of simvastatin was tested by incubating C2C12 cells that overexpress mLPL, hLPL or control cells with simvastatin-loaded lipoprotein fractions at pH relevant to physiological conditions, acidosis, and alkalosis. Simvastatin-loaded lipoprotein fractions were obtained by incubating the drug with hyperlipidaemic human plasma of different pH. The pH of plasma samples was adjusted as described previously (Section 2.4.1) to achieve physiological pH of 7.4, a pH of 6.8 corresponding to acidosis and a pH of 7.8 corresponding to alkalosis.

An appropriate volume of simvastatin lactone stock solution prepared in propylene glycol (9 mg/mL) was added to hyperlipidaemic human plasma of defined pH to achieve a final concentration of 90 μg/mL. Samples were incubated at 37°C for 1 hour with constant mixing. Following incubation, lipoprotein fractions were separated by density gradient ultracentrifugation as described above (Section 2.10). Following separation, individual lipoprotein fractions were transferred into Microsep™ advance centrifugal device with Omega™ membrane for removal of KBr salt used during lipoprotein separations. Since limited amount of lipoprotein fractions can be obtained from a single run, the above-mentioned experiment was repeated several times and lipoprotein fractions from different experiments were pooled together. Aliquots were used to determine the concentration of simvastatin in individual lipoprotein fractions by LC-MS/MS analysis. The remaining lipoprotein fractions were used to perform the uptake study with undifferentiated and differentiated C2C12 cells that overexpress mLPL, hLPL, or control cells.

Cells were cultured in 10 cm dishes at a density of 2×10^5/ mL and allowed to attach for 24 hours or to differentiate for 4 days by switching to differentiation medium containing 2% horse serum. Before starting the uptake study, the medium was replaced with a fresh one of different pH (relevant to the physiological pH, acidosis, and alkalosis) and the cells were allowed to equilibrate for 10 minutes. The uptake studies were initiated by applying simvastatin-loaded lipoprotein fractions (simvastatin-loaded triglyceride-rich lipoproteins [SV-TRL] or simvastatin-loaded low-density lipoprotein [SV-LDL]) to the cells and the cells were incubated for 3 hours at 37°C and 5% CO_2. Control cells were treated with a similar concentration of simvastatin applied as a solution directly into the cells without lipoprotein fractions. Simvastatin solution was prepared by diluting an appropriate volume of stock solutions of simvastatin hydroxy acid and lactone (prepared in DMSO) with PBS to final concentrations similar to that achieved within each lipoprotein fraction after ultracentrifugation.

Simvastatin uptake was terminated by suctioning off the medium containing the lipoprotein fraction and the cells were harvested by trypsinisation and re-suspended in 200 µL PBS (pH 7.4). Five replicates were tested at each pH level and the intracellular concentration of both simvastatin lactone and the corresponding hydroxy acid form were determined in cell suspension using fully validated LC-MS/MS assay as described previously (Section 2.3).[156] Cellular protein content was determined in the remaining cell suspension after pelleting and lysing the cells with RIPA lysis buffer, using Bradford protein assay and BSA as a standard.[149]

2.14.3 Determination of lipoprotein lipase activity

Lipoprotein lipase activity was determined in undifferentiated and differentiated C2C12 cells that overexpress mLPL, hLPL as well as in control cells transfected with empty vector using fluorometric assay kit. The kit utilizes a quenched substrate (fluorogenic triglyceride analogue) that hydrolyzes into a fluorescent fatty acid product in the presence of LPL enzyme (Figure 2.2). The fluorescence intensity is directly proportional to the LPL enzyme activity present in the sample.

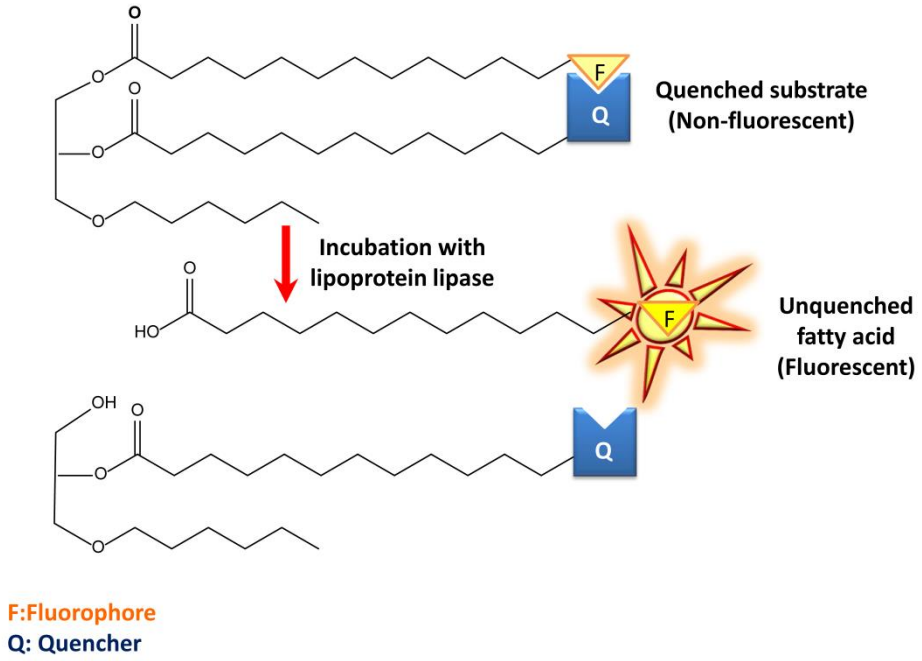


Figure 2.2: LPL activity assay principle.

C2C12 cells were cultured in 10 cm dishes at a density of 2×10^5/ mL and allowed to attach for 24 hours or to differentiate for 4 days by switching to differentiation medium containing 2% horse serum. After that, cells were incubated in a fresh medium of modified pH (relevant to the physiological pH, acidosis, and alkalosis) for 3 hours. Culture dishes were rinsed twice with ice-

cold PBS and the cells were harvested by trypsinisation and re-suspended in 200 µL PBS (pH 7.4). Cell suspensions were sonicated on ice for 15 seconds followed by centrifugation at 15,000g for 10 minutes at 4°C for removal of cell debris. Aliquots were taken for determination of cellular protein contents by Bradford assay and the remaining clear supernatant was held on ice and the enzyme activity was assayed within 30 minutes.

The assay was performed according to manufacturer's instructions. LPL enzyme standard (positive control) was diluted with dH_2O as described by the assay protocol and 4 µL was aliquoted into a separate well in a white, 96-well plate. Appropriate volumes of cell lysate samples (1-10 µL, determined by pilot experiment) were transferred into desired wells and the volume of the unknown and positive control samples was adjusted to 50 µL using dH_2O. Triglyceride substrate was diluted with LPL assay buffer and 50 µL was added to each well containing the unknown and positive control samples. A standard curve was prepared by mixing different volumes of the diluted substrate with diluted LPL enzyme standard to get final substrate amounts of 0-10 pmol/ well. The plate was mixed briefly and incubated at 37°C for 10 minutes protected from light to stabilize the signal. Fluorescence was measured kinetically for 1 hour at excitation/emission wavelengths of 482/515 nm using EnVision® Multilabel Plate Reader (PerkinElmer, USA). The standard curve was used to determine the amount of substrate consumed by each sample during the reaction time and sample LPL activity was determined according to the following equation:

Lipoprotein lipase activity (mU/mL) = A/(ΔT x V) x D *(eq. 7)*

Where, A is the amount of substrate calculated by the standard curve (pmol).

ΔT is the reaction time (minutes).

V is the sample volume added to the well (mL).

D is sample dilution factor.

2.15 Effect of Hyperlipidaemia Alone or in Combination with Acidosis/Alkalosis on Cytotoxicity of Simvastatin

The effect of simvastatin association with plasma lipoproteins on the viability of C2C12 cells was evaluated by MTT and LDH cytotoxicity assays. Undifferentiated and differentiated C2C12 that overexpress mLPL, hLPL and control cells were treated either with simvastatin-loaded lipoproteins factions or simvastatin applied directly in solution at concentrations identical to those achieved within the corresponding lipoprotein fractions. The incubation was done in culture medium of physiological pH (pH 7.4). In order to assess the combined effect of elevated plasma lipoprotein levels and medium pH alteration on the cytotoxicity of simvastatin, C2C12 cells were also incubated in culture media of modified pH (pH 6.8 corresponding to acidosis and 7.8 corresponding to alkalosis).

For MTT assay, cells were cultured at a density of 4,000 cells/ well in 96-well plates and allowed to attach for 24 hours or to differentiate for 4 days. Before starting treatment, the medium was replaced with a fresh one of different pH (relevant to the physiological pH, acidosis, and alkalosis) and the cells were allowed to equilibrate for 10 minutes. C2C12 cells were exposed to different concentrations of simvastatin either loaded into lipoprotein fractions

(SV-TRL or SV-LDL) or applied directly in solution (SV-Sol) for 72 hours and the metabolic activity of the cells was measured using the MTT assay. Control cells were treated with a constant volume of blank lipoprotein fractions without simvastatin and the results were used to normalize the data. Results were expressed as percentage of the vehicle control and IC_{50} values were calculated, when applicable, using Prism 6 (GraphPad Software Inc).

Membrane integrity of C2C12 cells was assessed by LDH assay kit. The test was performed at the final simvastatin concentrations that were obtained within the lipoprotein fractions after desalting process. Cells were cultured and treated as described early. Undifferentiated cells were treated for 72 hours whereas differentiated cells were maintained for 24 hours and manufacturer's instructions were followed. Results were expressed as the ratio of the amount of LDH released from the cells, per treatment, to the maximum amount released from control cells treated with the lysis buffer.

2.16 Statistical Analysis

Data were expressed as mean ± SD or SE as specified. Statistical differences between groups were determined by one-way ANOVA, two-way ANOVA or Kruskal-Wallis test followed by Tukey's, Bonferroni's, Dunn's or Fisher's post hoc test for multiple comparisons, as appropriate. Pearson's correlation coefficient (r) was used to describe the relationship between the amount of simvastatin-associated with plasma lipoproteins and the triglyceride or cholesterol contents of individual lipoprotein fractions. A *p*-value of less than 0.05 was considered to be statistically significant.

Chapter Three

3 Analytical Methods Development and Validation

3.1 Introduction

The use of statins has increased sharply in recent years and is expected to increase even further in the next few years following the new recommendations that offer statins for wider group of population.[21, 23] Such wide use of statins increases the risk of muscle-related side effects and necessitates the development of sensitive and reliable analytical methods that help in understanding the mechanism of statin-induced myotoxicity.

Quantification of statins in biological samples represents a huge challenge due to the fact that statins are unstable compounds and can undergo inter-conversion between the hydroxy acid and lactone forms.[173] Several analytical techniques have been developed to quantify statins in biological samples and pharmaceutical formulations. These methods involve the use of high-performance liquid chromatography (HPLC) with UV or fluorescence detector,[174-176] capillary Electrophoresis,[177, 178] gas chromatography-mass spectrometry (GC-MS)[179, 180] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) systems.[181, 182] The HPLC-UV methods are not often sensitive enough to determine low concentrations of the hydroxy acid and lactone form of statins. However, these methods are often available for routine analysis and still can be successfully employed for quantification of statins in pharmaceutical formulations where high concentrations are usually present.[175] On the other hand, GC-MS requires derivatisation steps with generally complicated sample preparation methods. The LC–MS/MS assay has

become the method of choice due to its high sensitivity, selectivity and robustness.[183]

The aim of this study is to develop analytical methods that allow the simultaneous quantification of the hydroxy acid and the lactone forms of simvastatin and pravastatin in a simple buffer solution, human plasma samples, cell culture medium, cell lysate samples and plasma lipoprotein fractions. HPLC-UV methods will be developed to analyse samples from inter-conversion studies where statin concentrations are relatively high. For samples with lower concentrations of statins (cell lysate and plasma lipoprotein samples), a more sensitive LC-MS/MS analysis will be employed.

3.2 Methods

3.2.1 Development and validation of HPLC-UV methods

HPLC-UV analyses were performed using Waters® Alliance 2965 HPLC system equipped with Waters® 996 Photodiode Array Detector (PDA). HPLC methods were developed, validated and used to determine the concentrations of the lactone and the corresponding hydroxy acid forms of simvastatin and pravastatin following inter-conversion studies in PBS, human plasma, and DMEM culture medium of different pH. Samples were prepared by protein precipitation followed by liquid-liquid extraction using MTBE. Detailed sample preparation processes, analytical conditions, and methods validation are provided in Section 2.2.

3.2.2 Development and validation of LC-MS/MS method

LC-MS/MS analysis was used to determine the intracellular concentrations of the lactone and hydroxy acid forms of simvastatin and pravastatin. Samples from cell suspension were prepared by protein precipitation followed by liquid-liquid extraction. LC-MS/MS analysis was performed using Quattro Ultima triple quadrupole mass spectrometer interfaced via an ESI probe with an Agilent HPLC system. More detailed description of the sample preparation methods, liquid chromatographic-mass spectrometric conditions, and assay validation process are presented in Section 2.3.

3.3 Results

3.3.1 Development and validation of HPLC-UV methods

Good chromatographic separations were achieved for all analytes under the developed conditions with retention times of 2.2, 4.2 and 8.5 minutes for simvastatin acid, simvastatin lactone, and DDT, respectively. Pravastatin acid, pravastatin lactone, and griseofulvin were separated at 1.6, 2.5 and 3.3 minutes, respectively. The degree of interference of the analytes and internal standards by endogenous substances was evaluated by inspecting chromatograms derived from processed blank samples. No significant interference of analytes' peaks with the endogenous constituents in blank samples at the retention times of the tested compounds (Figures 3.1 and 3.2). Calibration curves were found to be linear over a concentration ranging from 0.07-160 μmol/L for simvastatin, and 0.12-240 μmol/L for pravastatin

samples. The correlation coefficient (r^2) values were greater than 0.98 for all tested statins.

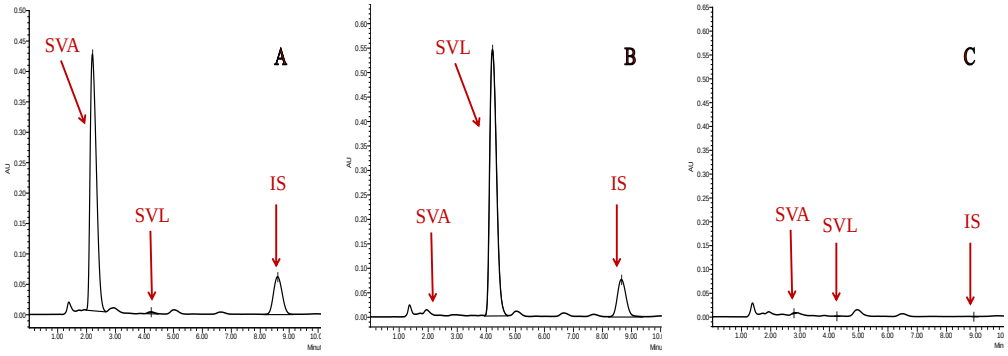


Figure 3.1: HPLC chromatograms of simvastatin hydroxy acid and simvastatin lactone. (A) simvastatin hydroxy acid (SVA) spiked into DMEM culture medium (pH 7.4) with internal standard (IS= DDT), (B) simvastatin lactone (SVL) spiked into DMEM (pH 7.4) with internal standard (IS= DDT), (C) blank culture medium.

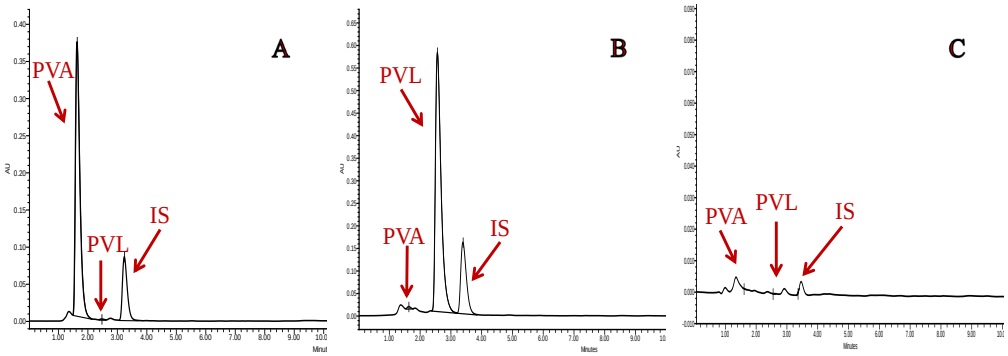


Figure 3.2: HPLC chromatograms of pravastatin hydroxy acid and pravastatin lactone. (A) pravastatin hydroxy acid (PVA) spiked into DMEM culture medium (pH 7.4) with internal standard (IS= griseofulvin), (B) pravastatin lactone (PVL) spiked into DMEM (pH 7.4) with internal standard (IS= griseofulvin), (C) blank culture medium.

Relevant results of precision and accuracy from PBS, human plasma, and DMEM samples are presented in Tables 3.1, 3.2, and 3.3, respectively. Both intra- and inter-day assay variability values were within acceptance criteria (i.e., RSD% and RE% less than 15%) for all tested compounds at the three quality control levels. The lower limit of quantification that produced intra- and inter- day precision and accuracy of < 20% were 0.156 µmol/L (65

ng/mL), 0.313 μmol/L (142 ng/mL), 0.234 μmol/L (95 ng/mL) and 0.469 μmol/L (209 ng/mL) for simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone and pravastatin hydroxy acid, respectively. The extraction recoveries were found to be satisfactory for all analytes at the three quality control levels (Table 3.4).

Table 3.1: Intra- and inter- day precision and accuracy of the HPLC methods used for determination of statins in PBS samples.

Statins	Quality control levels		Intraday (n = 6)		Interday (n = 6)	
	Concentration (μmol/L)	Concentration (μg/mL)	Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
Simvastatin lactone	LLOQ (0.156)	0.065	1.36	-7.04	7.91	15.80
	LQC (5)	2.09	4.51	-0.81	3.92	-11.04
	MQC (60)	25.12	11.89	-8.30	13.49	9.62
	HQC (120)	50.23	10.39	-6.69	10.16	-8.67
Simvastatin hydroxy acid	LLOQ (0.313)	0.142	3.75	-6.69	11.60	-19.38
	LQC (5)	2.26	8.39	-13.58	13.55	6.56
	MQC (60)	27.21	12.01	9.41	9.88	6.14
	HQC (120)	54.43	9.65	-1.8	10.75	-0.53
Pravastatin lactone	LLOQ (0.234)	0.095	4.16	7.06	19.92	-2.66
	LQC (10)	4.06	2.45	9.66	2.71	14.11
	MQC (100)	40.65	5.92	9.35	7.05	-3.10
	HQC (200)	81.30	5.28	14.57	6.18	-1.46
Pravastatin hydroxy acid	LLOQ (0.469)	0.209	2.12	19.99	14.32	-15.86
	LQC (10)	4.46	3.52	8.81	8.39	13.08
	MQC (100)	44.65	4.50	7.56	4.86	1.79
	HQC (200)	89.30	12.69	4.79	4.93	2.29

Abbreviations: HQC, high concentration quality control; LLOQ, lower limit of quantification; LQC, low concentration quality control; MQC, medium concentration quality control, PBS, phosphate buffer saline.

*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.

†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

Table 3.2: Intra- and inter-day precision and accuracy of the HPLC methods used for determination of statins in human plasma samples.

Statins	Quality control levels		Intraday (n = 6)		Interday (n = 6)	
	Concentration (µmol/L)	Concentration (µg/mL)	Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
Simvastatin lactone	LLOQ (0.156)	0.065	9.70	-5.94	14.56	-3.05
	LQC (5)	2.09	3.74	-10.13	14.79	-3.12
	MQC (60)	25.12	12.43	0.57	14.30	0.50
	HQC (120)	50.23	9.78	-1.36	14.44	5.90
Simvastatin hydroxy acid	LLOQ (0.313)	0.142	11.61	9.98	11.58	17.42
	LQC (5)	2.26	14.83	0.37	10.91	-1.33
	MQC (60)	27.21	6.54	-6.14	6.43	-2.45
	HQC (120)	54.43	6.72	-11.70	7.69	-8.93
Pravastatin lactone	LLOQ (0.234)	0.095	3.33	13.88	6.95	14.65
	LQC (10)	4.06	2.38	-3.17	6.99	-9.67
	MQC (100)	40.65	12.46	2.34	11.74	5.21
	HQC (200)	81.30	4.43	6.91	7.27	8.16
Pravastatin hydroxy acid	LLOQ (0.469)	0.209	17.39	-9.86	16.02	-4.53
	LQC (10)	4.46	8.35	12.51	8.17	11.50
	MQC (100)	44.65	11.84	-10.22	13.45	-10.71
	HQC (200)	89.30	9.85	-12.94	10.46	-11.32

Abbreviations: HQC, high concentration quality control; LLOQ, lower limit of quantification; LQC, low concentration quality control; MQC, medium concentration quality control.

*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.

†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

Table 3.3: Intra- and inter- day precision and accuracy of the HPLC methods used for determination of statins in DMEM culture medium samples.

Statins	Quality control levels		Intraday (n = 6)		Interday (n = 6)	
	Concentration (µmol/L)	Concentration (µg/mL)	Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
Simvastatin lactone	LLOQ (0.156)	0.065	2.06	7.36	17.28	-3.25
	LQC (5)	2.09	10.13	6.83	11.82	3.8
	MQC (40)	16.74	2.36	-3.34	9.35	11.15
	HQC (120)	50.23	6.71	-2.71	10.13	0.41
Simvastatin hydroxy acid	LLOQ (0.313)	0.142	3.99	-18.51	11.48	-15.85
	LQC (5)	2.26	11.97	6.67	12.7	1.97
	MQC (40)	18.14	11.47	-12.34	9.44	-14.98
	HQC (120)	54.43	2.68	-5.3	3.54	-4.16
Pravastatin lactone	LLOQ (0.234)	0.095	8.14	17.37	8.87	18.54
	LQC (10)	4.06	7.87	6.59	4.07	12.59
	MQC (100)	40.65	1.6	-6.71	1.5	-4.67
	HQC (200)	81.30	3.63	-5.66	4.2	-2.74
Pravastatin hydroxy acid	LLOQ (0.469)	0.209	7.28	-3.05	18.19	-8.12
	LQC (10)	4.46	3.42	-4.19	1.64	-7.99
	MQC (100)	44.65	1.54	2.01	1.19	1.29
	HQC (200)	89.30	7.13	-5.56	9.75	-3.37

Abbreviations: DMEM, Dulbecco's modified eagle medium; HQC, high concentration quality control; LLOQ, lower limit of quantification; LQC, low concentration quality control; MQC, medium concentration quality control.

*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.
†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

Table 3.4: Extraction recovery (%) of statins from PBS, human plasma, and DMEM samples.

Statins	Quality control levels	% of statin recovered (mean ± SD, n=6)		
		PBS samples	Plasma samples	DMEM samples
Simvastatin lactone	LQC	96.80 ± 6.04	80.75 ± 14.89	94.48 ± 2.76
	MQC	96.28 ± 11.12	89.14 ± 7.40	93.41 ± 2.94
	HQC	92.94 ± 12.23	87.52 ± 13.50	82.68 ± 7.48
Simvastatin hydroxy acid	LQC	90.15 ± 12.3	87.61 ± 16.11	94.43 ± 3.77
	MQC	86.66 ±14.98	84.61± 15.74	70.82 ± 6.81
	HQC	95.57 ± 3.32	88.13 ± 10.66	83.40 ± 20.31
Pravastatin lactone	LQC	88.52 ± 9.11	76.53 ± 16.27	78.81 ± 26.59
	MQC	86.55 ± 3.98	85.40 ± 16.22	91.46 ± 13.07
	HQC	88.43 ± 8.37	80.58 ± 8.47	90.11 ± 12.15
Pravastatin hydroxy acid	LQC	77.32 ± 5.06	78.56 ± 7.80	77.83 ± 11.90
	MQC	81.71 ± 26.71	83.08 ± 13.27	78.73 ± 5.33
	HQC	88.73 ± 31.67	81.95 ± 15.63	88.48 ± 5.36

Abbreviations: DMEM, Dulbecco's modified eagle medium; HQC, high concentration quality control; LLOQ, lower limit of quantification; LQC, low concentration quality control; MQC, medium concentration quality control; PBS, phosphate buffer saline.

3.3.2 Development and validation of LC-MS/MS analysis

Mass spectrometry (MS) detection was performed using both positive and negative ESI mode with MRM scan. Direct infusion experiments were carried out for all tested analytes to achieve the best MS tuning. The mass spectra of the hydroxy acid and lactone forms of simvastatin, pravastatin and internal standard (lovastatin) are shown in Figures 3.3 - 3.5, respectively. The most abundant ions observed during Q1 scanning were the sodium adduct $[M+Na]^+$ for the lactone forms and the deprotonated adduct $[M-H]^-$ adduct for the hydroxy acid forms. These adducts were selected as precursor ions to obtain the Q3 product ion spectra. Ion fragmentation was carried out by applying nitrogen as collision gas at sufficient flow rate and collision energy to

break the precursor ions. Profound optimization of mass spectrometry conditions was performed to achieve the highest signal intensity.

Ion transitions were monitored at *m/z* of 441.0→199.17 for simvastatin lactone, 429→183.22 for pravastatin lactone, 427.15→ 325.36 for lovastatin lactone, 435.49→ 319.43 for simvastatin hydroxy acid, 423.23→ 321.37 for pravastatin hydroxy acid, and 421.08→ 319.54 for lovastatin hydroxy acid.

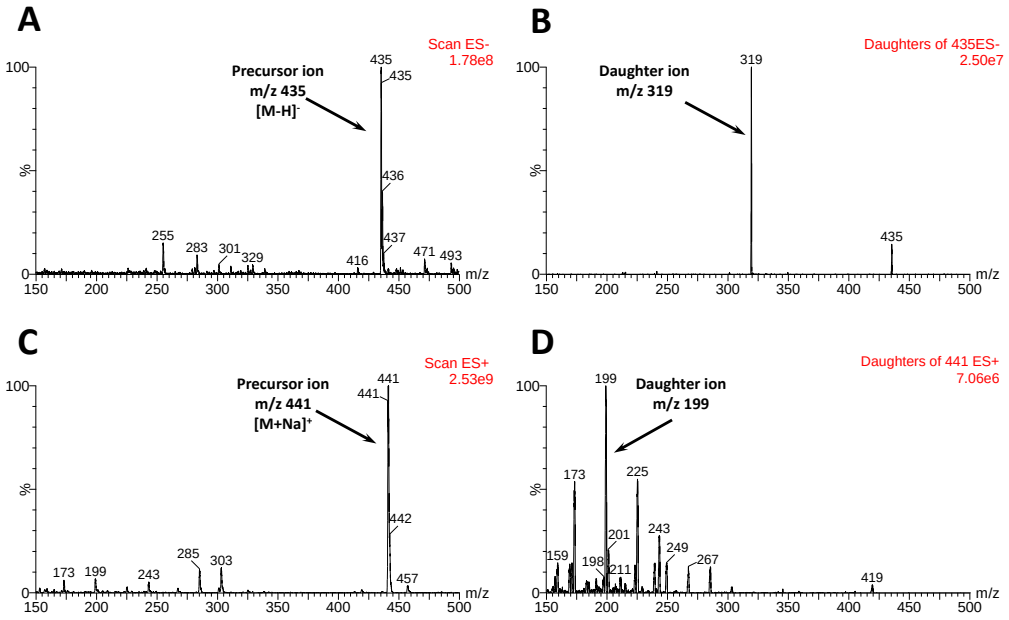


Figure 3.3: Ion mass spectra of simvastatin hydroxy acid precursor ion (A), simvastatin hydroxy acid product ion (B), simvastatin lactone precursor ion (C), and simvastatin lactone product ion (D).

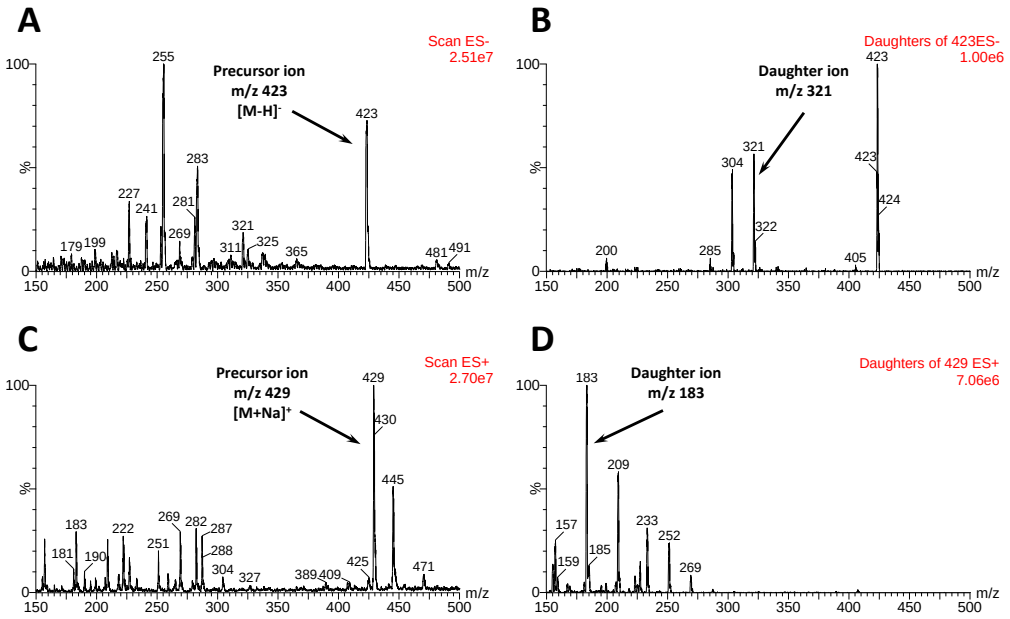


Figure 3.4: Ion mass spectra of pravastatin hydroxy acid precursor ion (A), pravastatin hydroxy acid product ion (B), pravastatin lactone precursor ion (C), and pravastatin lactone product ion (D).

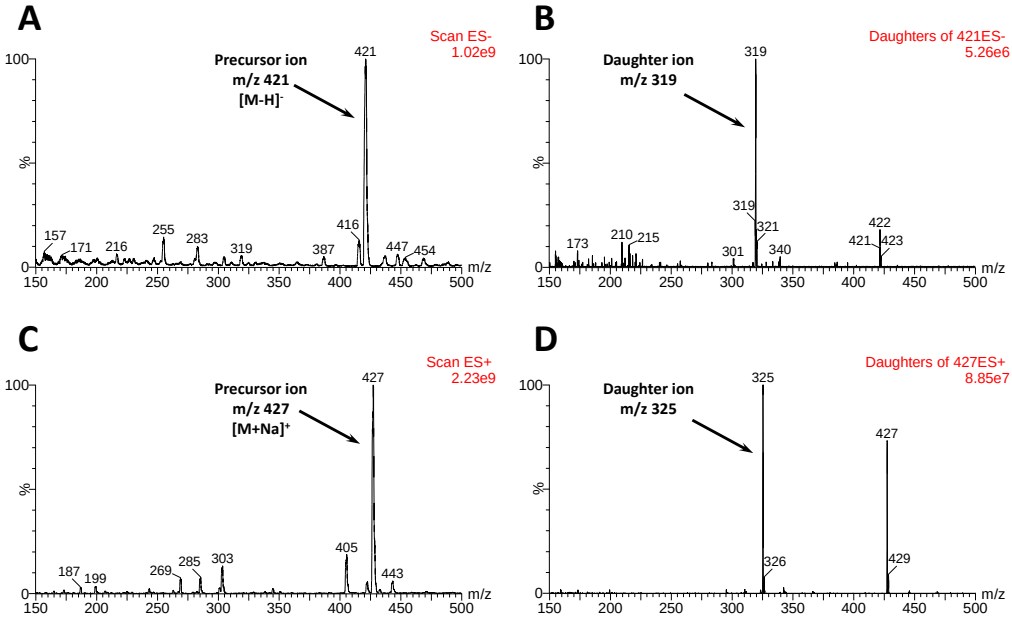


Figure 3.5: Ion mass spectra of lovastatin hydroxy acid precursor ion (A), lovastatin hydroxy acid product ion (B), lovastatin lactone precursor ion (C), and lovastatin lactone product ion (D).

Good chromatographic separations were achieved for all analytes under the developed conditions with retention times of 2.6, 5.8, 2.3 and 4.6 minutes

for simvastatin hydroxy acid, simvastatin lactone, lovastatin hydroxy acid, and lovastatin lactone, respectively (Figure 3.6). Under different chromatographic conditions, pravastatin hydroxy acid, pravastatin lactone, lovastatin hydroxy acid, and lovastatin lactone were separated at 1.6, 2.2, 3.4 and 7.2 minutes, respectively (Figure 3.8). The degree of interference of the analytes and internal standards with endogenous substances was evaluated by inspecting chromatograms derived from processed blank cell lysate samples. No significant interference of analytes' peaks with the endogenous constituents in blank samples was observed at the retention times of the tested compounds (Figures 3.7 and 3.9). Calibration curves were found to be linear over concentrations ranging from 1 - 2560 nmol/L with correlation coefficient (r^2) values of more than 0.99 for all tested statins.

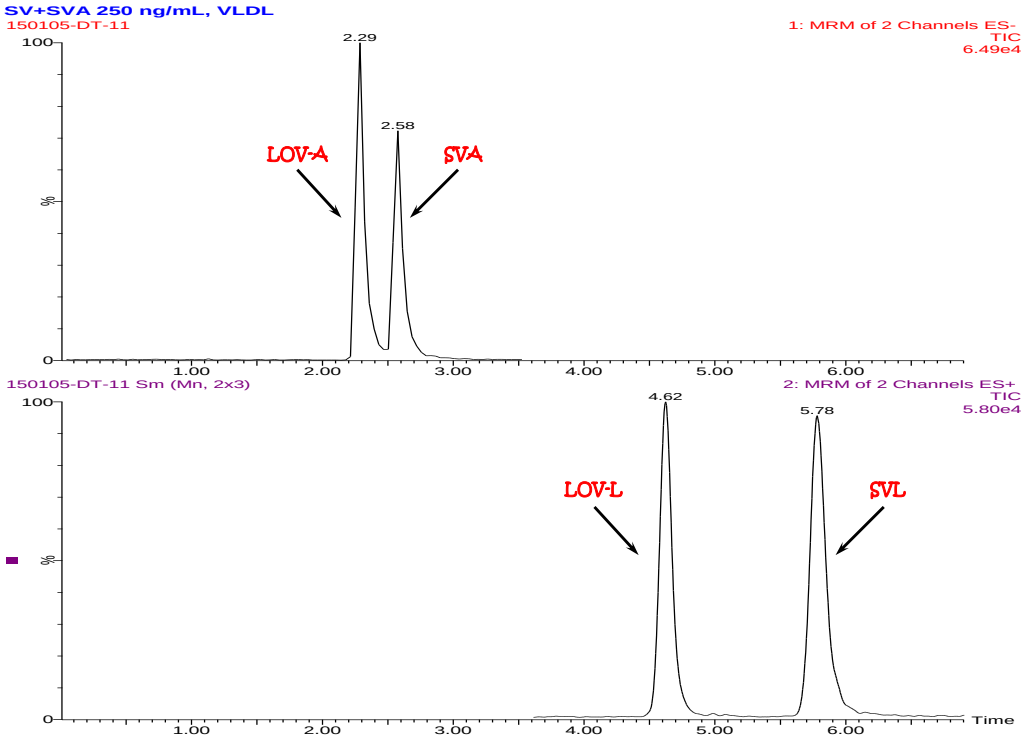


Figure 3.6: LC–MS/MS chromatograms of a mixture of measured analytes from cell lysate samples. LOV-A, lovastatin hydroxy acid (IS); SVA, simvastatin hydroxy acid; LOV-L, lovastatin lactone (IS); SVL, simvastatin lactone.

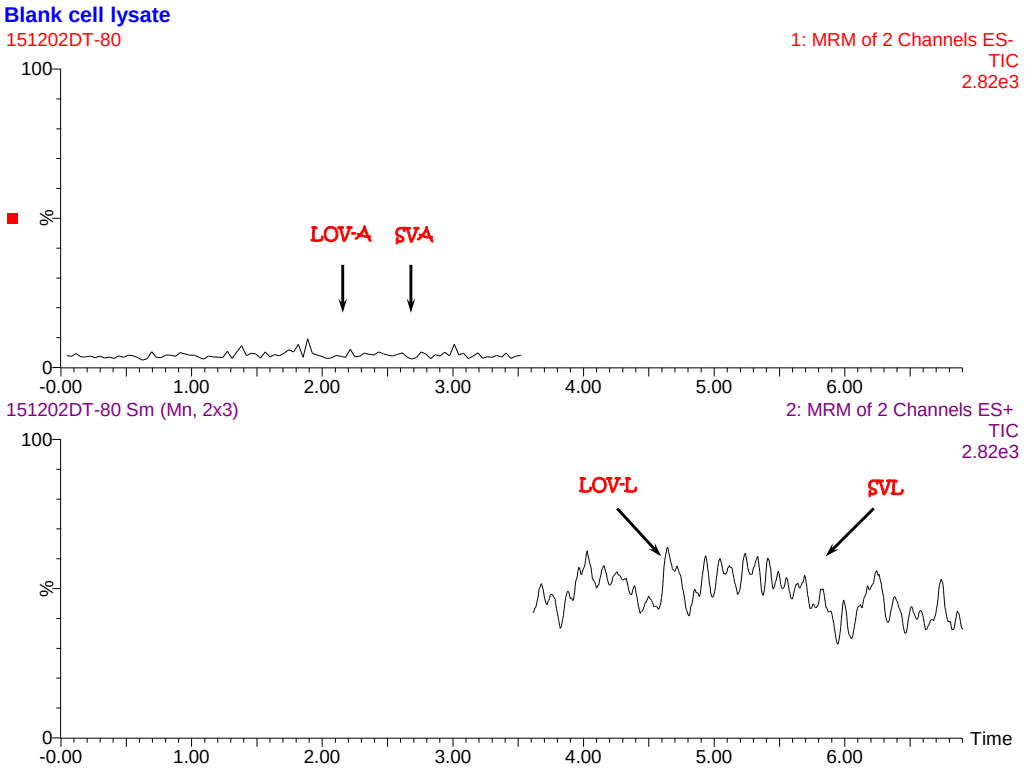


Figure 3.7: LC–MS/MS chromatograms of blank cell lysate sample. LOV-A, lovastatin hydroxy acid (IS); SVA, simvastatin hydroxy acid; LOV-L, lovastatin lactone (IS); SVL, simvastatin lactone.

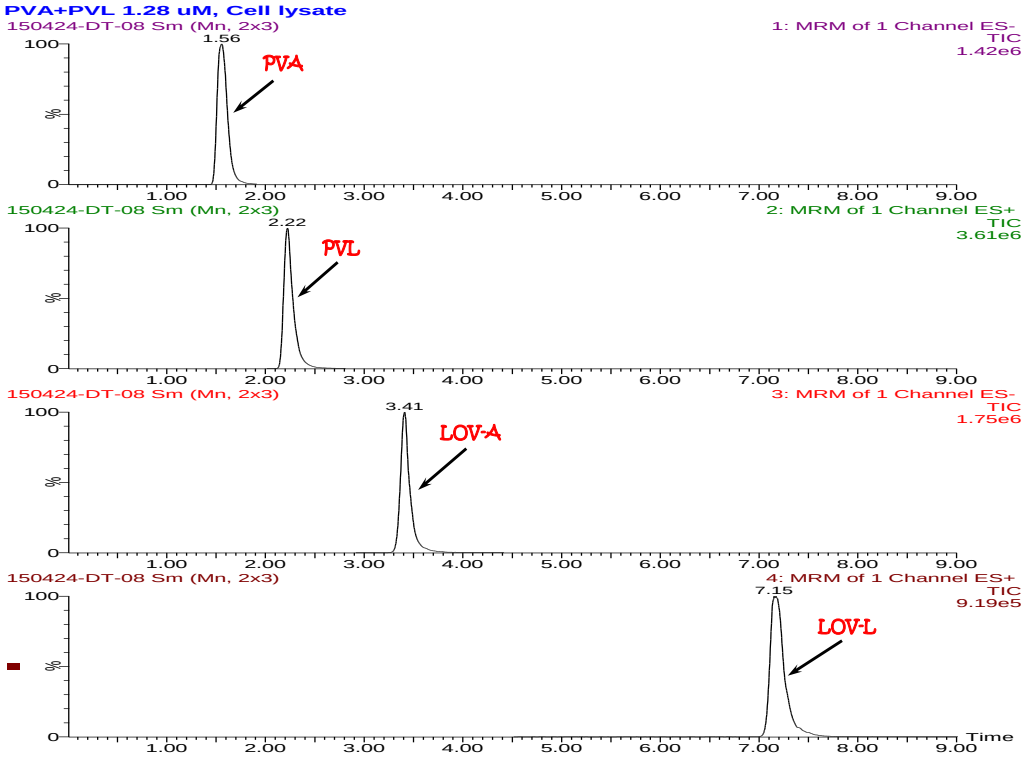


Figure 3.8: LC-MS/MS chromatograms of a mixture of measured analytes from cell lysate samples. PVA, pravastatin hydroxy acid; PVL, pravastatin lactone; LOV-A, lovastatin hydroxy acid (IS); LOV-L, lovastatin lactone (IS).

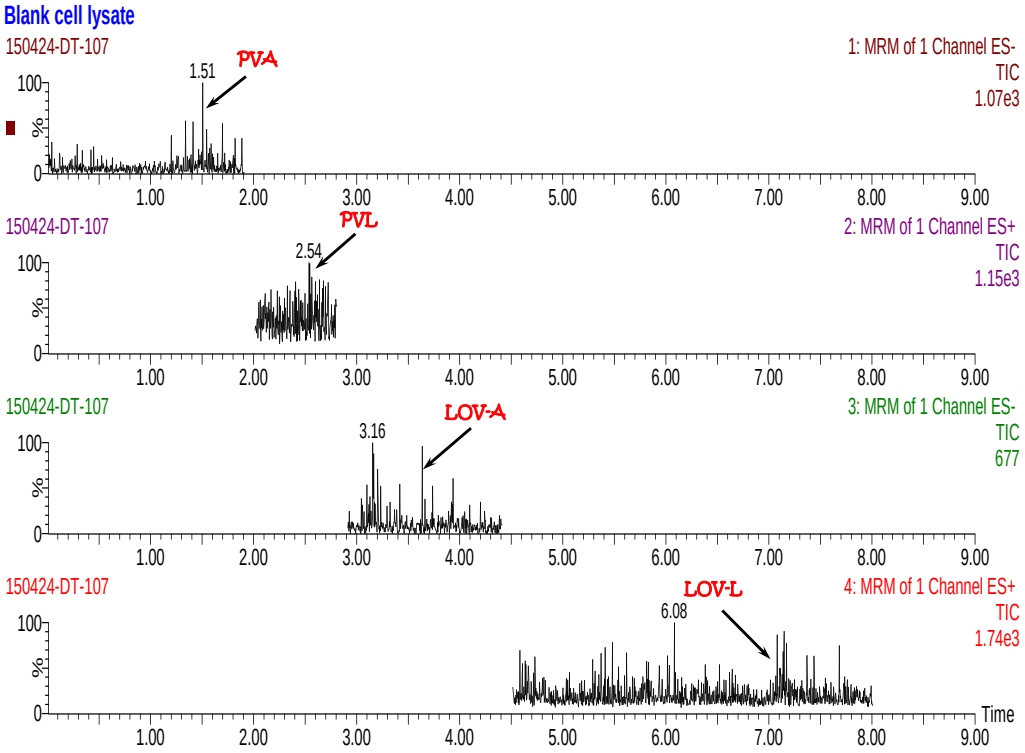


Figure 3.9: LC-MS/MS chromatograms of blank cell lysate sample. PVA, pravastatin hydroxy acid; PVL, pravastatin lactone; LOV-A, lovastatin hydroxy acid (IS); LOV-L, lovastatin lactone (IS).

The assay validation data of the tested analytes from cell lysate samples at four quality control levels are reported in Table 3.5. Both intra- and inter-day assay variability values were within acceptance criteria (i.e., RSD% and RE% less than 15%) for all tested compounds at the three quality control levels. The lower limit of quantification that produced intra- and inter- day precision and accuracy of < 20% were 2.5 nmol/L for simvastatin hydroxy acid and 5 nmol/L for simvastatin lactone, pravastatin lactone and pravastatin hydroxy acid.

Table 3.5: Intra- and inter- day precision and accuracy of the LC-MS/MS methods used for determination of statins in cell lysate samples.

Statins	Quality control levels		Intraday (n = 6)		Interday (n = 6)	
	Concentration (nmol/L)	Concentration (ng/mL)	Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
Simvastatin lactone	LLOQ (5)	2.09	14.34	10.06	15.89	9.60
	LQC (20)	8.37	12.35	7.44	13.70	-1.79
	MQC (200)	83.72	12.91	5.47	10.71	9.24
	HQC (2000)	837.17	12.45	8.30	10.52	3.93
Simvastatin hydroxy acid	LLOQ (2.5)	1.13	7.215	-3.17	9.30	0.16
	LQC (20)	9.07	4.69	8.75	5.18	9.52
	MQC (200)	90.72	5.12	3.51	6.59	4.77
	HQC (2000)	907.24	8.38	-5.44	8.19	-7.57
Pravastatin lactone	LLOQ (5)	2.03	9.98	14.16	6.96	10.00
	LQC (20)	8.13	11.46	-4.73	11.64	-2.20
	MQC (200)	81.3	11.36	0.03	9.43	3.72
	HQC (2000)	813	7.3	-0.20	6.28	-0.11
Pravastatin hydroxy acid	LLOQ (5)	2.23	17.04	7.30	12.93	11.62
	LQC (20)	8.93	11.76	-6.32	12.38	-6.87
	MQC (200)	89.30	12.28	-9.45	12.01	-3.61
	HQC (2000)	893.02	11.10	0.08	8.79	0.03

Abbreviations: HQC, high concentration quality control; LLOQ, lower limit of quantification; LQC, low concentration quality control; MQC, medium concentration quality control.
*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.
†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

The LC-MS/MS validation data of simvastatin hydroxy acid and simvastatin lactone from different plasma lipoprotein fractions are reported in Tables 3.6 and 3.7, respectively. The intra- and inter-day variability of both statins were obtained by running six different samples at four quality control levels on the same day and on 6 different days over a period of one month. Precision and accuracy results were within acceptance criteria of ±15% for the high, medium and low-quality control samples and ±20% for the lower limit of quantification. The assay method has a lower limit of quantification of 2.5 ng/mL for both forms of simvastatin in all lipoprotein fractions.

Table 3.6: Intra- and inter-day precision and accuracy of SVA from different lipoprotein fractions.

Lipoprotein fraction	Quality control (Concentration ng/mL)	Measured concentration (mean± SD)	Intra-day (n=6)		Inter-day (n=6)	
			Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
TRL	LLOQ (2.5)	2.42 ± 0.39	15.44	4.96	16.3	-3.20
	LQC (10)	10.1 ± 0.88	7.41	-0.41	8.65	1.19
	MQC (100)	92.7 ± 6.36	7.18	-7.74	6.86	-7.26
	HQC (1000)	1014.5 ± 46.15	4.22	4.22	4.55	1.45
LDL	LLOQ (2.5)	2.20 ± 0.35	15.63	-18.60	15.76	-11.86
	LQC (10)	10.12 ± 0.66	7.90	0.53	6.52	1.20
	MQC (100)	99.4 ± 3.86	3.90	1.81	3.88	-0.57
	HQC (1000)	916.22 ± 34.98	3.91	-7.92	3.82	-8.38
HDL	LLOQ (2.5)	2.44 ± 0.25	8.11	-4.57	10.27	-2.36
	LQC (10)	10.14 ± 0.54	3.10	0.76	5.28	1.43
	MQC (100)	95.22 ± 4.20	2.60	-5.14	4.41	-4.78
	HQC (1000)	927.3 ± 33.48	2.83	-4.92	3.61	-7.27
LPDP	LLOQ (2.5)	2.33 ± 0.30	6.35	-13.09	12.70	-6.99
	LQC (10)	10.28 ± 0.64	7.07	3.44	6.27	2.79
	MQC (100)	94.86 ± 3.43	3.29	-7.85	3.62	-5.14
	HQC (1000)	1058.7 ± 36.3	2.64	7.00	3.43	5.87

Abbreviations: HDL, high-density lipoprotein; HQC, high concentration quality control; LDL, low-density lipoprotein; LLOQ, lower limit of quantification; LPDP, lipoprotein-deficient plasma; LQC, low concentration quality control; MQC, medium concentration quality control; TRL, triglyceride-rich lipoproteins.

*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.

†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

Table 3.7: Intra- and inter-day precision and accuracy of SVL from different lipoprotein fractions.

Lipoprotein fraction	Quality control (Concentration ng/mL)	Measured concentration (mean± SD)	Intra-day (n=6)		Inter-day (n=6)	
			Precision (RSD%)*	Accuracy (RE%)†	Precision (RSD%)	Accuracy (RE%)
TRL	LLOQ (2.5)	2.48 ± 0.29	11.80	-0.68	13.65	-7.48
	LQC (10)	10.7 ± 1.36	14.67	11.99	12.67	7.07
	MQC (100)	95.7 ± 8.1	8.47	-4.30	11.21	-6.82
	HQC (1000)	1020.9 ± 61.1	5.98	2.09	8.72	9.90
LDL	LLOQ (2.5)	2.47 ± 0.43	10.54	8.40	17.34	-1.17
	LQC (10)	9.58 ± 1.29	9.10	-13.05	13.44	-4.15
	MQC (100)	101.2 ± 14.87	7.27	0.96	14.85	0.12
	HQC (1000)	1001.6 ± 44	4.34	2.06	10.92	0.16
HDL	LLOQ (2.5)	2.53 ± 0.19	7.63	1.23	9.64	-5.52
	LQC (10)	10.14 ± 1.19	13.25	-4.77	11.77	1.42
	MQC (100)	95.74 ± 12.36	10.86	3.33	12.91	-4.26
	HQC (1000)	924.95 ± 113.2	12.27	-12.30	12.24	-7.51
LPDP	LLOQ (2.5)	2.41 ± 0.43	12.40	-8.94	17.89	-3.71
	LQC (10)	10.34 ± 1.44	12.87	6.85	13.91	3.43
	MQC (100)	100.7 ± 11.5	8.10	-6.88	11.37	0.70
	HQC (1000)	1027.1 ± 93.3	9.38	-1.64	9.08	2.71

Abbreviations: HDL, high-density lipoprotein; HQC, high concentration quality control; LDL, low-density lipoprotein; LLOQ, lower limit of quantification; LPDP, lipoprotein-deficient plasma; LQC, low concentration quality control; MQC, medium concentration quality control; TRL, triglyceride-rich lipoproteins.

*****Relative standard deviation (RSD%) = (standard deviation [SD]/mean of obtained concentration) × 100.

†Relative error (RE%) = ([measured concentration−nominal concentration]/nominal concentration) × 100.

3.4 Discussion

3.4.1 Development and validation of HPLC-UV methods

One of the major technical challenges commonly encountered when developing bioanalytical methods for quantification of interconvertible compounds is to minimize the inter-conversion during the numerous steps of the bioanalytical process. Such inter-conversion may occur during sample collection, storage, extraction, evaporation, reconstitution, or even during sample analysis.[173, 175, 184] Several measures have been suggested to minimize the inter-conversion of statins during sample preparation. These include selecting a suitable extraction method with few operational procedures and short extraction time.[173, 185] Using suitable extraction solvents and controlling the pH of the solvents can further restrict statin inter-conversion during sample preparation. High temperature has been shown to accelerate statin inter-conversion. Therefore, it is necessary to maintain samples at low temperature during extraction and analysis procedures. Samples also need to be kept at -80 °C rather than -20°C especially when long term storage is required.[186]

Statin inter-conversion is a pH-dependent phenomenon and could affect the accuracy and precision of the analytical method [175]. It has been suggested that pH 4.5 is the optimum pH at which no inter-conversion of statins occurs[175, 182, 187]. Therefore, the pH of the samples was optimized immediately after sampling by the addition of ammonium acetate buffer (100 mM, pH 4.5).

Liquid-liquid extraction is one of the most common sample preparation techniques used for biological sample analysis. This extraction method generally provides cleaner extracts than solid phase extraction and protein precipitation. However, it has a disadvantage of low extraction recovery. In the

present analytical methods, samples were prepared by liquid-liquid extraction using acetonitrile, ammonium acetate buffer and methyl *tert*-butyl ether as the extraction solvents. Acetonitrile and ammonium acetate buffer were chilled in the refrigerator prior to addition in order to provide a low-temperature environment. Chilled acetonitrile was used to precipitate proteins from plasma and culture medium samples as it had previously been shown to be an efficient deproteinization agent,[188] and suitable for extracting of lipophilic compounds.[189-191] A volume of 250 µL acetonitrile for each 100 µL of sample was chosen as the lowest possible volume that can provide sufficient protein precipitation and good extraction recovery. Larger volume is expected to result in low extraction recovery. The use of ACN for protein precipitation has been suggested to improve the sensitivity of the analytical method and result in greater peak areas of the tested analytes. This increase in sensitivity is due to displacement of the analyte from plasma proteins resulting in better contact between the organic solvent and the analyte molecules. Internal standards were added to analytes containing samples prior to the addition of ACN in order to simulate the binding of analytes to plasma protein by ensuring satisfactory binding between internal standard molecule and plasma proteins.

The addition of ammonium acetate buffer (pH 4.5) not only minimizes the inter-conversion between the lactone and hydroxy acid forms but also creates an obvious separation between the aqueous and the organic layers.[192] Samples were evaporated and reconstituted during sample preparation. These steps have negligible effects on the inter-conversion of statins between the lactone and hydroxy acid forms.[185] Samples were then reconstituted with the

mobile phase that has been buffered to pH 4.5 in order to further minimize statin inter-conversion during sample storage and analysis.

In the present analytical methods, DDT and griseofulvin were selected as internal standards for simvastatin and pravastatin, respectively. These compounds have similar physicochemical properties, analytical behaviours, and extraction efficiencies as the analytes. Both compounds are stable and do not react with the sample or the mobile phase solutions. Other factor that affect our decision of using DDT and griseofulvin as internal standards is that they can elute after the analytes without interfering with any matrix or compound peaks. The use of internal standards is expected to enhance the method's ruggedness since such internal standards can track the analytes during the extraction procedure and compensate for any loss due to matrix effects.

Different mobile phase types and compositions have been tried for statins quantification. The best separation and peak shapes were obtained with acetonitrile: ammonium acetate buffer. The pH of the mobile phase was adjusted to 4.5 and the composition of the mobile phase was optimized using standard solutions and spiked matrix samples. The autosampler temperature was kept as low as possible (4^{o}C) in order to further minimize the inter-conversion process.

Validation results indicate that the developed HPLC-UV methods are both reliable and reproducible, with acceptable accuracy and precision. These methods have a lower limit of quantifications of 156, 313, 234 and 469 nmol/L for simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid, respectively. The low sensitivity of the developed

HPLC-UV methods in biological samples could be attributed to the poor UV-absorption properties of statin molecules.[175]

The developed HPLC-UV methods have been used to assess the inter-conversion of statins between hydroxy acid and lactone forms in PBS, human plasma, and cell culture medium at pH relevant to metabolic acidosis and alkalosis, in addition to physiological conditions.

3.4.2 Development and validation of LC-MS/MS analysis

LC-MS/MS is currently considered to be the method of choice for the determination of statin concentrations in biological samples.[175, 183] In this study, triple quadruple tandem mass spectrometry with ESI source was used for statins quantification as it was proven to be more sensitive than atmospheric-pressure chemical ionization (APCI) source.[182] Ionization response of the analytes was examined using both positive and negative mode. However, best tuning of the MS parameters was obtained with the positive mode for the lactone forms of statins and negative modes for the hydroxy acid forms. The use of ammonium acetate buffer as an aqueous mobile phase was found to further augment the signal and improve the selectivity of the analytical method. Acetonitrile was chosen as an organic phase because it showed lower background noise.

Data acquisition was performed using MRM mode. The collision energy was optimised for individual analytes and was found to be a critical step in selecting the product ions. $[M+Na]^+$ was chosen to monitor the lactone form of statins since it was the most abundant adduct. This adduct ion was derived from sodium ion present in biological matrix and glass containers used during

sample preparation and analysis.[193] $[M-H]^-$ was selected to analyse the hydroxy acid forms. The MS/MS spectrums were recorded by direct infusion of the tested analytes into the electrospray source via syringe pump at a flow rate of 20 µL/min (Figures 3.3 – 3.5).

Samples from cellular uptake studies are expected to contain both the hydroxy acid and lactone forms of statins. This necessitates polarity switching, during the run, from the negative to the positive ionization mode to allow the detection of the hydroxy acid and lactone forms, respectively. The intra-run polarity switching requires precise adjustment of the elution times of the hydroxy acid forms in order to separate them from the lactone forms. This was a challenging step since the lactone form may elute close to the acid form for some statins. Following optimization of the mobile phase composition and flow rate, we were able to achieve good separation between the two forms with sufficient time for the intra-run polarity switching (Figures 3.6 and 3.8).

Samples were prepared by protein precipitation using chilled ACN followed by liquid-liquid extraction with MTBE. This extraction method resulted in clean samples and help in minimizing ion suppression due to lower matrix components of the final extracts.[182] In the present analytical methods, lovastatin hydroxy acid and lactone were selected as internal standards for the hydroxy acid and lactone forms of simvastatin and pravastatin. These two compounds have the same analytical behaviours, ionization and extraction efficiencies as the analytes. Selection of such internal standards is expected to enhance the method's ruggedness since such internal standards can track the analytes during the extraction procedure and compensate for any loss due to matrix effects. Short retention times were achieved for all tested analytes and

internal standards making the developed methods suitable for routine analysis with high throughput. The developed LC-MS/MS method can also be applied to analyse the hydroxy acid and lactone forms of lovastatin using either simvastatin or pravastatin as internal standard.

Validation of the analytical methods was performed according to the US FDA guidelines.[194] Inter- and intra-day precision and accuracy were determined at the three quality control levels (LQC, MQC, and HQC). The inter- and intra-day precision and accuracy values of the hydroxy acid and lactone form of the tested analytes were within the acceptance criteria (Assay validation data of the tested analytes at four quality control levels are reported in Tables 3.5 -3.7). These results indicate that the developed methods are reproducible within the same day and over different days. The developed method has a lower limit of quantification of 5 nmol/L for simvastatin lactone, pravastatin lactone and pravastatin hydroxy acid and 2.5 nmol/L for simvastatin hydroxy acid.

In this study, we were able to develop LC-MS/MS methods that allow simultaneous determination of both hydroxy acid and lactone forms of simvastatin and pravastatin without any column or source changes. The methods have been proven to be sensitive, accurate, precise and reproducible with satisfactory lower limits of quantifications for the tested analytes. These methods have been successfully used to determine the intracellular concentrations of simvastatin and pravastatin hydroxy acid and lactone forms following uptake studies.

3.5 Conclusion

Accurate, precise and reproducible HPLC-UV and LC-MS/MS methods were developed and validated for the simultaneous quantification of the hydroxy acid and lactone forms of simvastatin and pravastatin. The HPLC methods were used to assess the inter-conversion of statins between hydroxy acid and lactone forms in PBS, human plasma, and cell culture medium at pH relevant to metabolic acidosis and alkalosis, in addition to physiological conditions. LC-MS/MS methods were successfully applied to determine the hydroxy acid and lactone forms of statins from C2C12 cell lysate samples and plasma lipoprotein fractions.

Chapter Four

4 pH-Dependent Inter-Conversion of Statins

4.1 Introduction

A number of lactone-containing compounds have been reported to undergo inter-conversion between their lactone and the corresponding hydroxy acid forms. These compound include statins, camptothecin anticancer drugs, and eplerenone.[173] Statins are commonly used to reduce morbidity and mortality associated with atherosclerotic cardiovascular disease.[195] Statin therapy has been recommended by the National Institute for Health and Care Excellence (NICE) guidelines for primary, as well as secondary prevention of cardiovascular disease.[196] In recent years, a significant increase in the number of statin prescriptions has been reported by the British Heart Foundation with more than seven million people currently taking statins in the UK.[19] Simvastatin, a highly lipophilic statin, is the most commonly prescribed cholesterol-lowering medication and 42% of American adults who are using cholesterol-lowering drugs are prescribed this drug.[20]

Statins are administered either as lactone (simvastatin and lovastatin) or hydroxy acid form (atorvastatin, cerivastatin, fluvastatin, pravastatin, and rosuvastatin).[75, 76] Chemically, lactones are cyclic esters of hydroxy carboxylic acids containing several carbon atoms and a single oxygen atom in a ring structure, with a ketone group replacing one of the carbons next to the oxygen atom (Figure 1.2).[173] The lactone form is pharmacologically inactive and is enzymatically converted to the hydroxy acid form in plasma, liver, and other tissues by the esterases and paroxinases in addition to CYP_{450s}.[75, 77] On the other hand, the hydroxy acid forms of statins can be converted to the

corresponding lactone forms mostly by glucuronidation mediated by UDP-glucuronosyltransferase enzymes and to a lesser extent by β-oxidation of the dihydroxy heptanoic acid side chain both *in vitro* and *in vivo*.[78-83] Both lactone and hydroxy acid forms were detected in the systemic circulation of humans and animals after oral administration of lovastatin,[84] atorvastatin,[85] simvastatin[86, 87] and cerivastatin[88] suggesting the presence of inter-conversion between both forms.

A marked difference in solubility exists between the lactone and hydroxy acid forms of statin. The lactone form is highly lipid soluble whereas hydroxy acid form has poor lipid solubility.[89] The lactone forms of statins are more potent in inducing myotoxicity compared to their corresponding acid forms with a 14-fold difference for atorvastatin lactone, 23-fold for pravastatin lactone, 26-fold for fluvastatin lactone and 37-fold for simvastatin lactone.[76]

There is limited information in the literature on the inter-conversion of statins between lactone and hydroxy acid forms in biological samples.[87, 197] Therefore, the aim of this study is to characterise the pH-dependent inter-conversion of statins between lactone and hydroxy acid forms over pH range relevant to physiological conditions, acidosis, and alkalosis. The specific objectives required to fulfil this aim include the following:

1- Assessment of the effect of pH changes on the inter-conversion of simvastatin and pravastatin between the lactone and hydroxy acid forms in simple buffer solution, human plasma, and cell culture medium.

2- Elucidation of the combined effect of pH alteration and hepatic microsomal enzymes on the conversion process of simvastatin lactone.

4.2 Methods

4.2.1 Inter-conversion of statins in PBS, human plasma, and DMEM culture medium

Statins inter-conversion studies were performed using the hydroxy acid and the lactone form of simvastatin and pravastatin. Initial studies were conducted by incubating either form of statins with PBS, human plasma or DMEM culture medium at three different pH levels corresponding to physiological condition (pH 7.4), acidosis (pH 6.8), and alkalosis (pH 7.8) for 24 hours. The concentration of the hydroxy acid and lactone form were determined at the end of the study by HPLC-UV analysis. Time course inter-conversion studies were then performed for the lactone forms of simvastatin and pravastatin. In these studies, the drug was incubated with PBS, human plasma, or DMEM culture medium at pH corresponding to both the extreme (pH 6.8 and 7.8) and less extreme (pH 7.0, 7.2, 7.4 and 7.6) levels. Time course inter-conversion of the lactone forms was tested over periods ranging from 30 minutes up to 48 hours. A more detailed description of the methodology used in statins inter-conversion studies is provided in Section 2.4.

4.2.2 Liver microsomal metabolic stability

Microsomal metabolic stability assay was performed for simvastatin lactone using human liver microsomes and assay buffer of three different pH levels to simulate physiological pH (7.4), acidosis (pH 6.8) and alkalosis (pH 7.8). A more detailed description of the methodology used in microsomal stability assay is provided in Section 2.5.

4.3 Results

4.3.1 Inter-conversion of statins in PBS, human plasma, and DMEM culture medium

To characterise the effect of pH alteration on inter-conversion of statins, the lactone and hydroxy acid forms of simvastatin and pravastatin were incubated with PBS, human plasma, and DMEM culture medium of pH relevant to acidosis, alkalosis, and physiological conditions for 24 hours. Figure 4.1 shows the results of simvastatin and pravastatin inter-conversion between lactone and hydroxy acid forms in PBS of different pH levels at a concentration of 50 μmol/L. Results indicate that the conversion of the lactone form of simvastatin and pravastatin to the corresponding hydroxy acid is strongly pH-dependent. At physiological and alkaline pH, substantial proportions of simvastatin lactone (~87% and 99%, respectively) and pravastatin lactone (~98% and 99%, respectively) were converted to the active hydroxy acid forms after 24 hours of incubation at 37°C. At acidic pH, this conversion occurs to a lower extent, resulting in a greater proportion of statins remaining in the more lipophilic lactone form (Figure 4.1, *A* and *C*). On the other hand, pH alteration has not influenced the conversion of the hydroxy acid form to the corresponding lactone form (Figure 4.1, *B* and *D*). Results of statins inter-conversion between lactone and hydroxy acid forms in human plasma and DMEM culture medium are shown in Figures 4.2 and 4.3, respectively.

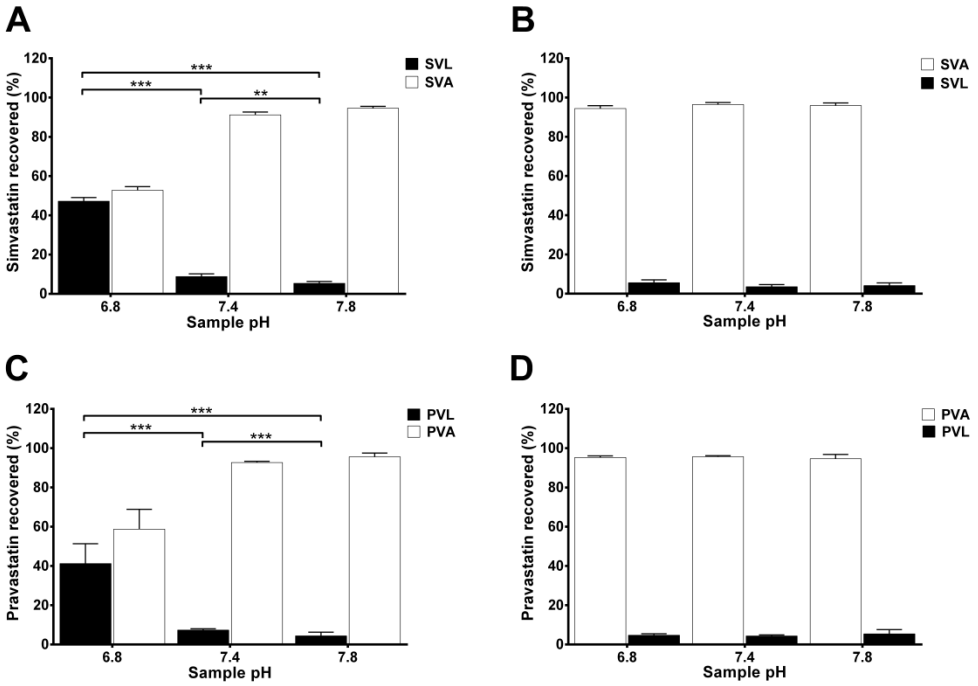


Figure 4.1: Inter-conversion of statins between lactone and hydroxy acid forms in phosphate buffer saline at different pH levels. Simvastatin lactone (A), simvastatin hydroxy acid (B), pravastatin lactone (C), and pravastatin hydroxy acid (D) were incubated with PBS of different pH levels (6.8–7.8) at a concentration of 50 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p < 0.001$; ** $p < 0.01$). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid; PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.

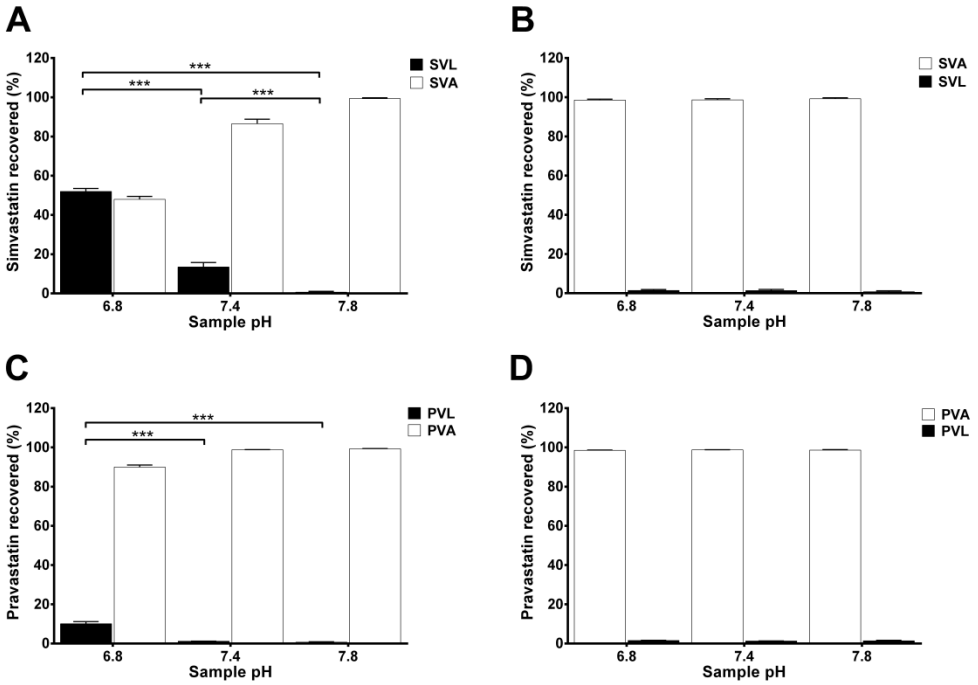


Figure 4.2: Inter-conversion of simvastatin and pravastatin between lactone and hydroxy acid forms in human plasma of different pH levels. Simvastatin lactone (A), simvastatin hydroxy acid (B), pravastatin lactone (C), and pravastatin hydroxy acid (D) were incubated with human plasma of different pH levels (6.8–7.8) at a concentration of 50 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid; PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.

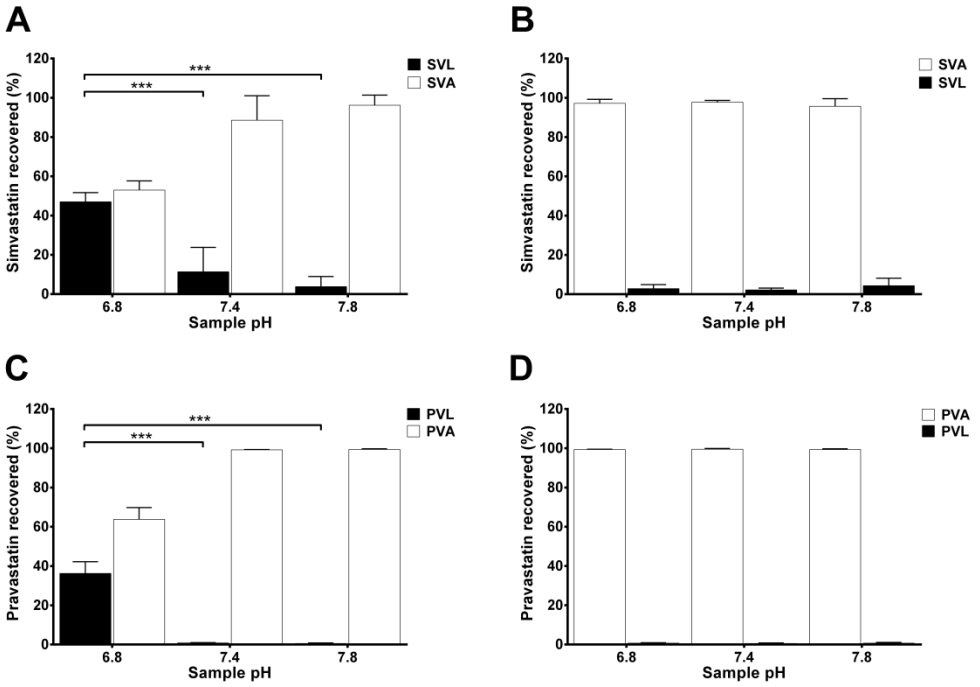


Figure 4.3: Inter-conversion of statins between lactone and hydroxy acid forms in DMEM culture medium at different pH levels. Simvastatin lactone (A), simvastatin hydroxy acid (B), pravastatin lactone (C), and pravastatin hydroxy acid (D) were incubated with DMEM culture medium of different pH levels (6.8–7.8) at a concentration of 50 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid; PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.

Since only the lactone form of statins was found to be susceptible to the pH-dependent conversion (as shown in Figures 4.1 – 4.3), time course conversion studies were performed for only this form of simvastatin and pravastatin at a concentration of 50 µmol/L and pH of 6.8–7.8 over 48 hours in PBS (Figure 4.4), human plasma (Figure 4.5), and DMEM culture medium (Figure 4.6).

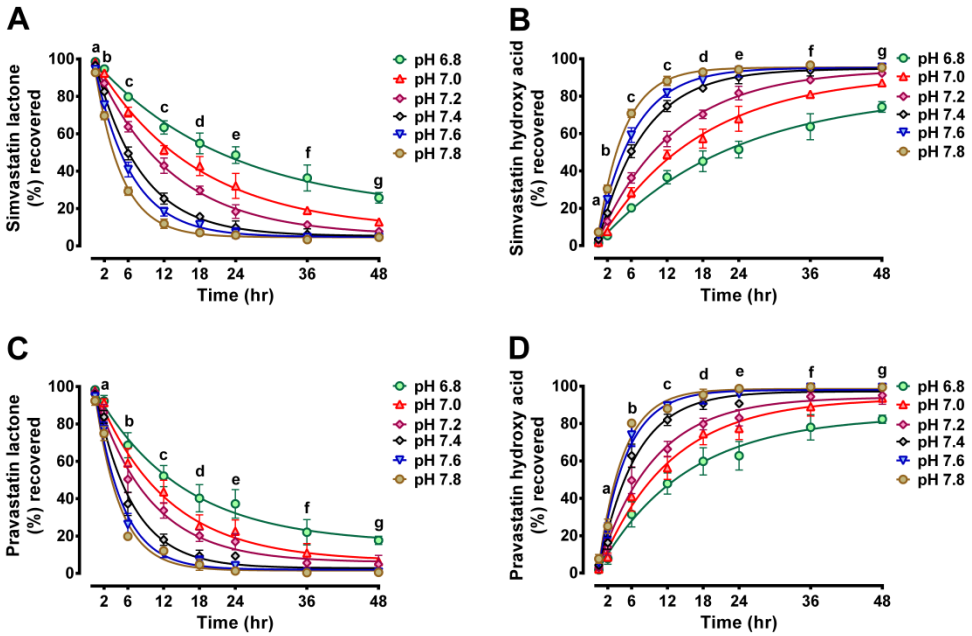


Figure 4.4: Time course inter-conversion of simvastatin lactone and pravastatin lactone in PBS at different pH levels. The percentages of the lactone and hydroxy acid forms recovered at different time points were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

The inter-conversion of simvastatin lactone shows disappearance of the lactone form (A) and formation of the hydroxy acid form (B).

a = pH 7.8 vs pH 6.8, 7.0 ($p < 0.001$); pH 7.8 vs pH 7.2 ($p < 0.05$). b = pH 6.8 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8; pH 7.6 vs pH 7.8 ($p < 0.001$). c = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8; pH 7.6 vs pH 7.8 ($p < 0.001$). d = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.8 ($p < 0.001$); pH 7.4 vs pH 7.6 ($p < 0.01$).e = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8 ($p < 0.001$); pH 7.4 vs pH 7.8 ($p < 0.05$). f = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8 ($p < 0.001$). g = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.4, 7.6, 7.8 ($p < 0.001$).

The inter-conversion of pravastatin lactone shows disappearance of the lactone form (C) and formation of the hydroxy acid form (D).

a = pH 6.8 vs pH 7.6, 7.8; pH 7.0 vs pH 7.6, 7.8; pH 7.2 vs pH 7.8 ($p < 0.001$); pH 7.4 vs pH 6.8, 7.8; pH 7.2 vs pH 7.6 ($p < 0.01$). b = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8 ($p < 0.001$). c = pH 6.8 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.0 vs 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8 ($p < 0.001$); pH 6.8 vs pH 7.0 ($p < 0.01$). d = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8 ($p < 0.001$). e = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.4, 7.6, 7.8; pH 7.2 vs pH 7.6, 7.8, ($p < 0.001$); pH 7.4 vs pH 7.8 ($p < 0.01$). f = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.4, 7.6, 7.8 ($p < 0.001$). g = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8 ($p < 0.001$).

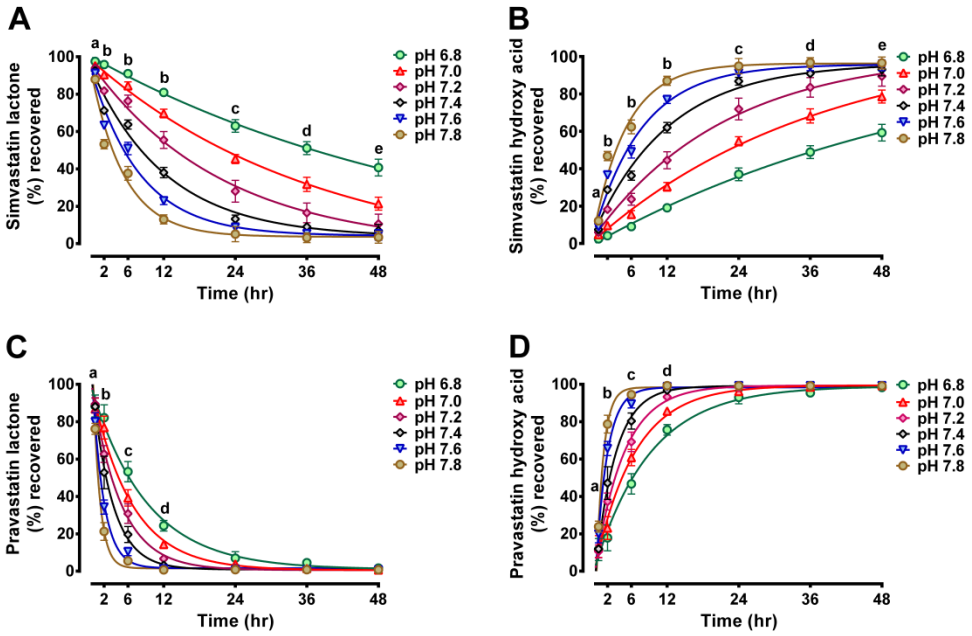


Figure 4.5: Time course inter-conversion of simvastatin lactone and pravastatin lactone in human plasma at different pH levels. The percentages of the lactone and hydroxy acid forms recovered at different time points were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

The inter-conversion of simvastatin lactone shows disappearance of the lactone form (A) and formation of the hydroxy acid form (B).

a = pH 7.0 vs pH 7.6 ($p < 0.05$); pH 7.4 vs pH 6.8, 7.8 ($p < 0.01$); pH 6.8 vs pH 7.6, 7.8; pH 7.8 vs pH 7.0, 7.2 ($p < 0.001$).

b = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8; pH 7.6 vs pH 7.8 ($p < 0.001$).

c = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8 ($p < 0.001$); pH 7.6 vs pH 7.8 ($p < 0.05$).

d = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8 ($p < 0.001$); pH 7.4 vs pH 7.6 ($p < 0.05$).

e = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.6, 7.8 ($p < 0.001$); pH 7.4 vs pH 7.2, 7.8 ($p < 0.05$).

The inter-conversion of pravastatin lactone shows the disappearance of the lactone form (A) and the formation of the hydroxy acid form (B).

a = pH 7.6 vs pH 6.8, 7.4 ($p < 0.01$); pH 7.8 vs pH 6.8, 7.0, 7.2, 7.4; pH 7.2 vs pH 7.6 ($p < 0.001$).

b = pH 6.8 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8; pH 7.6 vs pH 7.8 ($p < 0.001$).

c = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.2, 7.4, 7.6, 7.8; pH 7.2 vs pH 7.4, 7.6, 7.8; pH 7.4 vs pH 7.6, 7.8 ($p < 0.001$).

d = pH 6.8 vs pH 7.0, 7.2, 7.4, 7.6, 7.8; pH 7.0 vs pH 7.4, 7.6, 7.8 ($p < 0.001$); pH 7.0 vs pH 7.2 ($p < 0.01$).

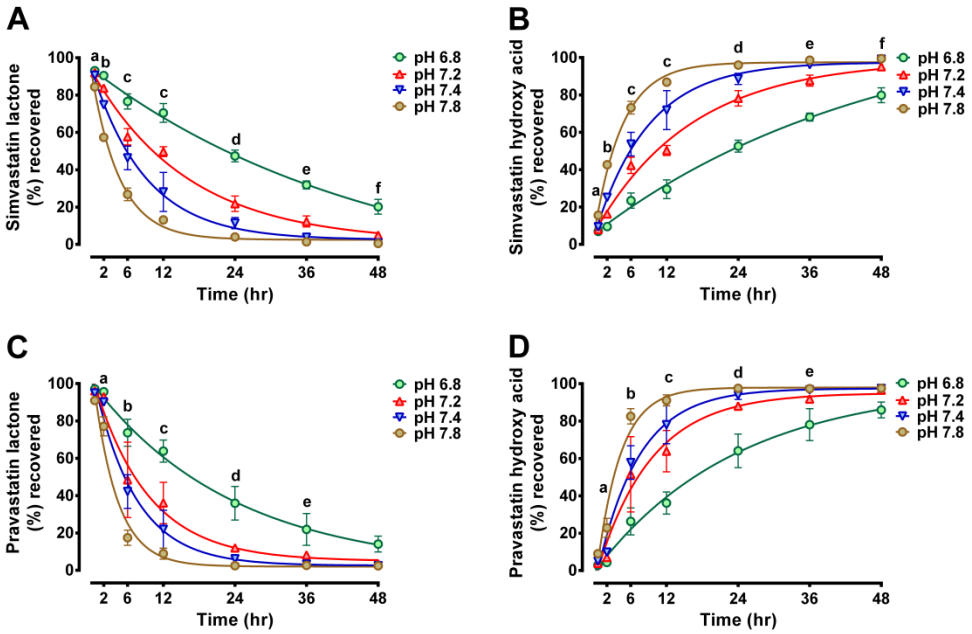


Figure 4.6: Time course inter-conversion of simvastatin lactone and pravastatin lactone in DMEM culture medium at different pH levels. The percentages of the lactone and hydroxy acid forms recovered at different time points were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

The inter-conversion of simvastatin lactone shows the disappearance of the lactone form (A) and the formation of the hydroxy acid form (B).

a = pH 7.8 vs pH 6.8, 7.2 ($p < 0.01$).

b = pH 6.8 vs pH 7.2 ($p < 0.05$); pH 6.8 vs pH 7.4, 7.8; pH 7.2 vs pH 7.4, 7.8; pH 7.4 vs pH 7.8 ($p < 0.001$).

c = pH 6.8 vs pH 7.2, 7.4, 7.8; pH 7.2 vs pH 7.4, 7.8; pH 7.4 vs 7.8 ($p < 0.001$).

d = pH 6.8 vs pH 7.2, 7.4, 7.8; pH 7.2 vs pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs 7.8 ($p < 0.01$).

e = pH 6.8 vs pH 7.2, 7.4, 7.8; pH 7.2 vs pH 7.4, 7.8 ($p < 0.001$).

f = pH 6.8 vs pH 7.2, 7.4, 7.8 ($p < 0.001$).

The inter-conversion of pravastatin lactone shows the disappearance of the lactone form (C) and the formation of the hydroxy acid form (D).

a = pH 6.8 vs pH 7.8 ($p < 0.01$); pH 7.2 vs pH 7.8 ($p < 0.05$).

b = pH 6.8 vs pH 7.2, 7.4, 7.8; pH 7.8 vs pH 7.2, 7.4 ($p < 0.001$).

c = 6.8 vs pH 7.2, 7.4, 7.8; pH 7.2 vs 7.8 ($p < 0.001$); pH 7.2 vs pH 7.4 ($p < 0.05$).

d = pH 6.8 vs pH 7.2, 7.4, 7.8 ($p < 0.001$).

e = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$); pH 6.8 vs pH 7.2 ($p < 0.05$).

The results show that lactone hydrolysis is both pH- and time-dependent. Even a slight increase in pH is accompanied by a substantial increase in the rate of hydrolysis to the less lipophilic hydroxy acid form. This conversion was observed as early as 30 minutes of incubation (Figures 4.4 – 4.6). After 48 hours of incubation with any matrix of alkaline pH, the lactone form of simvastatin and pravastatin was almost completely converted to the hydroxy acid form. However, at acidic pH, substantial parts of simvastatin and pravastatin from different matrices remained in the lactone form. The exception is pravastatin lactone incubated with human plasma which almost completely converted to the hydroxy acid form after 48 hours even under acidic condition. Faster rate of hydrolysis was observed for pravastatin lactone incubated with human plasma compared to its incubation with DMEM and PBS. Contrary to this, the rate of hydrolysis of simvastatin lactone was slower in plasma samples than in DMEM and PBS samples. This was demonstrated by comparing the hydrolysis half-life of the lactone form of statins under different pH levels (Table 4.1).

Pravastatin lactone showed the shortest half-life when incubated with human plasma, whereas simvastatin lactone demonstrated the longest half-life. Identical inter-conversion patterns were reported for each statin over all tested concentrations (12.5, 25, 50, and 100 µmol/L for simvastatin lactone and simvastatin hydroxy acid and 25, 50, 100, and 200 µmol/L for pravastatin lactone and pravastatin hydroxy acid) and among different matrices (Section 8.2).

Table 4.1: pH dependence of the hydrolysis of the lactone form of statins.

Sample pH	Simvastatin lactone (half-life, hours)			Pravastatin lactone (half-life, hours)		
	PBS	Human plasma	DMEM	PBS	Human plasma	DMEM
6.8	26.68 ± 0.97	36.51 ± 4.37[*,†]	21.42 ± 2.35[*]	21.22 ± 1.75	8.84 ± 0.31[*,†]	17.30 ± 4[‡]
7.0	16.92 ± 0.86	21.30 ± 2.50[*]	-	12.96 ± 1.38	7.39 ± 0.22[‡]	-
7.2	13.68 ± 0.71	14.46 ± 3.55	11.70 ± 0.50	9.36 ± 1.57	6.61 ± 0.9[§,†]	11.89 ± 2.71
7.4	12.86 ± 0.31	10.45 ± 0.38	7.40 ± 0.37[*]	6.89 ± 0.41	6.85 ± 0.54[γ]	9.72 ± 1.42[§]
7.6	9.58 ± 1.67	9.16 ± 1.77	-	5.59 ± 0.49	NA	-
7.8	10.23 ± 1.18	8.61 ± 3.38	7.09 ± 0.30	4.98 ± 0.44	NA	10.66 ± 0.81[‡]

Abbreviations: DMEM, Dulbecco's modified eagle medium; NA, nonapplicable; PBS, phosphate buffer saline.
Data are presented as mean ± SD of 6 replicates. Statistical analysis was done by 2-way ANOVA followed by Bonferroni's post hoc test.
[*]Significant difference from PBS samples ($p < 0.001$).
[†]Significant difference from DMEM samples ($p < 0.001$).
[‡]Significant difference from PBS samples ($p < 0.01$).
[§]Significant difference from DMEM samples ($p < 0.05$).
[γ]Significant difference from DMEM ($p < 0.05$).

4.3.2 Liver microsomal metabolic stability

Microsomal stability profiles of simvastatin lactone at different pH levels and concentration-time profiles of simvastatin hydroxy acid form in corresponding reaction mixtures are depicted in Figure 4.7, *A* and *B*, respectively. Simvastatin lactone was metabolised at a slower rate in the microsomal reaction mixture at pH 6.8 compared with other pH levels. The half-lives (mean ± SD) were 2.61 ± 0.07, 1.28 ± 0.05, and 1.23 ± 0.01 minutes at pH 6.8, 7.4, and 7.8, respectively. It is worth mentioning that the concentration-time profiles of simvastatin hydroxy acid form seen in Figure 4.7, *B* do not correspond to the rate of metabolic loss of the lactone, indicating that the hydroxy acid is not the main metabolite of simvastatin lactone in the liver microsomal reaction mixture.

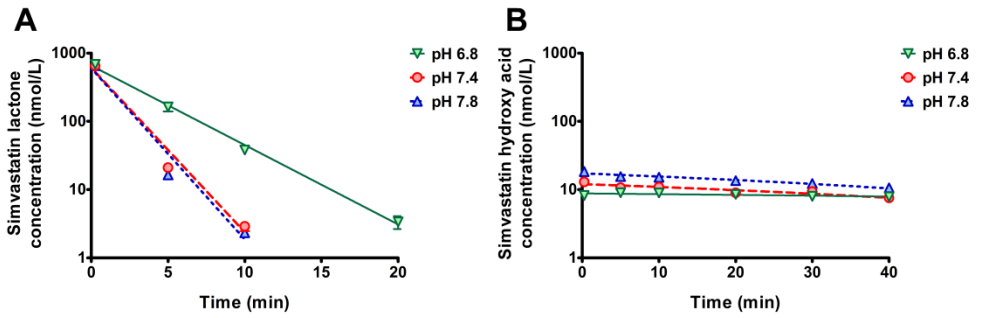


Figure 4.7: Liver microsomal stability of simvastatin lactone at 3 different pH levels. (A) Microsomal stability of simvastatin lactone as a function of time at different pH levels; (B) concentration-time profiles of simvastatin hydroxy acid form detected in liver microsomal stability reaction mixtures at 3 different pH levels. Results are expressed as mean ± SD, (n = 3). Solid line denotes exponential regression of samples at pH 6.8; dashed line denotes exponential regression of samples at pH 7.4; whereas dotted line denotes exponential regression samples at pH 7.8. This data was generated with the help of Jong Bong Lee.

4.4 Discussion

4.4.1 Inter-conversion of statins in PBS, human plasma, and DMEM culture medium

Simvastatin has been selected in this work as a model lipophilic statin because of its wide clinical use, high lipophilicity, and high incidence of muscle toxicity.[20, 135] Pravastatin was selected as a model hydrophilic statin with expected lower cellular uptake and myotoxicity. The inter-conversion of statins between lactone and hydroxy acid forms was investigated over concentrations ranging from 12.5 to 100 µmol/L for simvastatin lactone and simvastatin hydroxy acid, and concentrations of 25 to 200 µmol/L for pravastatin lactone and pravastatin hydroxy acid. These concentrations have been selected based on a review of literature that described the *in vitro* experiments of statin-induced myotoxicity.[38, 71, 72, 198-200]

In the current study, the lactone forms of simvastatin and pravastatin were found to be highly susceptible to pH-dependent conversion to the less lipophilic hydroxy acid forms. Acidification of PBS, human plasma, and DMEM culture medium significantly reduced the conversion of the lactone forms of both statins to the corresponding acid forms. On the other hand, alkalinization resulted in almost complete conversion of the lactone form to the active and less myotoxic hydroxy acid form (Figures 4.1 – 4.3; *A* and *C*). It is worth noting that regardless of the different pH conditions, the hydroxy acid forms of simvastatin and pravastatin were more stable than the lactone forms (Figures 4.1 – 4.3; *B* and *D*).

Statins are known to have relatively short half-lives in the range of 1-5 hours.[201] However, because the effects of pH alteration on statin inter-conversion were observed as early as within the first 30 minutes of incubation (Figures 4.4 – 4.6), changes in plasma pH are expected to affect statins inter-conversion within the general circulation before statin is cleared. A slower rate of hydrolysis was reported when simvastatin lactone was incubated with human plasma compared with DMEM and PBS samples as shown by longer half-life observed under acidic conditions (Table 4.1). This finding could be attributed to the binding of simvastatin lactone to albumin and other plasma proteins. It has been reported that simvastatin lactone is extensively bound to plasma proteins (95%–98%).[201] Such binding reduces the free (unbound) fraction of simvastatin lactone, makes it less susceptible to pH-dependent inter-conversion, and consequently slows down its hydrolysis. Similar results were reported with camptothecin (an anticancer drug which undergoes a similar pH-dependent inter-conversion between the lactone and hydroxy acid

forms).[102, 103] On the other hand, the rate of hydrolysis of pravastatin lactone in plasma was found to be faster than that in DMEM and PBS. The likely reason is that pravastatin lactone has low plasma protein binding (50%) which makes it readily available for chemical and enzymatic hydrolysis (by plasma esterases and paraoxonases).[202, 203] Although pravastatin is usually administered as hydroxy acid, it is expected that approximately 15% of the administered dose to be converted to the more lipophilic lactone form by the time the drug transit the stomach due to acid catalysed lactonisation (Section 8.1).

4.4.2 Liver microsomal metabolic stability

Metabolic stability studies were performed using human liver microsomes and potassium phosphate buffer at different pH levels to simulate physiological pH, acidosis, and alkalosis. Simvastatin lactone was tested in microsomal stability experiments because it was found to be the most cytotoxic statin as described in Section 5.3.4. Simvastatin lactone underwent extensive metabolism at all pH levels. However, at acidic pH, the metabolic rate was 2-fold slower than that at physiological pH. This result suggests that at pathophysiological conditions of acidosis, the metabolism of the lactone form by liver enzymes could be slower. Therefore, the slower rates of metabolism and chemical conversion of simvastatin at acidic pH would result in higher levels of the lactone form in general circulation. Interestingly, Figure 4.7, *B* shows that the levels of formation of simvastatin hydroxy acid do not correspond to the metabolic loss of the lactone form. This suggests that the hydroxy acid form is not the main metabolite formed in the microsomal reaction mixture. It is also evident that simvastatin hydroxy acid is not

metabolised extensively by the liver microsomes, which is in agreement with previously reported studies.[204]

Several studies have shown that hydroxy acid form of statins is susceptible to enzymatic lactonisation mostly by glucuronidation by UDP-glucuronosyltransferase enzymes and to a lesser extent by β-oxidation of the dihydroxy heptanoic acid side chain both *in vitro* and *in vivo*.[78-83] Although the enzymatic stability of the hydroxy acid form has not been tested in the current work, it is expected that such enzymatic lactonisation would render the hydroxy acid form of statins to be present in the more myotoxic lactone form inside the body especially in the presence of acidosis which could further slow down their chemical and enzymatic conversion.

4.5 Conclusions

The inter-conversion of statins between lactone and corresponding hydroxy acid form is strongly pH-dependent process and is affected by disturbance in acid-base balance. At physiological and alkaline pH, the lactone form of statins undergoes substantial conversion to the corresponding hydroxy acid form, while at acidic pH this conversion occurs to a lower extent causing substantial proportion of statin to remain in the lipophilic lactone form. Moreover, the rate of metabolism of the lactone form of statins is slower acidic pH compared to physiological and alkaline pH. On the other hand, the conversion of the hydroxy acid form to the corresponding lactone form is negligible at all tested pH levels.

Chapter Five

5 Influence of Acid-Base Imbalance on Cellular Uptake and Myotoxicity of Statins

5.1 Introduction

Statins are generally well tolerated, but muscular adverse effects considerably influence drug tolerability and patient adherence especially with long term use.[195] It has been estimated that in clinical practice about 10% of patients receiving statin therapy develop statin-induced muscle toxicity (mainly in the form of myalgia or myositis).[25-27] The exact mechanism by which these drugs induce their myotoxic effects is not fully understood. It has been postulated that lipophilic statins are more myotoxic than hydrophilic ones, most probably because of their ability to penetrate skeletal muscle tissues and alter membrane structure.[16, 66, 76] Nonetheless, the ability of lipophilic statins to penetrate hepatic cells makes them more potent in reducing elevated cholesterol levels.[66] This property might explain their wider use comparing to hydrophilic statins.

Several risk factors have been suggested to predispose patients to statin-associated myotoxicity including advanced age, high dose, female gender, drug interactions, genetic variability of drug metabolising enzymes and transporters, lipophilicity of statins, and coincident morbidities.[64]

Statins are administered either as lactone or hydroxy acid form. The lactone form is pharmacologically inactive, while the hydroxy acid is the active form that lowers plasma cholesterol levels.[76] Substantial differences exist between these forms in term of their lipophilicity. The lactone form is highly lipophilic, whereas hydroxy acid has poor lipid solubility.[66, 89] It has

been reported that lactone form is more myotoxic than the active acid form owing to its lipophilicity.[49, 76, 205] *In vivo*, inter-conversion between both forms is mediated by enzymatic as well as pH-dependent chemical reaction in plasma, liver, and other tissues.[80, 82, 87, 197] Therefore, acid-base imbalance can potentially alter the lipophilicity of statins by affecting their inter-conversion between the lactone and hydroxy acid forms. The higher lipophilicity of statins in the lactone form can potentially facilitate their penetration into muscle cells and consequently induce high local drug concentrations within skeletal muscle tissues.

Disturbance in acid-base imbalance is quite common among statin users. Many patients receiving statins are elderly and have multiple co-morbidities such as diabetes mellitus, cardiovascular and renal diseases, or using diuretics. These conditions have been reported to lead to development of metabolic acidosis or alkalosis.[206] Normally, the pH of blood plasma is maintained within narrow limits of 7.35 to 7.45. Most often, acidosis or alkalosis develops when there is a mild disturbance in blood plasma pH outside this range.[96] Nevertheless, extreme disturbances in acid-base balance with blood pH of less than 7 or greater than 7.65 have been reported in intensive care units among patients with long-term diuretic abuse,[157] prolonged vomiting,[158] diabetic ketoacidosis,[159, 160] lactic acidosis,[161, 162] renal tubular acidosis,[163] hypovolemic shock,[164, 165] and ethylene glycol intoxication.[166, 168] Muscle tissue acidosis is also likely in exercising patients. Indeed, exercise has been reported as a risk factor for statin-associated muscle toxicity.[93, 207] Thus, statin-associated myalgia could discourage exercise.

Although acid-base imbalance has been proposed to be a possible risk factor for statin-induced myotoxicity, the effect of disturbances in acid-base balance on the development and potentiation of statin-related myotoxicity has received only limited investigation.[59, 107, 108, 208] Limited numbers of *in vitro* studies have been performed to evaluate the myotoxicity pattern of statins at pH relevant to acidosis and alkalosis using a number of skeletal muscle cell lines. Results from these studies indicate enhanced cytotoxicity of statins under acidic conditions. However, the relationship between the pH-dependent inter-conversion of statins and their myotoxicity has not been addressed as a possible cause in these studies.[59, 107, 108, 208]

Therefore, the overall aim of this work was to elucidate the role of acid-base imbalance in statin-induced muscle toxicity. Simvastatin was selected as a model lipophilic statin in this study because of its high lipophilicity, wide clinical use and high incidence of reported simvastatin-associated muscle toxicity.[20, 135] Pravastatin was selected as a model hydrophilic statin with expected lower myotoxicity. The specific objectives required to fulfil this aim include the following:

1- Differentiation of C2C12 mouse myoblast cells into muscle myotubes and testing the markers of myogenic differentiation by quantitative polymerase chain reaction analysis.

2- Elucidation of the effect of disturbances in acid-base balance on statins uptake by muscle cells under conditions relevant to acidosis, alkalosis, and physiological pH.

3- Evaluation of the role of drug transporters in the cellular uptake of statins.

4- Assessment of the effect of acidosis/alkalosis on the development and potentiation of statin-induced muscle toxicity.

5.2 Methods

5.2.1 Characterisation of myogenic differentiation of C2C12 cells

C2C12 cell line was selected as a model for the *in vitro* studies of the effects of acid-base imbalance on the cellular uptake and myotoxicity of lipophilic and hydrophilic statins. The differentiation of the cells was induced by growing the cells in low serum culture medium. Cell differentiations were followed by daily microscopic examination and the mRNA expression of two myogenic markers of differentiation (myogenin and MHC) was monitored at different stages of cell differentiation using qPCR analysis. Total RNA extraction, cDNA synthesis, gene amplification, and assay validation are described in details in Section 2.6.2.

5.2.2 Cellular uptake of statins in response to medium pH alteration

The effect of acid-base imbalance on cellular uptake of statins was examined by incubating undifferentiated and differentiated C2C12 cells with the hydroxy acid or lactone form of simvastatin and pravastatin in cell culture medium of different pH (6.8-7.8). At the end of incubation period, cells were harvested, lysed and the intracellular concentrations of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone and pravastatin hydroxy acid were determined by the developed LC-MS/MS methods. Detailed descriptions of statins uptake studies at different medium pH levels are provided in Section 2.6.

5.2.3 Expression of cellular transporters

The expression of two influx transporters (Oatp1a4 and Oatp2b1) and three efflux transporters (Mrp1, Mrp4, and Mrp5) was examined in undifferentiated and differentiated C2C12 cells maintained under different pH conditions. The mRNA levels of transporters genes were determined using qPCR analysis as described in Section 2.7.

5.2.4 *In vitro* cytotoxicity of statins at medium with different pH levels

The cytotoxicity of the hydroxy acid and lactone forms of simvastatin and pravastatin to C2C12 cells were evaluated by MTT and LDH cytotoxicity assays in response to medium pH alteration. Undifferentiated and differentiated C2C12 cells were treated with increasing concentrations of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone and pravastatin hydroxy acid in cell culture medium of different pH (corresponding to physiological conditions, acidosis, and alkalosis) and cytotoxicity assays were performed as described in Section 2.8.

5.3 Results

5.3.1 Characterisation of myogenic differentiation of C2C12 cells

C2C12 cell line was selected as a model for the *in vitro* studies of the effects of acid-base imbalance on the cellular uptake and myotoxicity of lipophilic and hydrophilic statins.

In the present study, C2C12 cells were induced to differentiate for 9 days by switching them to a medium containing 2% horse serum. Myotubes formation was followed by daily microscopic examination and pictures were taken at different stages of differentiation by AMG EVOS digital microscope. Figure 5.1 shows both undifferentiated and differentiated C2C12 cells within 5 days of differentiation. There are marked differences in the morphology of both cells. The undifferentiated cells appear star-shaped or fusiform, and upon differentiation, they become elongated and fused together.

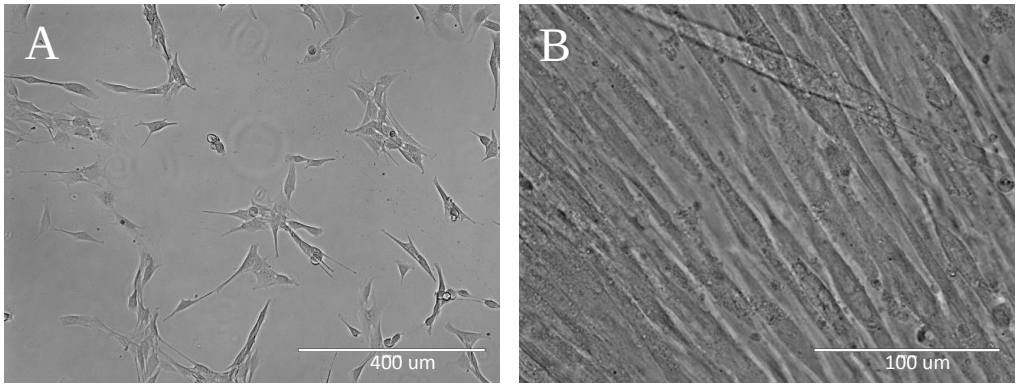


Figure 5.1: Differentiation of C2C12 mouse myoblasts. (A) Undifferentiated C2C12 myoblasts; (B) Differentiated C2C12 myotubes after 5 days of differentiation. The undifferentiated cells appear star-shaped or fusiform, and upon differentiation, they become elongated and fuse together. Pictures were taken by AMG EVOS digital microscope.

Analysis of real-time qPCR results revealed successful differentiation of C2C12 myoblast into myotubes as shown by marked increase in the expression of myogenin and MHC genes during cell differentiation (Figure 5.2, *A*). Myogenin expression was increased in the early stage of myoblast differentiation, reached a maximum of 36-fold the baseline levels after 3 days and remained elevated for the subsequent days of differentiation. On the other hand, a gradual increase in gene expression was observed for MHC, and the levels of expression reached a maximum of 70-fold by day 7. No significant changes were reported in the expression of Gapdh, Hprt and at most time points of Rps12 reference genes during C2C12 cell differentiation (Figure 5.2, *B*).

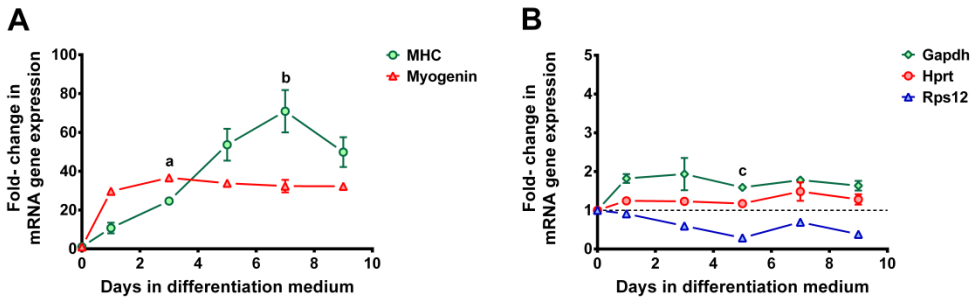


Figure 5.2: qPCR expression profiles of (A) myogenin and myosin heavy chain (MHC) at days 0-9 of C2C12 differentiation; (B) glyceraldehyde-3-phosphate (Gapdh), hypoxanthine-guanine phosphoribosyltransferase (Hprt), and ribosomal protein S12 (Rps12) reference genes at days 0–9 of C2C12 differentiation. Analysis of gene expression was done after normalization to a single reference gene (TATA box-binding protein) and the fold changes in gene expression were expressed relative to gene expression in undifferentiated cells. Results are expressed as mean ± SD, (n = 3). Data were analysed by Kruskal-Wallis nonparametric test followed by Dunn's test for multiple comparisons. a = significant difference in myogenin expression between day 0 and day 3 ($p < 0.01$), b = significant difference in myosin heavy chain expression between day 0 and day 7 ($p < 0.01$), c = significant difference in ribosomal protein S12 expression between day 0 and day 5 ($p < 0.05$).

To ensure that the used primers gave rise to only single DNA fragment, PCR products were analysed on 1.2% agarose gels after staining with SYBR safe stain. Figure 5.3 shows that there was only one band for each primer set and that the bands were within the expected size. Such single band with no primer dimer formation indicates the specificity and efficiency of the used primers.

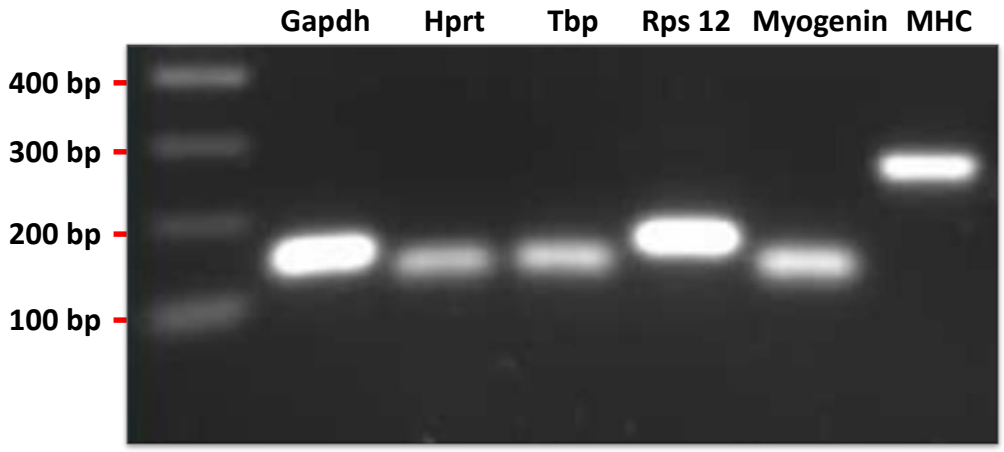


Figure 5.3: Agarose gel electrophoreses of qPCR amplification products of 2 target genes and 4 reference genes. 1.2% of agarose gel were stained with SYBR safe stain and visualized with UV transilluminator.

5.3.2 Cellular uptake of statins in response to medium pH alteration

To gain a better understanding of the influence of pH alteration on statin-induced myotoxicity, time course uptake studies of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid were performed using undifferentiated and differentiated C2C12 cells over pH relevant to acidosis, alkalosis, and physiological conditions.

The effect of medium pH alteration on cellular simvastatin uptake was assessed by comparing the total concentration of simvastatin (lactone and hydroxy acid forms combined) found in muscle cells maintained under different pH conditions. Figures 5.4 shows the uptake of simvastatin by

undifferentiated and differentiated C2C12 after treatment with 1 µmol/L simvastatin lactone or simvastatin hydroxy acid over a period of 6 hours. The maximum cellular uptake of simvastatin lactone by undifferentiated cells was reached within 2 hours of treatment. This initial increase in cellular simvastatin lactone uptake by muscle cells was followed by a gradual decline over time (Figure 5.4, *A*). During the first 2 hours of treatment, no significant differences in simvastatin lactone uptake by undifferentiated cells were found between different pH levels. However, incubating cells for a longer period of time resulted in significant differences in the cellular simvastatin lactone uptake at different pH levels as shown by the changes in the concentration of total simvastatin (lactone and hydroxy acid forms) recovered in response to medium pH modifications (Figure 5.4, *A*).

The total simvastatin recovered in undifferentiated cells maintained at physiological pH after 3, 5, and 6 hours of treatment was 170.5 ± 23.7, 84.8 ± 4.1, and 99.7 ± 21.4 nmol/mg protein, respectively. Reducing extracellular pH to acidic range resulted in a marked increase in total simvastatin uptake by approximately 50%, 78%, and 40%, respectively, compared to total simvastatin found in cells grown under physiological pH. On the other hand, no significant differences in simvastatin lactone uptake were detected between cells grown under alkaline and physiological conditions (Figure 5.4, *A*).

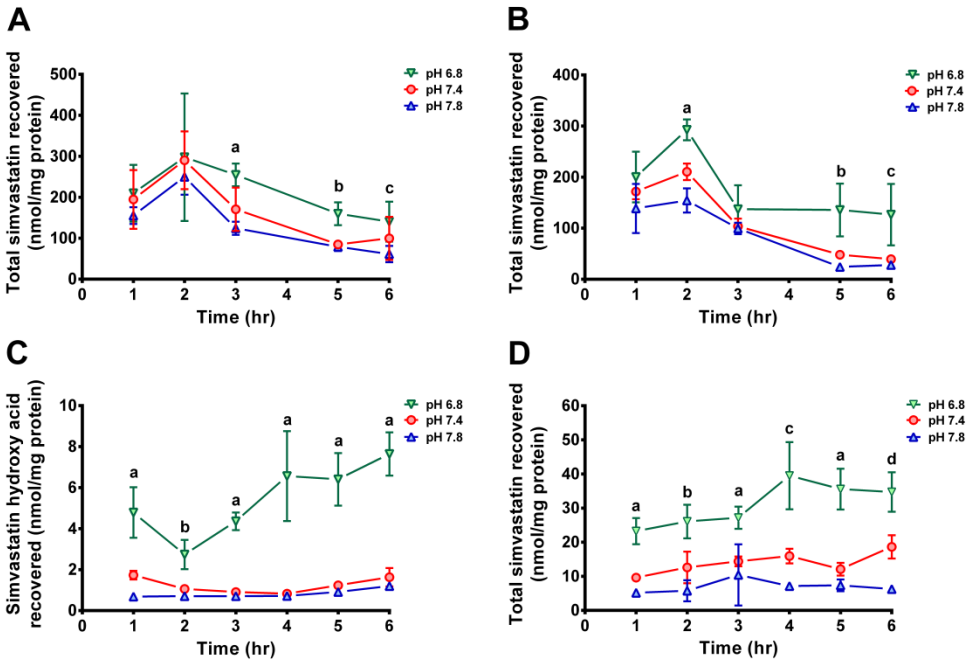


Figure 5.4: Uptake of simvastatin lactone (SVL) and simvastatin hydroxy acid (SVA) by undifferentiated and differentiated C2C12 cells. The uptake was determined after incubation of the cells with 1 µmol/L of SVL or SVA in Dulbecco's modified eagle medium culture medium of different pH levels (6.8-7.8) at 37°C for 6 hours. Results are expressed as nanomoles per milligram of protein ± SD, (n = 5). Data were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

(A) Total simvastatin (lactone + hydroxy acid) recovered in undifferentiated C2C12 cells after treatment with 1 µmol/L SVL.

a = pH 6.8 vs pH 7.4 ($p < 0.01$); pH 6.8 vs pH 7.8 ($p < 0.001$).

b = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$).

c = pH 6.8 vs pH 7.8 ($p < 0.05$).

(B) Total simvastatin recovered in differentiated C2C12 cells after treatment with 1 µmol/L SVL.

a = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs pH 7.8 ($p < 0.01$).

b = pH 6.8 vs pH 7.4 ($p < 0.01$); pH 6.8 vs pH 7.8 ($p < 0.001$).

c = pH 6.8 vs pH 7.4, 7.8 ($p < 0.01$).

(C) SVA recovered in undifferentiated C2C12 cells after treatment with 1 µmol/L SVA (no SVL was recovered in this experiment).

a = pH 6.8 vs pH 7.4 ($p < 0.01$); pH 6.8 vs pH 7.8 ($p < 0.001$).

b = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$).

(D) Total simvastatin recovered in differentiated C2C12 cells after treatment with 1 µmol/L SVA.

a = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$).

b = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs pH 7.8 ($p < 0.05$).

c = pH 6.8 vs pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs pH7.8 ($p < 0.01$).

d = pH 6.8 vs pH 7.4, 7.8; pH 7.4 vs pH 7.8 ($p < 0.001$).

The intracellular concentrations of simvastatin lactone recovered in undifferentiated C2C12 cells were significantly higher than those of the hydroxy acid form at all tested pH levels (Figure 5.5, *A*). Unlike undifferentiated cells, short-term treatment of differentiated C2C12 cells with simvastatin lactone for 2-hour period resulted in significant changes in simvastatin uptake in response to medium pH alteration. At physiological pH, after 2 hours of treatment, simvastatin lactone uptake by differentiated C2C12 cells was 210.4 ± 7.2 nmol/mg protein. Acidification of culture medium to a pH of 6.8 significantly increased simvastatin lactone uptake by 40% relative to physiological pH, whereas medium alkalinization to a pH of 7.8 resulted in a significant reduction in simvastatin lactone uptake by 27% (Figure 5.4, *B*).

Results of cellular uptake of simvastatin hydroxy acid by undifferentiated and differentiated C2C12 cells under different pH conditions are shown in Figure 5.4, *C* and *D*, respectively. The uptake of simvastatin hydroxy acid by these cells was much lower than that of simvastatin lactone at all-time points. Significantly more simvastatin hydroxy acid (~2.5-5 folds) was found in undifferentiated C2C12 cells maintained under acidic pH compared with cells treated under physiological conditions. Increasing the extracellular pH to alkaline level of 7.8 has not been shown to further affect the uptake of simvastatin hydroxy acid compared to pH 7.4 (Figure 5.4, *C*). All intracellular simvastatin found in undifferentiated cells was in the hydroxy acid form, and no simvastatin lactone was detected at any pH level.

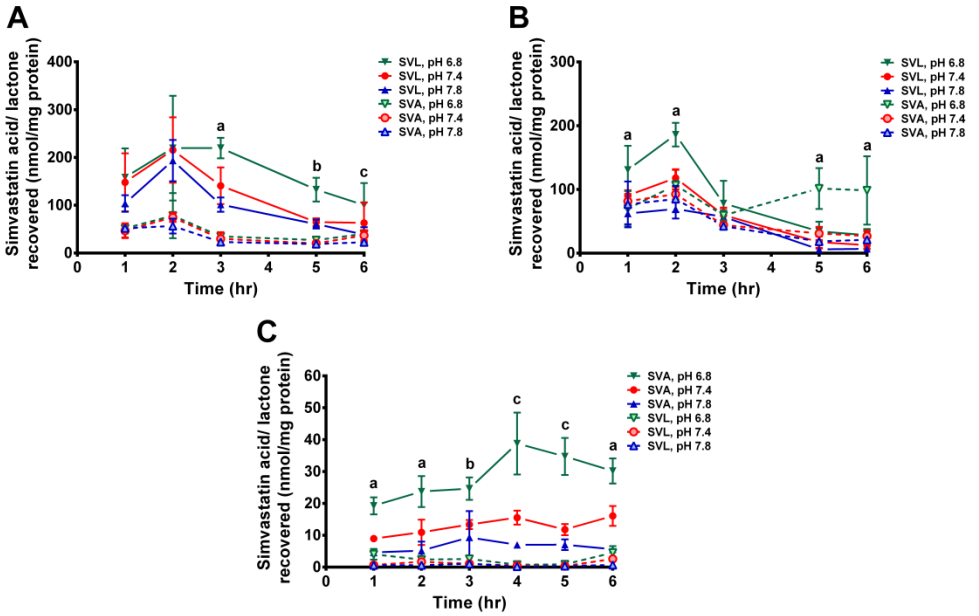


Figure 5.5: Uptake of simvastatin by undifferentiated and differentiated C2C12 cells. The uptake was determined after incubation of the cells with 1 µmol/L of simvastatin lactone (SVL) or simvastatin hydroxy acid (SVA) in Dulbecco's modified eagle medium of different pH levels (6.8-7.8) at 37°C for 6 hours. Results are expressed as nanomoles per milligram of protein ± SD, (n = 5). Data were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

(A) Intracellular concentration of simvastatin acid and lactone recovered in undifferentiated cells after treatment with 1 µmol/L SVL.

a = SVL, pH 6.8 vs SVA, pH 6.8; SVL, pH 7.4 vs SVA, pH 7.4 ($p < 0.001$).

b = SVL, pH 6.8 vs SVA, pH 6.8; SVL, pH 7.4 vs SVA, pH 7.4; SVL, pH 7.8 vs SVA, pH 7.8 ($p < 0.001$).

c = SVL, pH 6.8 vs SVA, pH 6.8; SVL, pH 7.4 vs SVA, pH 7.4 ($p < 0.001$); SVL, pH 7.8 vs SVA, pH 7.8 ($p < 0.01$).

(B) Intracellular concentration of simvastatin acid and lactone recovered in differentiated cells after treatment with 1 µmol/L SVL.

a = SVL, pH 6.8 vs SVA, pH 6.8 ($p < 0.001$).

(C) Intracellular concentration of simvastatin acid and lactone recovered in differentiated cells after treatment with 1 µmol/L SVA.

a = SVA, pH 6.8 vs SVL, pH 6.8; SVA, pH 7.4 vs SVL, pH 7.4 ($p < 0.001$).

b = SVA, pH 6.8 vs SVL, pH 6.8; SVA, pH 7.4 vs SVL, pH 7.4; SVA, pH 7.8 vs SVL, pH 7.8 ($p < 0.001$).

c = SVA, pH 6.8 vs SVL, pH 6.8; SVA, pH 7.4 vs SVL, pH 7.4 ($p < 0.001$); SVA, pH 7.8 vs SVL, pH 7.8 ($p < 0.01$).

The uptake of simvastatin hydroxy acid by differentiated C2C12 was several folds higher than its uptake by undifferentiated cells. Reducing the extracellular pH to acidic levels significantly increased the uptake of simvastatin hydroxy acid by differentiated C2C12 cells by more than 2-folds, whereas maintaining the cells under alkaline pH significantly reduced its uptake by approximately 50% (Figure 5.4, *D*).

Unlike undifferentiated cells, minor inter-conversion of simvastatin hydroxy acid to the corresponding lactone form was found in differentiated cells treated under different pH levels (Figure 5.5, *C*). Uptake study was also performed by incubating undifferentiated and differentiated C2C12 cells with pravastatin lactone or pravastatin hydroxy acid (1 µmol/L). As expected, the intracellular levels of pravastatin were extremely low (below the limit of quantification of the analytical assay; data not shown).

5.3.3 Expression of cellular transporters

In the present study, the expression of mRNA of 2 influx transporters (Oatp1a4 and Oatp2b1) and 3 efflux transporters (Mrp1, Mrp4, and Mrp5) was assessed in undifferentiated and differentiated C2C12 cells maintained under different pH conditions. These transporters have been reported to be involved in statin uptake.[209-211] As shown in Figure 5.6, Mrp1, Mrp4, and Mrp5 were expressed in both cell types.

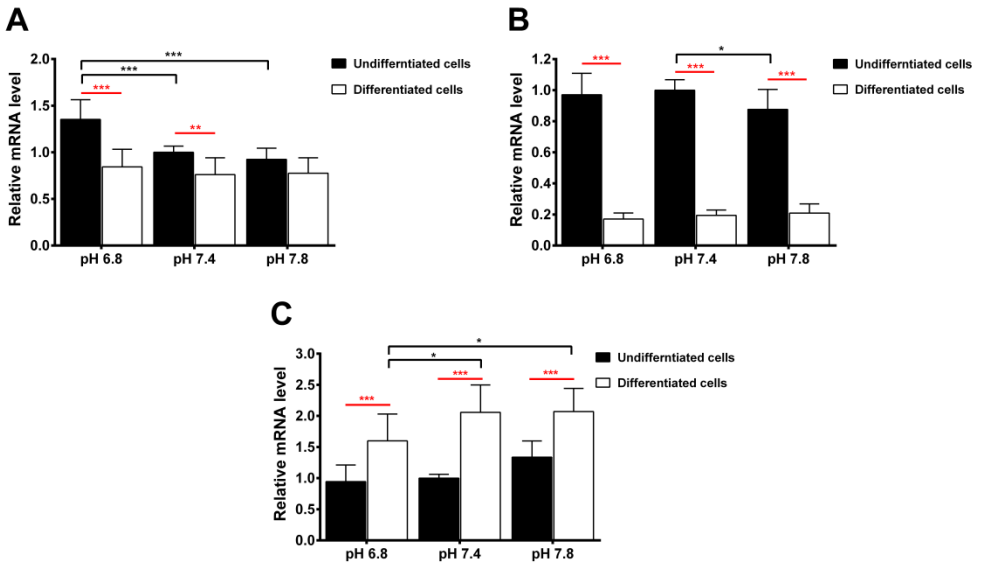


Figure 5.6: mRNA expression of (A) Mrp1, (B) Mrp4 and (C) Mrp5 in undifferentiated and differentiated C2C12 cells maintained at different pH levels**.** Results are expressed as fold changes in gene expression relative to the baseline level observed with undifferentiated C2C12 maintained at physiological pH and were normalized relative to Gapdh. Data are presented as mean ± SD, (n=4) and were analysed by 2-way ANOVA followed by Bonferroni's post hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Red straight lines denote differences in relative mRNA levels between undifferentiated and differentiated cells. Black arched lines denote differences within each cell type maintained at different pH levels. This data was generated with the help of Raj Gandhi.

However, neither Oatp1a4 nor Oatp2b1 influx transporters were detected in either undifferentiated or differentiated C2C12 cells. This is consistent with previous reports that showed no expression of these influx transporters in undifferentiated and differentiated C2C12 cells lines.[209, 211] The mRNA of both Mrp1 and Mrp4 efflux transporters were found to be expressed at higher levels in undifferentiated cells, whereas Mrp5 showed higher expression in differentiated cells. The pH dependency was found with Mrp1 in undifferentiated cells and with Mrp5 in differentiated cells. Mrp1 showed higher expression at acidic pH in undifferentiated cells, whereas Mrp5

exhibited increased levels with alkaline pH in differentiated cells. Results of mRNA expression of reference genes are presented in Figure 5.7.

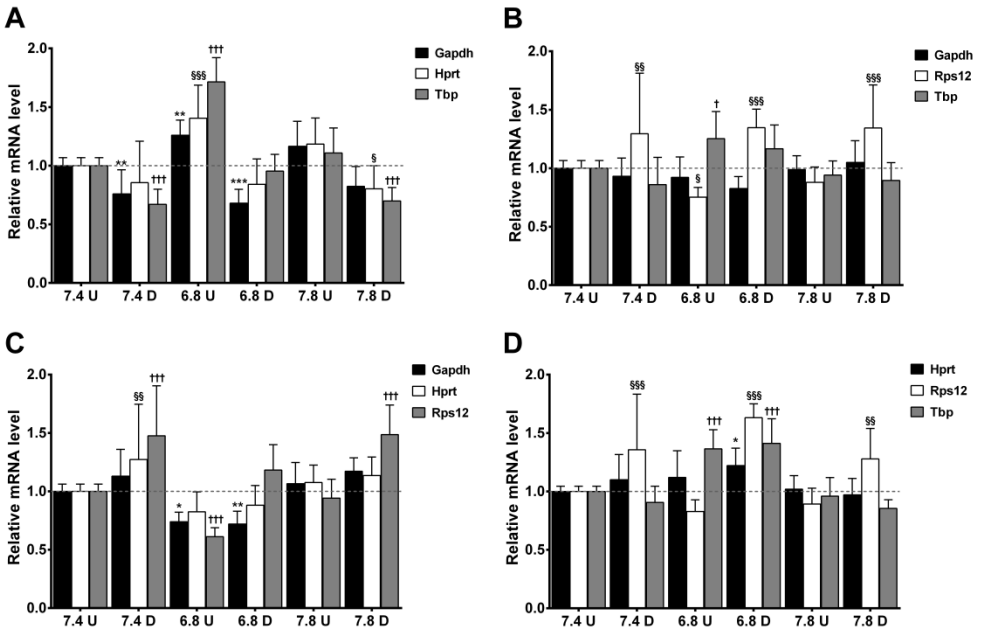


Figure 5.7: mRNA expression of (A) ribosomal protein S12 (Rps12), (B) hypoxanthine phosphoribosyltransferase (Hprt), (C) TATA box-binding protein (Tbp), and (D) glyceraldehyde-3-phosphate dehydrogenase (Gapdh) reference genes in undifferentiated (U) and differentiated (D) C2C12. Results are expressed as fold changes in gene expression relative to the baseline level observed with undifferentiated C2C12 maintained at physiological pH. Each reference gene was normalized to the other 3 reference genes. Data are presented as mean ± SD, (n = 4) and were analysed 2-way ANOVA followed by Bonferroni's post hoc test, $^{***, §§§, †††}$ $p < 0.001$, $^{**, §§, ††}$ $p < 0.01$, $^{*, §, †}$ $p < 0.05$ compared to mRNA expression level in undifferentiated cell samples of pH 7.4. This data was generated with the help of Raj Gandhi.

To ensure that the used primers gave rise to only single DNA fragment, PCR products were analysed on 1.2% agarose gels after staining with SYBR safe stain. Figure 5.8 showed that there was only one band for each primer set and that the bands were within the expected size. Such single band with no primer dimer formation indicates the specificity and efficiency of the used primers.

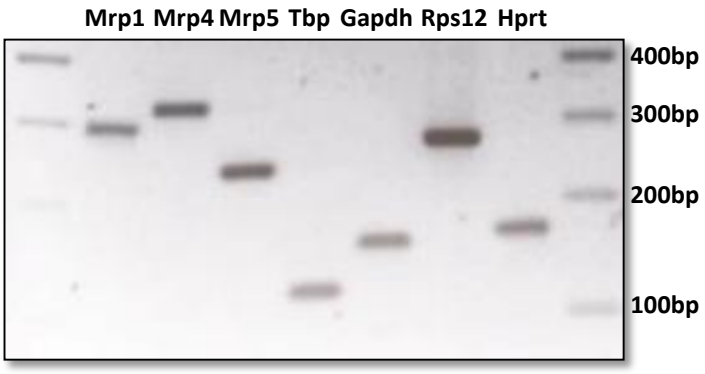


Figure 5.8: Agarose gel electrophoreses of qPCR amplification products of 3 drug transporter genes and 4 reference genes. 1.2% of agarose gel were stained with SYBR safe stain and visualized with UV transilluminator.

5.3.4 *In vitro* cytotoxicity of statins at different medium pH levels

The effect of medium pH changes on cytotoxicity of statins was investigated using both undifferentiated and differentiated C2C12 skeletal muscle cells. Cells were exposed to increasing concentrations of simvastatin lactone, simvastatin hydroxy acid, pravastatin lactone, or pravastatin hydroxy acid for 72 hours, and the metabolic activity of the cells was measured using the MTT test (Figures 5.9 and 5.10).

The IC_{50} of simvastatin lactone in undifferentiated C2C12 cells grown in DMEM culture medium of physiological pH was found to be 0.58 ± 0.02 µmol/L. Medium acidification to a pH of 6.8 significantly reduced the IC_{50} to 0.44 ± 0.02 µmol/L, whereas alkalinizing the medium to pH 7.8 resulted in significant increase in IC_{50} to 1.13 ± 0.04 µmol/L. Similar cytotoxicity pattern was observed with differentiated C2C12 cells treated with simvastatin lactone under different pH conditions (Table 5.1). Likewise, incubating C2C12 cells with simvastatin hydroxy acid resulted in cytotoxic response comparable with that observed with simvastatin lactone in response to medium pH alteration

(Figure 5.9). However, the IC_{50} values of simvastatin hydroxy acid were several folds higher than that of simvastatin lactone (Table 5.1).

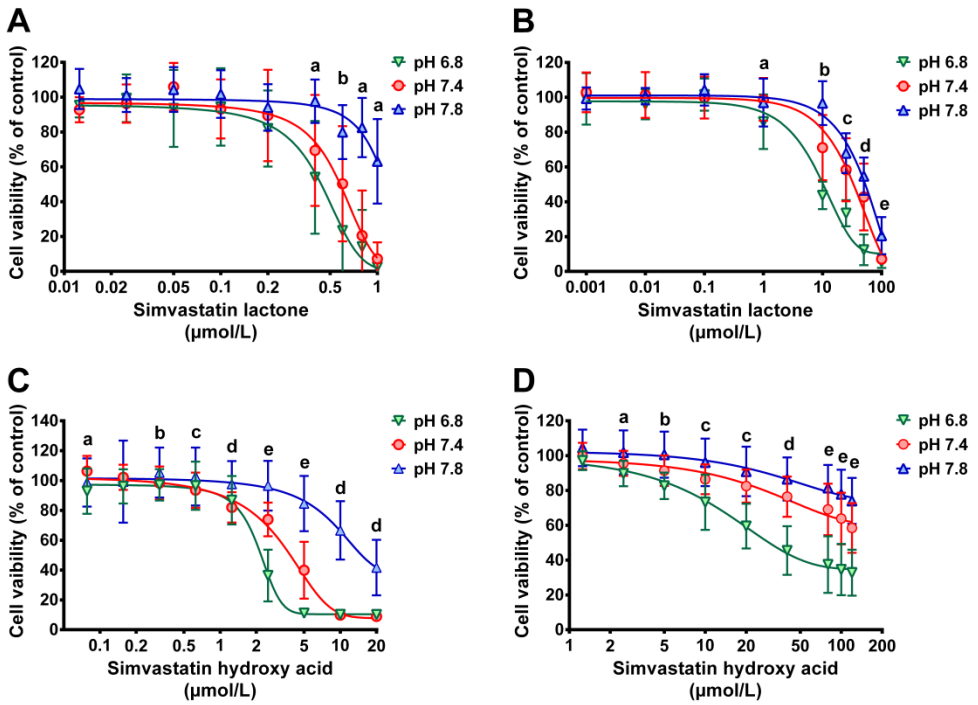


Figure 5.9: Effect of simvastatin lactone (SVL) and simvastatin hydroxy acid (SVA) on the viability of undifferentiated and differentiated C2C12. Cells were cultured at a density of 4,000 cells/well and allowed to attach for 24 hours or to differentiate for 4 days, then exposed to increasing concentrations of SVL or SVA under acidic, neutral, and alkaline medium pH for 72 hours. Results are presented as mean ± SD of 3 experiments, 8 replicates per experiment. Data were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.
(A) Effects of SVL on cell viability of undifferentiated C2C12 myoblasts.
a= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$); b= pH 7.8 vs. pH 6.8, 7.4; pH 6.8 vs. pH 7.4 ($p < 0.001$).
(B) Effects of SVL on cell viability of differentiated C2C12 myocytes.
a= pH 6.8 vs. pH 7.4 ($p < 0.01$); pH 6.8 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); d= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.01$); e= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$).
(C) Effects of SVA on cell viability of undifferentiated C2C12 myoblasts.
a= pH 6.8 vs. pH 7.4 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.05$); b= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.05$); c= pH 7.4 vs. pH 7.8 ($p < 0.01$); d= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$); e= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).
(D) Effects of SVA on cell viability of differentiated C2C12 myocytes.
a= pH 6.8 vs. pH 7.8 ($p < 0.001$); b= pH 7.4 vs. pH 6.8, 7.8 ($p < 0.05$); pH 6.8 vs. pH 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.05$); d= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.01$); e= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).

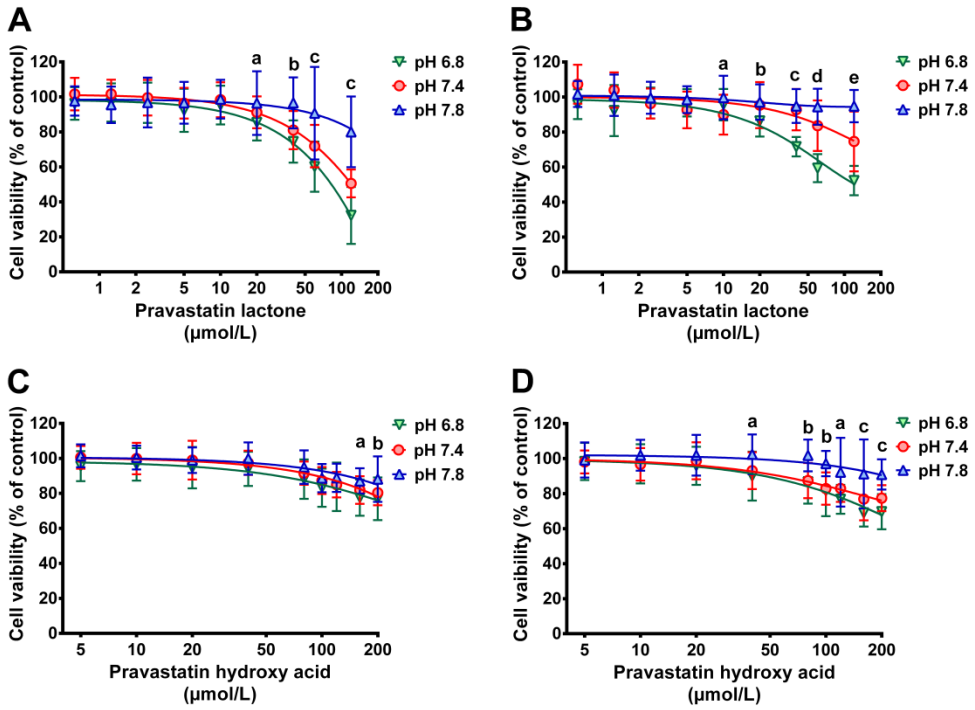


Figure 5.10: Effect of pravastatin lactone (PVL) and pravastatin hydroxy acid (PVA) on the viability of undifferentiated and differentiated C2C12. Cells were cultured at a density of 4,000 cells/well and allowed to attach for 24 hours or to differentiate for 4 days, then exposed to increasing concentrations of PVL or PVA under acidic, neutral, and alkaline medium pH for 72 hours. Results are presented as mean ± SD of 3 experiments, 8 replicates per experiment. Data were analysed by 2-way ANOVA followed by Bonferroni's post hoc test.

(A) Effects of PVL on cell viability of undifferentiated C2C12 myoblasts.
a= pH 6.8 vs. pH 7.8 ($p < 0.001$); b= pH 6.8 vs. pH 7.4 ($p < 0.05$); pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8, pH 7.4 vs. pH 7.8 ($p < 0.001$).

(B) Effects of PVL on cell viability of differentiated C2C12 myocytes.
a= pH 7.4 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.8 ($p < 0.01$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); d= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$), pH 7.4 vs. pH 7.8 ($p < 0.01$); e= pH 6.8 vs. pH 7.4, 7.8, pH 7.4 vs. pH 7.8 ($p < 0.001$).

(C) Effect of PVA on cell viability of undifferentiated C2C12 myoblasts.
a= pH 6.8 vs. pH 7.8 ($p < 0.01$); b= pH 6.8 vs. pH 7.8 ($p < 0.001$).

(D) Effect of PVA on cell viability of differentiated C2C12 myocytes.
a= pH 6.8 vs. pH 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.01$); b= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$); c= pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$); pH 6.8 vs. pH 7.4 ($p < 0.05$).

Table 5.1: Cytotoxicity of statins to C2C12 cells after 72 hours of treatment in medium of different pH.

Statins	C2C12 cells	IC_{50} values of statins (μmol/L)		
		Acidic medium	Neutral medium	Alkaline medium
Simvastatin lactone	Undifferentiated cells	0.44 ± 0.02[*]	0.58 ± 0.02[†]	1.13 ± 0.04[‡]
	Differentiated cells	15.79 ± 0.77[*,§]	40.76 ± 1.75[†, §]	56.99 ± 1.86[‡,§]
Simvastatin hydroxy acid	Undifferentiated cells	2.22 ± 0.04[*]	4.35 ± 0.09[†]	16.85 ± 0.48[‡]
	Differentiated cells	65.41 ± 2.69[§]	NA	NA
Pravastatin lactone	Undifferentiated cells	85.59 ± 2.05	NA	NA
	Differentiated cells	109.9 ± 3.74[§]	NA	NA

Abbreviations: NA, non-applicable; SD, standard deviation.
Data are presented as mean ± SD of three experiments, eight replicates per experiment. Statistical analysis was done by 2-way ANOVA followed by Bonferroni's post hoc test.
[*]Significant difference from the neutral and alkaline medium ($p < 0.001$).
[†]Significant difference from the acidic and alkaline medium ($p < 0.001$).
[‡]Significant difference from the acidic and neutral medium ($p < 0.001$).
[§]Significant difference from the undifferentiated cells ($p < 0.001$).

The higher cytotoxicity of simvastatin observed at acidic pH was associated with enhanced cellular uptake. The cytotoxicity of pravastatin lactone and pravastatin hydroxy acid in both undifferentiated and differentiated C2C12 was much lower than that of simvastatin lactone and simvastatin hydroxy acid after 72 hours of treatment under different pH conditions (Figures 5.9 and 5.10). Interestingly, undifferentiated C2C12 cells were found to be more sensitive to statin-induced myotoxicity compared with differentiated cells (Table 5.1).

The effect of medium pH alteration on cell membrane integrity was also tested using LDH assay. Results from LDH release assay are shown in Figures 5.11 and 5.12. The results show that simvastatin (applied either as lactone or hydroxy acid form) induces higher LDH leakage from cell membranes of undifferentiated and differentiated C2C12 cells than pravastatin over the tested concentrations. Compared with their hydroxy acid counterparts, the more lipophilic lactone forms of simvastatin and pravastatin were shown to induce higher LDH release especially when cells were maintained under acidic

conditions. The cytotoxicity results obtained by means of LDH assay correspond well with the cell viability data observed using MTT assay.

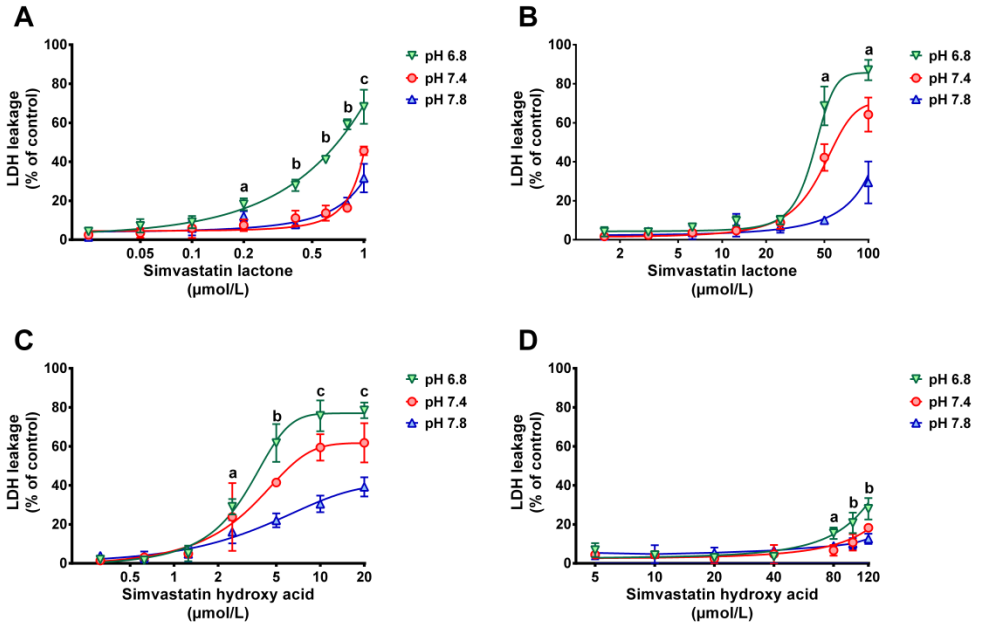


Figure 5.11: Effect of simvastatin lactone (SVL) and simvastatin hydroxy acid (SVA) on lactate dehydrogenase (LDH) release from undifferentiated and differentiated C2C12 cells maintained under different pH levels. C2C12 cells were cultured at a density of 4,000 cells/well and allowed to attach for 24 hours or to differentiate for 4 days, then exposed to increasing concentrations of SVL or SVA under acidic, neutral, and alkaline medium pH. Undifferentiated cells were treated for 72 hours, whereas differentiated cells were maintained for 24 hours. Data are presented as mean ± SD, (n=3) and analysed by 2-way ANOVA followed by Bonferroni's post hoc test.
(A) LDH release from undifferentiated C2C12 cells treated with SVL.
a= pH 6.8 vs. pH 7.4 ($p < 0.001$); b= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).
(B) LDH release from differentiated C2C12 cells treated with SVL.
a= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).
(C) LDH release from undifferentiated C2C12 cells treated with SVA.
a= pH 6.8 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4 ($p < 0.01$); pH 7.8 vs. pH 6.8, 7.4 ($p < 0.001$).
(D) LDH release from differentiated C2C12 cells treated with SVA.
a= pH 6.8 vs. pH 7.4 ($p < 0.01$); pH 6.8 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$).

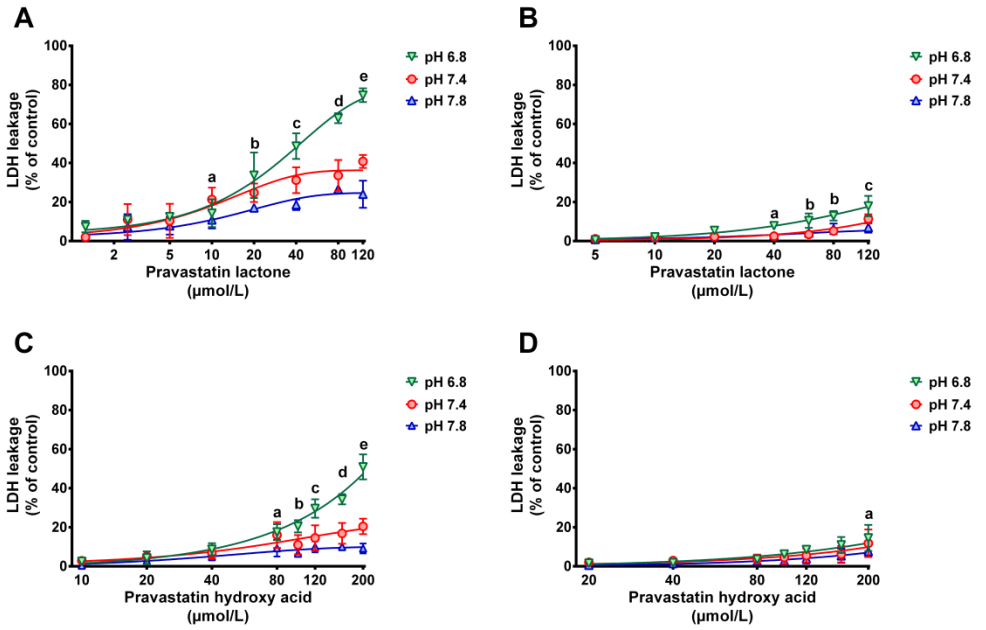


Figure 5.12: Effect of pravastatin lactone (PVL) and pravastatin hydroxy acid (PVA) on lactate dehydrogenase (LDH) release from undifferentiated and differentiated C2C12 cells maintained under different pH levels. C2C12 cells were cultured at a density of 4,000 cells/well and allowed to attach for 24 hours or to differentiate for 4 days, then exposed to increasing concentrations of PVL or PVA under acidic, neutral, and alkaline medium pH. Undifferentiated cells were treated for 72 hours, whereas differentiated cells were maintained for 24 hours. Data are presented as mean ± SD, (n=3) and analysed by 2-way ANOVA followed by Bonferroni's post hoc test.
(A) LDH release from undifferentiated C2C12 cells treated with PVL.
a= pH 7.4 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.05$); d= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); e= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).
(B) LDH release from differentiated C2C12 cells treated with PVL.
a= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.01$); b= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.05$).
(C) LDH release from undifferentiated C2C12 cells treated with PVA.
a= pH 6.8 vs. pH 7.8 ($p < 0.05$); b= pH 6.8 vs. pH 7.4 ($p < 0.01$); pH 6.8 vs. pH 7.8 ($p < 0.001$); c= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); d= pH 6.8 vs. pH 7.4, 7.8 ($p < 0.001$); pH 7.4 vs. pH 7.8 ($p < 0.05$); e= pH 6.8 vs. pH 7.4, 7.8; pH 7.4 vs. pH 7.8 ($p < 0.001$).
(D) LDH release from differentiated C2C12 cells treated with PVA.
a= pH 6.8 vs. pH 7.8 ($p < 0.05$).

5.4 Discussion

5.4.1 Characterisation of myogenic differentiation of C2C12 cells

C2C12 cells are widely used as a model to study the myotoxicity of drugs.[209, 212] These are mouse myoblast cells capable of spontaneous differentiation into myotubes under low serum conditions.[145] In the present study, both undifferentiated and differentiated cells were utilised to elucidate the effect of acid-base imbalance on the myotoxicity of lipophilic and hydrophilic statins.

It has been shown that depriving C2C12 cells of serum resulted in arresting of the cell cycle, cell elongation, fusion and formation of multinucleated myotubes.[213] This myogenic differentiation is controlled by a number of myogenic regulatory factors such as Myo-D, Mrf4, Myf5, myogenin and other transcription factors.[214] These myogenic regulatory factors induce the expression of other muscle specific proteins, such as alpha-actin and myosin heavy chains.[145]

Successful differentiation of C2C12 myoblast cells into functioning and integrated myotubes was confirmed by examining the expression of 2 markers of myogenic differentiation (myogenin and MHC). The expression of myogenin started at day 1 after induction of differentiation and reached high levels at day 3, a stage at which the cells start fusing and form multinucleated myotubes (Figure 5.2, *A*). Myogenin expression is an early marker for the entry of C2C12 myoblasts into the differentiation phase. The expression of myogenin is followed by skeletal myogenesis through a highly organised sequence of events that involve withdrawal from cell cycle, expression of

contractile proteins such as myosin heavy chain, and finally cell fusion resulting in the formation of multinucleated myotubes (Figure 5.13).[213]

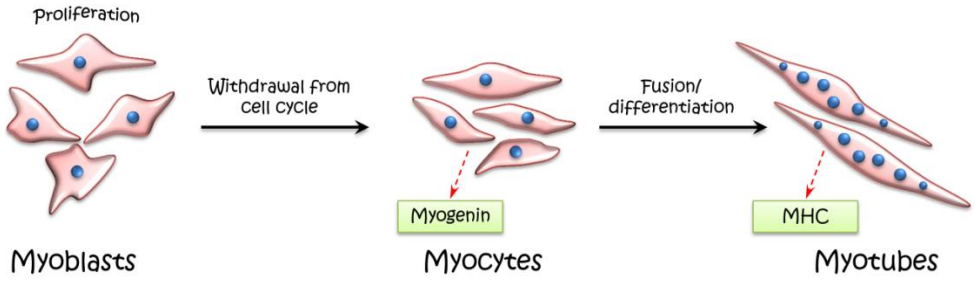


Figure 5.13: Stages of C2C12 differentiation**.**

The expression of MHC showed a steady increase over time and reached a maximum of 70-fold the baseline level by the seventh day (Figure 5.2, *A*). In previously reported study, western blot analysis of a number of myogenic markers during C2C12 differentiation revealed that the expression of myogenin preceded the induction of MHC, whose expression showed a steady increase up to 4 days in the differentiation medium.[213]

5.4.2 Cellular uptake of statins in response to medium pH alteration

High statin levels in plasma have been considered to be a risk factor for statin-induced myotoxicity. However, there are patients who exhibit high statin plasma levels but do not develop myopathy, suggesting that other factors, including skeletal muscle fibre statin concentration, may have an impact on side effect risk.[211] In the present study, the alteration of extracellular pH as a result of acidosis and alkalosis has been proposed to contribute to the variable uptake of statins by skeletal muscle cells.

No pravastatin lactone or hydroxy acid forms were detected in either undifferentiated and differentiated C2C12 cells at any pH levels after different incubation times. On the other hand, the cellular uptake of simvastatin was found to be strongly affected by the pH level of the incubation medium. Incubating undifferentiated C2C12 cells with simvastatin lactone for a period exceeding 2 hours resulted in significantly higher total simvastatin accumulation within the cells under acidic condition compared with neutral and alkaline conditions (Figure 5.4, *A*). These findings could be attributed to the greater proportion of simvastatin that remains in the more lipophilic lactone form at acidic pH. The lactone form has higher ability to cross the cell membranes of muscle cells and achieve high intracellular concentrations. This could explain the higher cytotoxicity observed under acidic condition compared with alkaline and neutral conditions (Table 5.1).

In contrast to acidic conditions, medium alkalinization did not influence the cellular uptake of simvastatin significantly in comparison to the uptake at physiological pH. Simvastatin lactone is less stable at alkaline pH and therefore dynamic equilibrium favours hydrolysis of the lactone ring to yield the hydrophilic hydroxy acid form. Higher intracellular concentrations of the lactone than its corresponding acid form were found in undifferentiated cells at all pH levels indicating that cellular accumulation of simvastatin is associated with its lipophilic nature (Figure 5.5, *A*). A marked difference in lipophilicity exists between lactone and hydroxy acid forms of statins, with the lactone form being more lipophilic than the corresponding acid form.[66, 89] The lipid-enriched membranes of muscle cells act as a barrier to hydrophilic statins but allow passive diffusion of the lipophilic ones.[65] It has been suggested that

intracellular statin concentrations are also controlled by the dynamic interplay between uptake and efflux transporter activities.[211] However, it is not yet clear whether these transporters have differential selectivity to the acid or lactone forms of statins. It is worth noting that the intracellular concentrations of the acid form of simvastatin found in undifferentiated cells were not significantly different at various pH levels studied, suggesting that intracellular microenvironment was not influenced by the extracellular pH changes (Figure 5.5, *A*).

Similarly, medium acidification increased simvastatin uptake by differentiated C2C12 cells as a result of greater proportion of the highly lipophilic lactone form preserved under acidic conditions Figure 5.4, *B*). Less simvastatin lactone was taken up by differentiated C2C12 cells compared with undifferentiated cells. Although the reasons for this difference are not entirely clear, the denser organisation of cells as they differentiate could contribute to lower uptake of the statins by differentiated C2C12 cells. More studies will be needed to clarify this phenomenon.

Measurement of the intracellular concentrations of both forms of simvastatin revealed a faster rate of hydrolysis of the lactone form within differentiated C2C12 cells compared to undifferentiated cells (Figure 5.5, *A* and *B*). The higher metabolic rate of differentiated cells could explain the faster rate of simvastatin lactone hydrolysis.

Simvastatin hydroxy acid was taken up by undifferentiated and differentiated C2C12 cells to much lower extent than simvastatin lactone (Figure 5.4, *C* and *D*). The higher uptake of simvastatin hydroxy acid observed under acidic conditions could be attributed to the passive diffusion of the more

lipophilic unionised form of simvastatin hydroxy acid which becomes more predominant at acidic pH compared with physiological and alkaline pH. No attempt has been made in this study to quantify the percentage of the hydroxy acid and lactone forms of simvastatin that remained in the culture medium after the uptake study. Such measurement is expected to provide a clear explanation of weather the hydroxy acid and lactone forms that recovered inside the cells is the result of direct intracellular conversion of parent form after being taken up by the cells or it is due to statin conversion within culture medium followed by the uptake of both forms into the cells.

5.4.3 Expression of cellular transporters

The potential contribution of drug transporters to cellular uptake and cytotoxicity of statins over different pH levels was assessed in this study by measuring the mRNA expression of influx and efflux transporters that are known to influence statin uptake. A recent study of statin myotoxicity has shown that differentiated human skeletal muscle cells constitutively express MRP transporters (namely MRP1, MRP4, and MRP5) which mediate the efflux of statins from skeletal muscle fibres. Expression of these efflux transporters combined with the inability of differentiated muscle cells grown *in vitro* to express OATPs influx transporters could potentially play protective roles against intracellular statin accumulation.[211] Similar to previously reported studies,[209, 211] the levels of mRNA expression of influx transporters (Oatp1a4 and Oatp2b1) were not detectable in our experiments. Mrp5 was found to have higher expression in differentiated cells compared to undifferentiated cells which could explain the relative resistance of former cells to statin-induced

cytotoxicity. Moreover, it was found to be expressed at a lower level at pH 6.8, where higher cytotoxicity was observed (Figure 5.6). On the other hand, the mRNA expression of Mrp1 and Mrp4 transporter genes appear to be greater in undifferentiated compared with differentiated C2C12 cells.

Taken together, the results suggest that although Mrp5 could play some role in the intracellular accumulation of statin, overall, the results of expression of transporters do not explain the intracellular accumulation of statins. Therefore, it is likely that passive diffusion of the more lipophilic lactone form is the primary mechanism of intracellular accumulation of statins, especially in acidic conditions.

5.4.4 *In vitro* cytotoxicity of statins in different medium pH levels

5.4.4.1 *MTT assay*

For the cells to have a normal function and metabolism, the pH should be maintained within narrow limits of 7.35–7.45. Disturbances in acid-base balance impose profound effects on many aspects of drug action.[97] Our results demonstrated that alteration in culture medium pH greatly influenced the cytotoxicity of statins (Figures 5.9 and 5.10). The higher cytotoxicity of simvastatin lactone found in C2C12 cells treated under acidic conditions could be attributed to the higher proportion of statin that remained in the more lipophilic lactone form. Low cytotoxicity under alkaline condition could be due to inability of statin to achieve adequate intracellular concentrations as a result of conversion to the hydrophilic acid form. It has been reported that simvastatin lactone is about 3 times more lipophilic than its corresponding hydroxy acid form[66] and, therefore, can achieve higher intracellular

concentrations as observed in Section 5.3.2. Enhanced cytotoxicity of simvastatin hydroxy acid has also been demonstrated at acidic pH, which could be due to the passive transport mechanism of the predominantly unionised hydroxy acid form in acidic environment.[215] Hydrophilic pravastatin was shown to be less cytotoxic than lipophilic simvastatin. Undifferentiated C2C12 cells were found to be more sensitive to simvastatin-induced myotoxicity compared with differentiated cells (Table 5.1). Undifferentiated cells are rapidly proliferating cells and their cholesterol demands is expected to be much higher than the less proliferating differentiated cells. This could explain the relative high sensitivity of the undifferentiated cells to simvastatin therapy. These findings suggest that multinuclear skeletal muscle cells are more resistant to statin-induced myotoxicity compared with mononuclear stem cells. This could imply that statins might primarily block muscle repair and regeneration by reducing the pool of muscle stem cells.

Although simvastatin lactone is inactive with respect to lipid-lowering effect, it may still mediate muscular side effects, either through a direct toxic effect or through intracellular conversion to the HMG-CoA reductase inhibiting acid form.[205]

5.4.4.2 LDH cytotoxicity assay

In the present study, LDH has been used to reflect the cell membrane integrity in response to cell exposure to statin therapy. The amounts of LDH release induced by statins treatment over different pH levels are shown in Figures 5.11 and 5.12. The cytotoxicity detected with LDH assay was in agreement with the cell viability profiles obtained with MTT. Similar LDH

release pattern was observed with both simvastatin and pravastatin in response to medium pH alteration. A significant increase in LDH release was observed when cells were exposed to statins under acidic conditions compared with physiological and alkaline conditions. The LDH release was higher from C2C12 cells treated with simvastatin compared with pravastatin-treated cells. Furthermore, the lactone forms of both statins have induced higher LDH release than the corresponding hydroxy acid forms. The higher release of LDH by statins under acidic conditions is expected to be due to the higher cellular uptake of the more lipophilic lactone forms.

5.5 Conclusions

This study demonstrated that acidosis can enhance the cellular uptake of statins by the skeletal muscle cells by minimizing the hydrolysis of the lactone form resulting in potentiation of muscle toxicity. On the other hand, alkalosis increases the hydrolysis of the lactone ring rendering statins less lipophilic and therefore limits their penetration into the skeletal muscles. Lipophilic simvastatin lactone was found to be more cytotoxic to skeletal muscle cells than the more hydrophilic simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid. Furthermore, undifferentiated C2C12 cells were more sensitive to statin-induced myotoxicity than differentiated muscle cells.

Chapter Six

6 Influence of Hyperlipidaemia and Acid-Base Imbalance on Statin-Induced Myotoxicity

6.1 Introduction

Statins are essential pharmacotherapy for patients with ischemic heart disease and dyslipidaemia.[21] However, these drugs have the potential to cause serious side effects such as muscle-related toxicity and hepatotoxicity. Many of the side effects of statins are related to their chemical nature. Statins are classified into hydrophilic or lipophilic compounds based on their physicochemical properties and tissue selectivity.[66, 73] These drugs are available either as lactone or hydroxy acid form. In general, the lactone forms of statins are more lipophilic, and indeed, more myotoxic than the corresponding hydroxy acid forms owing to their lipophilicity.[49, 76]

The exact mechanism of statin-induced myotoxicity remains largely unclear. However, the likelihood of developing myotoxicity from statin use appears to be greater in the presence of certain risk factors. Comorbidities including multisystem diseases such as hepatic or renal failure, hypothyroidism, and diabetes mellitus are among the most important risk factors that have been shown to predispose patients to statin-induced myotoxicity.[64] We have previously reported that disturbances in acid-base balance, such as acidosis or alkalosis, could have a significant influence on the toxicity profile of statins. Acidosis increases the proportion of lipophilic lactone form, promotes accumulation of statins in skeletal muscle cells and enhances statin-induced myotoxicity *in vitro*.[156]

The interaction of lipophilic drugs with plasma lipoproteins has been shown to modify the pharmacokinetics, tissue distribution and pharmacological activity of these compounds.[122, 216] For example, an increase in drug association with LDL has been linked to increased nephrotoxicity of amphotericin and ciclosporin.[123, 125, 217]

Transient elevation in TRL, mainly chylomicrons, occurs following the ingestion of a high-fat meal.[128] These lipoproteins are the major carriers of lipids in the systemic circulation, and can also serve as potential carriers for lipophilic statins.[129, 130] Similarly, pathological hyperlipidaemia is associated with elevated levels of some lipoproteins (VLDL and LDL).

It is conceivable that lipophilic statins can associate with plasma lipoproteins in the general circulation, and this, in turn, can facilitate their delivery to peripheral tissues. Lipoprotein lipase (LPL) present at the surface of the capillary endothelium of cardiac muscle, skeletal muscle, and adipose tissue mediates the hydrolysis of triglycerides within the TRL to fatty acids and monoacylglycerol. Released free fatty acids are then transported across cell membranes to the tissues of LPL synthesis and secretion.[131] It is reasonable to assume that this process could be accompanied by an increase in the uptake of lipoprotein-associated lipophilic statins by the skeletal muscle and consequently contribute to increased muscle toxicity. In addition, LPL also mediates the internalisation of LDL into cells (as reported in several studies[132-134]), which could be an additional mechanism of enhanced intracellular accumulation and toxicity of statins in hyperlipidaemic state. Therefore, the administration of lipophilic statins to patients with pathological or postprandial hyperlipidaemia could potentially contribute to enhanced muscle toxicity. In

support of this hypothesis, it has been shown recently that forcibly making a muscle to usilise carbohydrates as a fuel alleviates statin-induced muscle toxicity in rats.[218]

The coincidence of hyperlipidaemia with acidosis could augment the risk of statin-induced myotoxicity even further. This is because acidosis, as we have previously demonstrated,[156] increases the proportion of lipophilic lactone form of statins in the general circulation and skeletal muscles. This not only can increase the toxicity on its own, but can also contribute to statin-induced muscle toxicity by increasing the association of lactone form with plasma lipoproteins, and increasing the intracellular concentration of statins by LPL-mediated mechanism. It has been reported that professional athletes suffering from familial hypercholesterolaemia rarely tolerate statin treatment because of muscle toxicity (muscle tissue acidosis is likely in exercising patients).[219]

Both acidosis and hyperlipidaemia are quite common among statin users. Most patients using statins have abnormal plasma lipoproteins levels.[113] There is also a general consensus by clinicians to recommend such patients to use unsaturated fat and fatty acids such as olive oil for prevention of cardiovascular and atherosclerotic diseases.[220] The use of these oils is associated with transient but significant elevation in plasma TRL (mainly chylomicrons).[221] In addition, many patients on statins have multiple co-morbidities such as diabetes mellitus, cardiovascular and renal diseases. These conditions are risk factors for development of metabolic acidosis.[206]

To the best of our knowledge, there are no previously reported studies which examined the influence of hyperlipidaemia, or a combination of hyperlipidaemia and acidosis, on the myotoxic potential of statins. Therefore,

the aim of this study is to assess the effect of hyperlipidaemia alone and in combination with acidosis/alkalosis on development and severity of statin-induced myotoxicity. The specific objectives required to fulfil this aim include the following:

1- Prediction of statins association with chylomicrons using *in silico* model.

2- Assessment of statins association with chylomicron-like particles and lipoprotein fractions from hyperlipidaemic and normolipidaemic plasma at different pH levels.

3- Assessment of the effects of statin association with plasma lipoproteins on the cellular uptake using skeletal muscle cells that express different levels of lipoprotein lipase under conditions of altered pH levels.

4- Elucidation of the effects of statin association with plasma lipoproteins on their cytotoxicity to skeletal muscle cells that express different levels of lipoprotein lipase under conditions of altered pH levels.

6.2 Methods

6.2.1 Association of statins with plasma lipoproteins

The degree of association of statins with plasma lipoproteins was assessed using *in silico* multi-regression analysis model, artificial chylomicrons-like lipid particles, natural rats and human chylomicrons and human lipoproteins from hyperlipidaemic and normolipidaemic plasma samples. The association studies were conducted at pH relevant to physiological conditions (pH 7.4), acidosis (pH 6.8), and alkalosis (pH 7.8). Lipoprotein fractions were separated from plasma using density gradient ultracentrifugation and the amount of the hydroxy acid and lactone forms of statin recovered within individual lipoprotein was determined by LC-MS/MS analysis. Detailed explanations of statins association studies with plasma lipoproteins are provided in Sections 2.9, 2.10, and 2.11.

6.2.2 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cellular uptake of lipophilic statin

Simvastatin was selected in these studies as a model lipophilic statin due to its high lipophilicity, wide clinical use and high incidence of reported simvastatin-associated muscle toxicity.[16, 172] Recently, atorvastatin has become a more frequently used lipophilic statin in developed world. However, simvastatin is still a prevalent statin in developing countries.[222] The combined effect of hyperlipidaemia and acidosis/alkalosis on cellular uptake of simvastatin was tested by incubating C2C12 cells with simvastatin-loaded lipoprotein fractions (SV-TRL and SV-LDL) at pH relevant to physiological conditions, acidosis, and alkalosis. The cells used in these studies express

different levels of lipoprotein lipase (C2C12 cells transfected with mLPL, hLPL or empty vector). Simvastatin-loaded lipoprotein fractions were obtained by incubating the drug with hyperlipidaemic human plasma of different pH followed by lipoprotein separations using density gradient ultracentrifugation. Control cells were treated with simvastatin solution at concentrations similar to that achieved within lipoprotein fractions. Following incubation, cells were harvested, lysed and the intracellular concentrations of the hydroxy acid and lactone forms of simvastatin were determined by LC-MS/MS system. More detailed explanations of cellular uptake studies of simvastatin from plasma lipoproteins at different medium pH levels are provided in Section 2.14.

6.2.3 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cytotoxicity of simvastatin

The effect of simvastatin association with plasma lipoproteins on the viability of undifferentiated and differentiated C2C12 cells was evaluated by MTT and LDH cytotoxicity assays. C2C12 cells that overexpress mLPL, hLPL, and control cells were treated either with simvastatin-loaded lipoproteins factions (SV-TRL or SV-LDL) or simvastatin applied directly in solution at concentrations similar to that achieved within the corresponding lipoprotein fractions. The incubation was performed in cell culture medium of physiological pH (pH 7.4) or disturbed pH (pH 6.8 corresponding to acidosis and 7.8 corresponding to alkalosis) and cytotoxicity assays were conducted as described in Section 2.15.

6.3 Results

6.3.1 *In silico* prediction of statins association with chylomicrons

The predictive values of statins association with chylomicrons obtained using the *in silico* model are presented in Table 6.1. Simvastatin hydroxy acid, pravastatin lactone and pravastatin hydroxy acid showed no predicted association with chylomicrons. Simvastatin lactone was the only form that showed a considerable predicted association with chylomicrons (9.08%).

Table 6.1: *In silico* prediction of statins association with chylomicrons.

Physicochemical Property	Unscaled regression coefficients	Simvastatin lactone	Simvastatin hydroxy acid	Pravastatin lactone	Pravastatin hydroxy acid
Log$D_{7.4}$	0.299879	4.69	1.34	2.79	-0.67
Log$P$ - Log$D_{7.4}$	-0.238127	-0.27	2.72	-1.08	2.02
PSA	-0.00855215	72.83	104.06	93.06	124.29
H-acceptors	-0.184359	5	6	6	7
FRB	0.0805226	7	11	7	11
Density	1.45337	1.11	1.13	1.18	1.21
Molar volume	0.00545912	376.5	383.9	341.7	349.1
H-donors	0.0823094	1	3	2	4
Constant	-5.24138				
Predicted association with Chylomicrons %		9.08%	0.24%	1.78%	0.04%

Abbreviations: Log *D*, distribution coefficient; Log *P*, partition coefficient; PSA, polar surface area; H-acceptors, hydrogen-bond acceptors; FRB, freely rotatable bonds; H-donors, hydrogen-bond donors.

6.3.2 Association of statins with protein-free artificial lipid particles and plasma-derived isolated chylomicrons

Similar to *in silico* prediction, simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid showed no association with human chylomicrons *ex vivo* and therefore were not selected for subsequent studies. The results of association of simvastatin lactone with artificial chylomicron-like lipid particles and plasma-derived isolated chylomicrons are summarised in Figure 6.1. At physiological pH, the degree of simvastatin association with artificial lipid particles, rat, and human chylomicron emulsions were found to be 15.5, 16.4%, and 18.5%, respectively. Changing the pH of Intralipid® emulsion above or below the physiological range has not resulted in

statistically significant differences in the degree of simvastatin association. On the other hand, when simvastatin association was tested with human chylomicrons of different pH levels, statistically significant differences were found between these pH levels. The association value was the lowest in alkaline condition (14.2%) while neutral pH and acidic pH resulted in higher association values (18.5% and 19.3%, respectively). As expected, no simvastatin lactone was detected in the upper 1 mL layer of the ultracentrifuge tube in the control (PBS) samples.

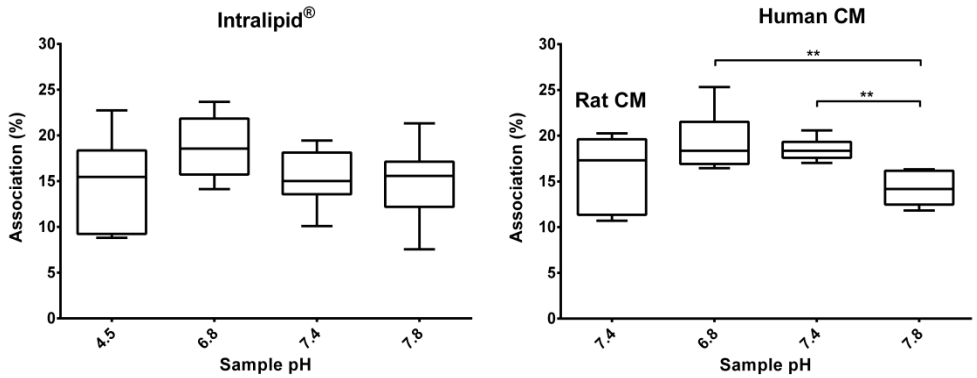


Figure 6.1: Association of simvastatin lactone with Intralipid®, rat, and human chylomicrons (CM) at different pH levels. Results are presented as box and whiskers, (n=6). No simvastatin lactone was detected in the upper 1 mL layer of the ultracentrifuge tube (corresponding to chylomicron fraction) in the control (PBS) samples. Data were analysed by one-way ANOVA followed by Tukey's post hoc test, $^{**}p < 001$. This data was generated with the help of Jong Bong Lee and Atheer Zgair.

6.3.3 Effect of hyperlipidaemia alone or in combination with acidosis/ alkalosis on simvastatin association with plasma lipoproteins

The distribution of simvastatin lactone into different lipoprotein fractions from hyperlipidaemic and normolipidaemic human plasma are presented in Figure 6.2. The concentrations of simvastatin lactone and hydroxy acid forms

recovered in individual lipoprotein fractions were determined by fully validated LC-MS/MS analysis (validation data are provided in Section 3.3.2).

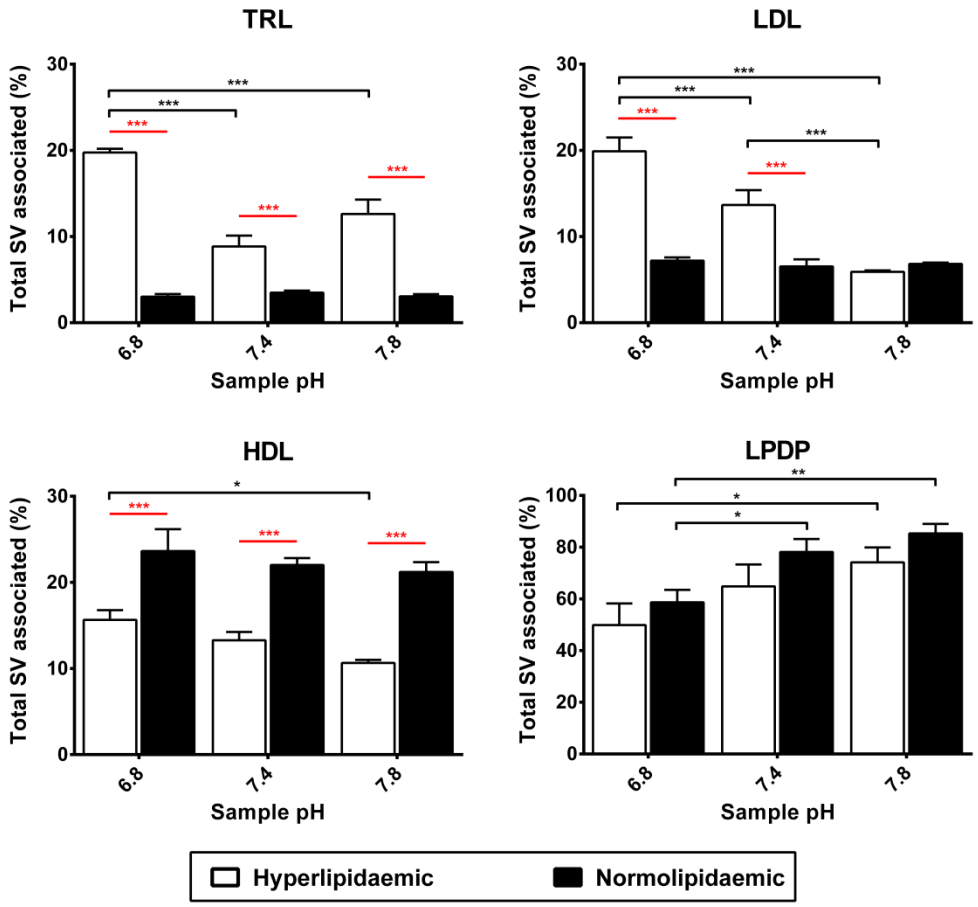


Figure 6.2: Distribution of simvastatin lactone into different lipoprotein fractions from hyperlipidaemic and normolipidaemic plasma. Simvastatin lactone was incubated with hyperlipidaemic or normolipidaemic plasma at 37°C over physiological, acidic, and alkaline pH with constant mixing for 1 hour. Lipoprotein fractions were separated by density gradient ultracentrifugation and the total amount of simvastatin (both acid and lactone forms) recovered in each fraction were presented as percentage of the initial amount of simvastatin lactone applied into plasma samples. Data are presented as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, *$p < 0.05$, **$p < 001$, ***$p < 0.001$. Red straight lines denote differences in % of simvastatin recovered in lipoprotein fractions from hyperlipidaemic and normolipidaemic plasmas. Black arched lines denote differences in simvastatin association within individual lipoprotein fractions as a function of pH changes. TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma.

Simvastatin lactone showed considerably higher association with the non-polar lipoproteins fractions (TRL and LDL) extracted from hyperlipidaemic plasma, compared to its association with lipoproteins from normolipidaemic plasma. On the other hand, more simvastatin was found to associate with HDL from normolipidaemic plasma compared to hyperlipidaemic plasma.

Overall, simvastatin association with different plasma lipoproteins in hyperlipidaemic plasma was greater at acidic pH compared to physiological and alkaline pH. At acidic pH, more than 50% of simvastatin distributed to different lipoprotein fractions in hyperlipidaemia plasma (20% in TRL, 20% in LDL, and 16% in HDL). The remaining simvastatin (around 45%) appeared in the LPDP fraction. However, at physiological and alkaline pH, most simvastatin was recovered in the LPDP fraction and only small proportions were associated with plasma lipoproteins (Figure 6.2).

To investigate the factors that influence simvastatin association with plasma lipoproteins, cholesterol, and triglyceride levels were determined in each lipoprotein fraction from hyperlipidaemic and normolipidaemic plasma samples (Figures 6.3 and 6.4).

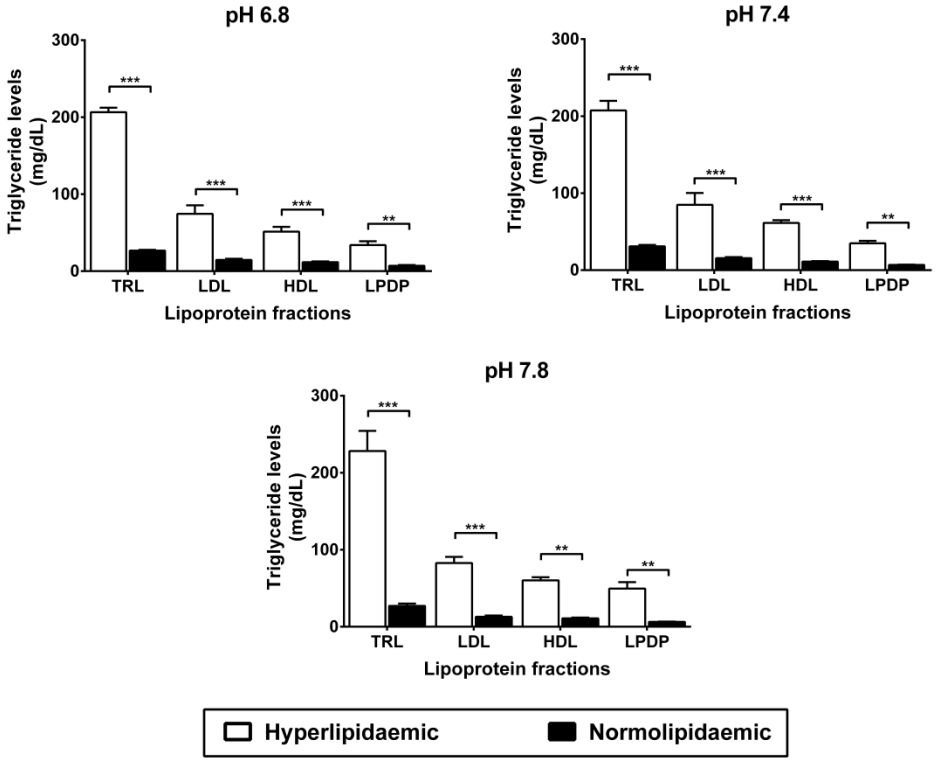


Figure 6.3: Triglyceride content of individual lipoprotein fractions following ultracentrifugation of hyperlipidaemic and normolipidaemic plasma samples of different pH. Results are expressed as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, ** $p <$ 001, *** $p < 0.001$. TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma.

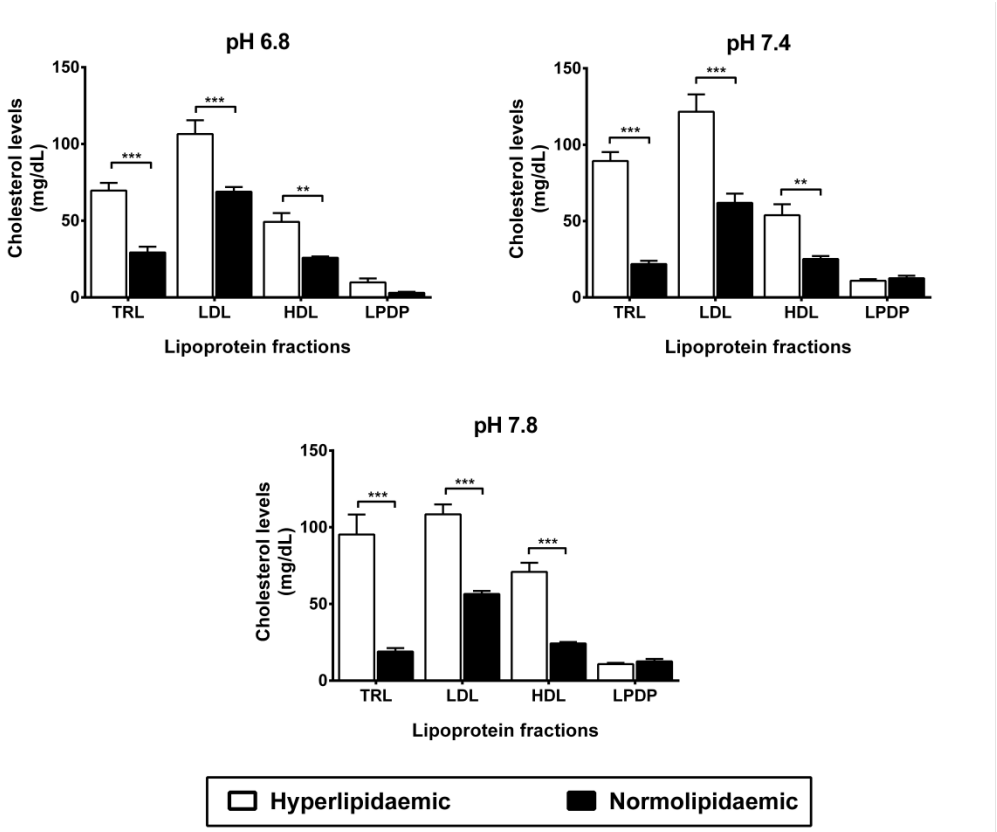


Figure 6.4: Cholesterol content of individual lipoprotein fractions following ultracentrifugation of hyperlipidaemic and normolipidaemic plasma samples of different pH. Results are expressed as mean ± SE, (n=5). Data were analysed by 2-way ANOVA followed by Fisher's LSD post hoc test, ** $p < 001$, *** $p < 0.001$. TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma.

In addition, the correlation between the amount of simvastatin recovered in individual lipoprotein fractions and the levels of triglyceride and cholesterol present in each fraction were examined (Figures 6.5 and 6.6). Positive correlations ($p < 0.01$) were observed between the levels of triglyceride or cholesterol and the amount of simvastatin-associated with TRL and LDL. On the other hand, negative correlations ($p < 0.01$) were found between the amount of simvastatin recovered in HDL and the triglyceride or cholesterol contents of this fraction. As expected, there was no correlation between the triglyceride or cholesterol contents of LPDP fraction and the amount of simvastatin found in this fraction ($p > 0.05$) (Figures 6.5 and 6.6).

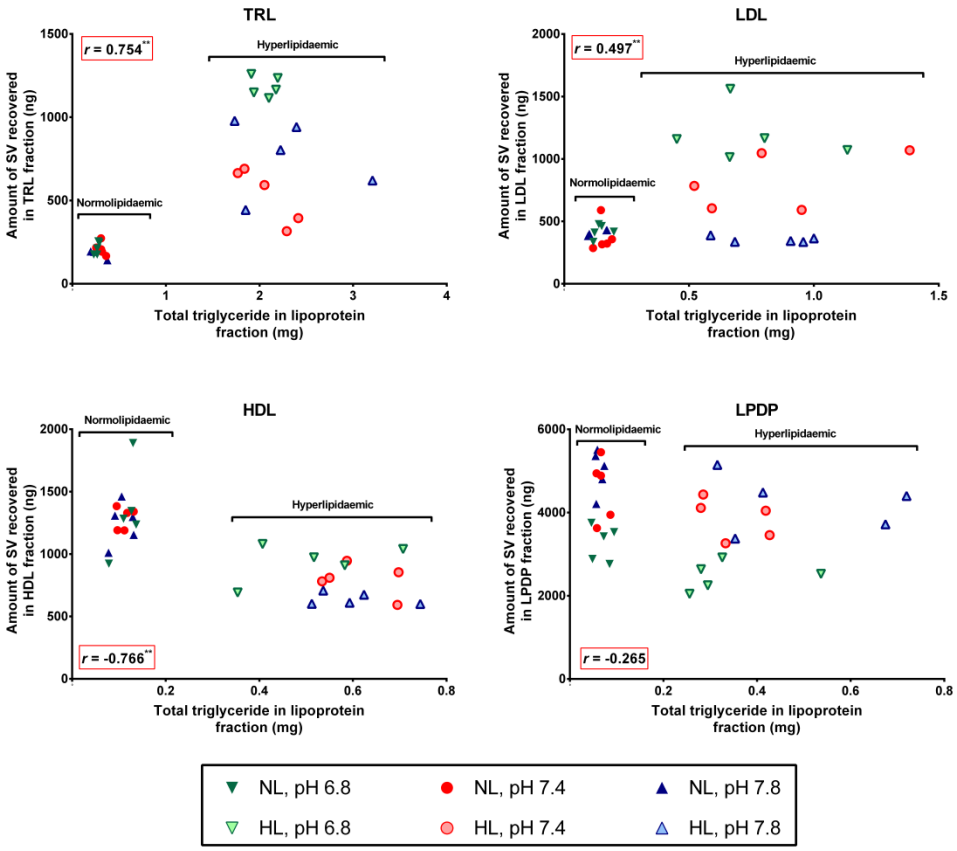


Figure 6.5: Correlation between the amounts of simvastatin recovered in individual lipoprotein fractions and the triglyceride content of the lipoproteins following incubation of hyperlipidaemic and normolipidaemic plasma of different pH with simvastatin lactone. Correlation was determined by Pearson's correlation coefficient (r), ** $p < 0.01$. HL, hyperlipidaemic plasma; NL, normolipidaemic plasma; TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma.

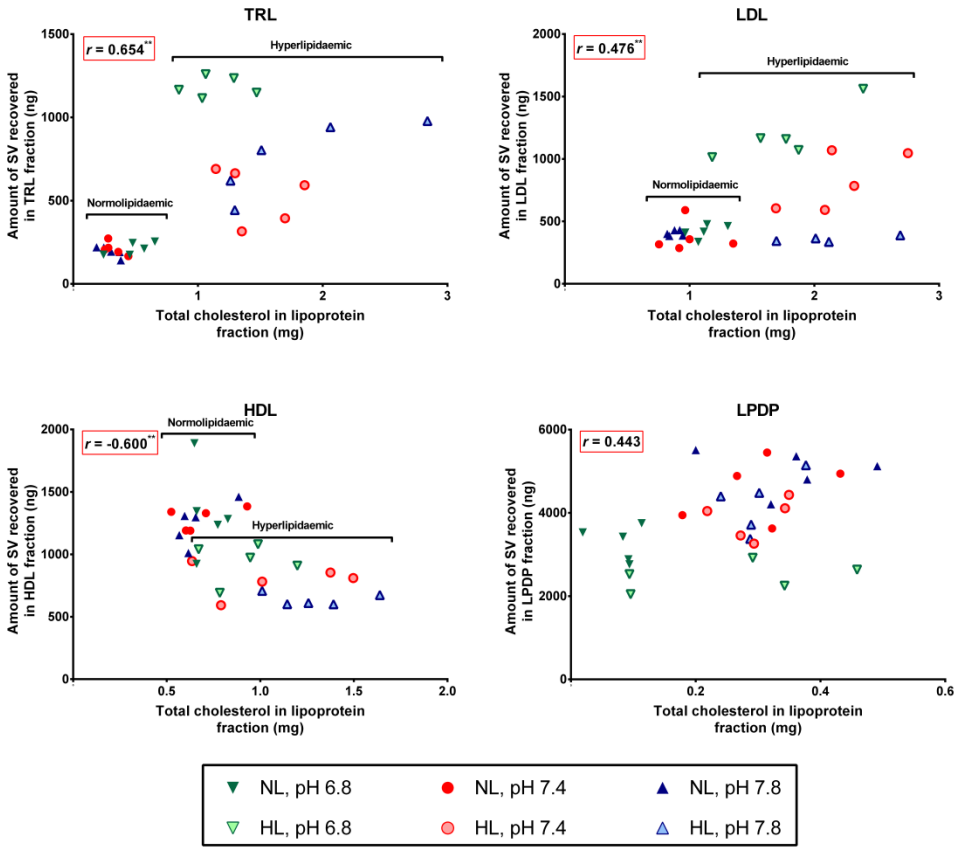


Figure 6.6: Correlation between the amounts of simvastatin recovered in individual lipoprotein fractions and the cholesterol content of the lipoproteins following incubation of hyperlipidaemic and normolipidaemic plasma of different pH with simvastatin lactone. Correlation was determined by Pearson's correlation coefficient (r), ** $p < 0.01$. HL, hyperlipidaemic plasma; NL, normolipidaemic plasma; TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma.

Measuring the concentrations of simvastatin lactone and the corresponding hydroxy acid within each lipoprotein fraction provided a picture of the effect of plasma pH alteration on simvastatin distribution into different lipoprotein fractions (Figures 6.7 and 6.8). At acidic pH, the majority of simvastatin recovered in lipoprotein fractions was in the lipophilic lactone form. As the pH increases, more simvastatin lactone starts to convert to the more hydrophilic hydroxy acid form, resulting in simvastatin redistribution to the more polar LPDP fraction.

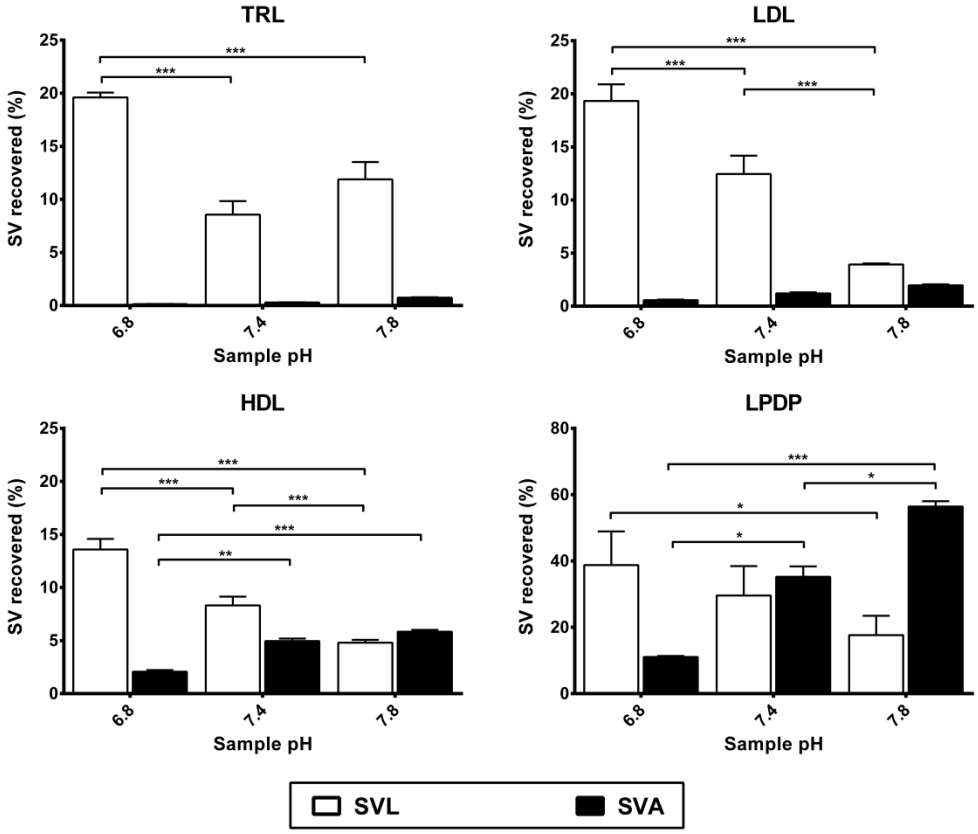


Figure 6.7: Percentage of simvastatin lactone and corresponding hydroxy acid forms recovered in individual lipoprotein fractions of hyperlipidaemic plasma. Results are expressed as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, * $p < 0.05$, ** $p < 001$, *** $p < 0.001$. TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma; SVL, simvastatin lactone; SVA, simvastatin hydroxy acid.

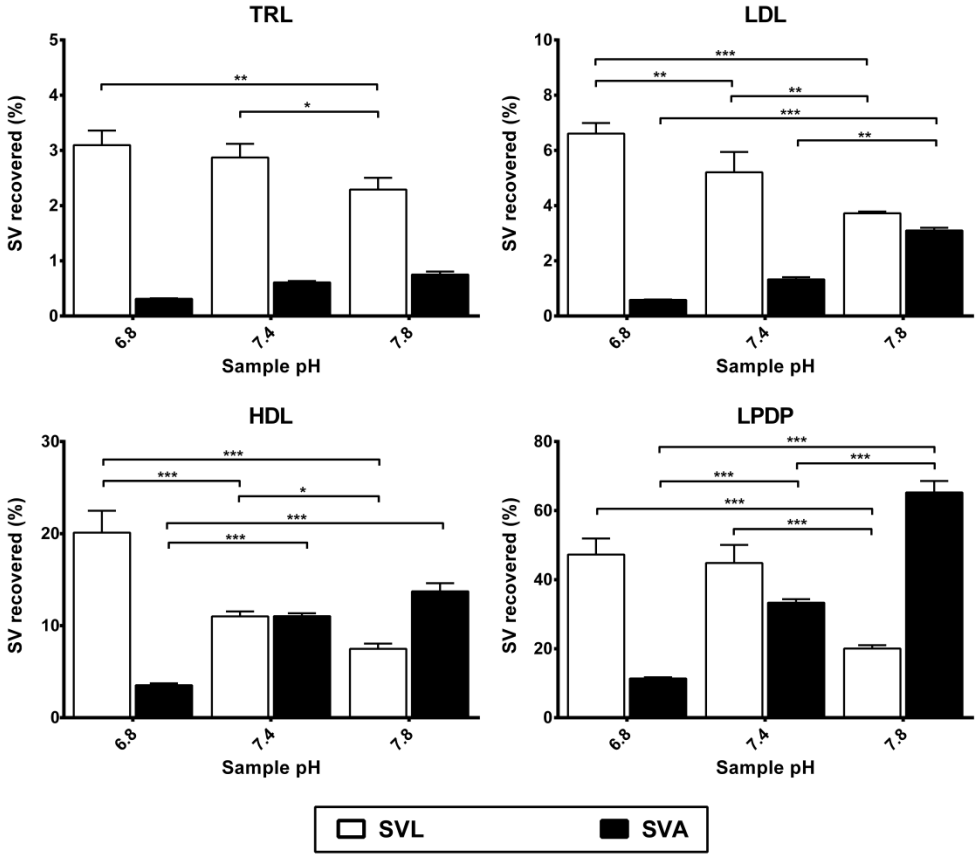


Figure 6.8: Percentage of simvastatin lactone and corresponding hydroxy acid forms recovered in individual lipoprotein fractions of normolipidaemic plasma. Results are expressed as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, * $p < 0.05$, ** $p < 001$, *** $p < 0.001$. TRL, triglyceride-rich lipoproteins; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma; SVL, simvastatin lactone; SVA, simvastatin hydroxy acid.

The amount of simvastatin lactone and hydroxy acid forms recovered in individual lipoprotein fractions of hyperlipidaemic and normolipidaemic human plasma at different pH levels are shown in Table 6.2.

Table 6.2: Amount of simvastatin lactone and hydroxy acid forms recovered in individual lipoprotein fractions of hyperlipidaemic and normolipidaemic human plasma at different pH levels (n=5).

Sample	Sample pH	Statin	Amount of statins recovered in individual lipoprotein fractions (ng)				Total statin recovered (%)
			TRL	LDL	HDL	LPDP	
Hyperlipidaemic plasma	6.8	SVL	1177± 27	990 ± 200	815 ± 60	2193 ± 149	86.23
		SVA	8 ± 1	34 ± 3	124 ± 10	282 ± 11	7.45
	7.4	SVL	514 ± 76	747 ± 104	499 ± 50	2828 ± 203	76.48
		SVA	17 ± 1	72 ± 6	298 ± 14	1032 ± 44	23.65
	7.8	SVL	713 ± 99	236 ± 6	288 ± 16	2197 ± 254	57.23
		SVA	44 ± 2	118 ± 5	351 ± 10	2027 ± 82	42.32
Normolipidaemic plasma	6.8	SVL	186 ± 16	397 ± 23	1207 ± 143	2836 ± 279	77.09
		SVA	19 ± 1	35 ± 1	212 ± 12	681 ± 22	15.77
	7.4	SVL	172 ± 15	312 ± 44	660 ± 33	2689 ± 315	63.90
		SVA	37 ± 2	80 ± 5	661 ± 19	1999 ± 61	46.28
	7.8	SVL	138 ± 13	233 ± 4	449 ± 34	1202 ± 57	33.54
		SVA	45 ± 3	186 ± 6	824 ± 53	3915 ± 200	82.83

Abbreviations: SVL, simvastatin lactone; SVA, simvastatin hydroxy acid; TRL, triglyceride-rich lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoprotein; LPDP, lipoprotein-different plasma.

6.3.4 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cellular uptake of lipophilic statin

To examine the effect of plasma lipoprotein association on cellular uptake of simvastatin, C2C12 cells were incubated with simvastatin loaded into lipoprotein fractions (TRL or LDL) or applied directly as a solution. In addition to physiological pH, uptake study was also conducted at pH corresponding to acidosis and alkalosis to elucidate the role of hyperlipidaemia and acid-base imbalance on cellular uptake of simvastatin. Figure 6.9 shows the total simvastatin (lactone and hydroxy acid forms combined) taken up by C2C12 cells that overexpress mLPL, hLPL, or control cells following treatment with simvastatin-loaded TRL. The same amounts of simvastatin were found in undifferentiated cells that have low endogenous LPL expression ability (cells transfected with empty vector) following treatment with either TRL bound simvastatin or free simvastatin in solution. On the other hand,

higher simvastatin uptake was observed in undifferentiated cells that overexpress mLPL or hLPL upon treatment with simvastatin-preloaded TRL fractions compared to simvastatin in solution (Figure 6.9).

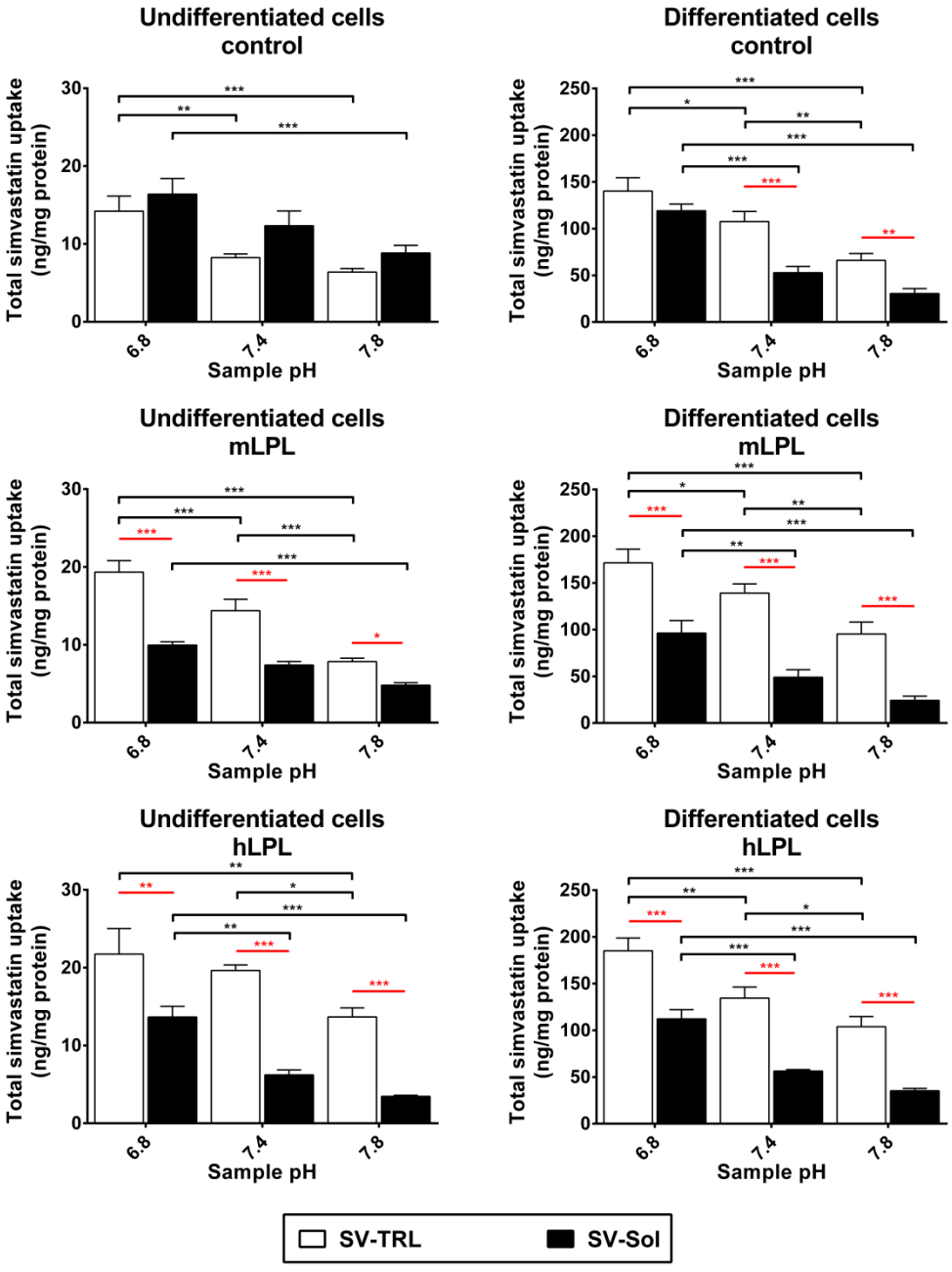


Figure 6.9: Total simvastatin uptake by undifferentiated and differentiated C2C12 after 3 hours of incubation with simvastatin either loaded into TRL fractions or applied directly in solution. Data are presented as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, *$p < 0.05$, **$p < 001$, ***$p < 0.001$. Red straight lines denote differences in simvastatin uptake between cells treated with SV-TRL and SV-Sol. Black arched lines denote differences in simvastatin uptake by cells maintained at different pH.

Results of simvastatin uptake by C2C12 cells treated with simvastatin-loaded LDL are shown in Figure 6.10. Undifferentiated C2C12 cells that overexpress mLPL or hLPL showed higher simvastatin uptake when treated with simvastatin-loaded LDL compared to their treatment with simvastatin solution. No significant differences in simvastatin uptake were detected in control undifferentiated C2C12 cells following treatment with either simvastatin-loaded LDL or simvastatin applied directly in solution at physiological and alkaline pH. However, more simvastatin was found in these cells under acidic conditions when treated with simvastatin solution compared to simvastatin-loaded LDL (Figure 6.10). Overall, higher cellular uptake was observed when undifferentiated cells were treated with simvastatin-loaded LDL compared to their treatment with simvastatin-loaded TRL (Figures 6.9 and 6.10).

Differentiated C2C12 cells that overexpress mLPL or hLPL showed higher simvastatin uptake upon treatment with simvastatin-loaded lipoprotein fractions compared to their treatment with free simvastatin solution. It is worth noting that differentiated control cells (transfected with empty vector) also showed higher simvastatin uptake when treated with simvastatin-loaded LDL compared to undifferentiated cells (Figure 6.10).

A remarkable point is that the amounts of simvastatin found in differentiated cells were approximately ten-fold higher than that in undifferentiated cells following treatment with simvastatin-associated with lipoprotein fractions. The data also show that maintaining both undifferentiated and differentiated cells in acidic medium further increases the cellular uptake

of simvastatin whether it is bound to lipoprotein fractions or applied as free simvastatin in solution (Figures 6.9 and 6.10).

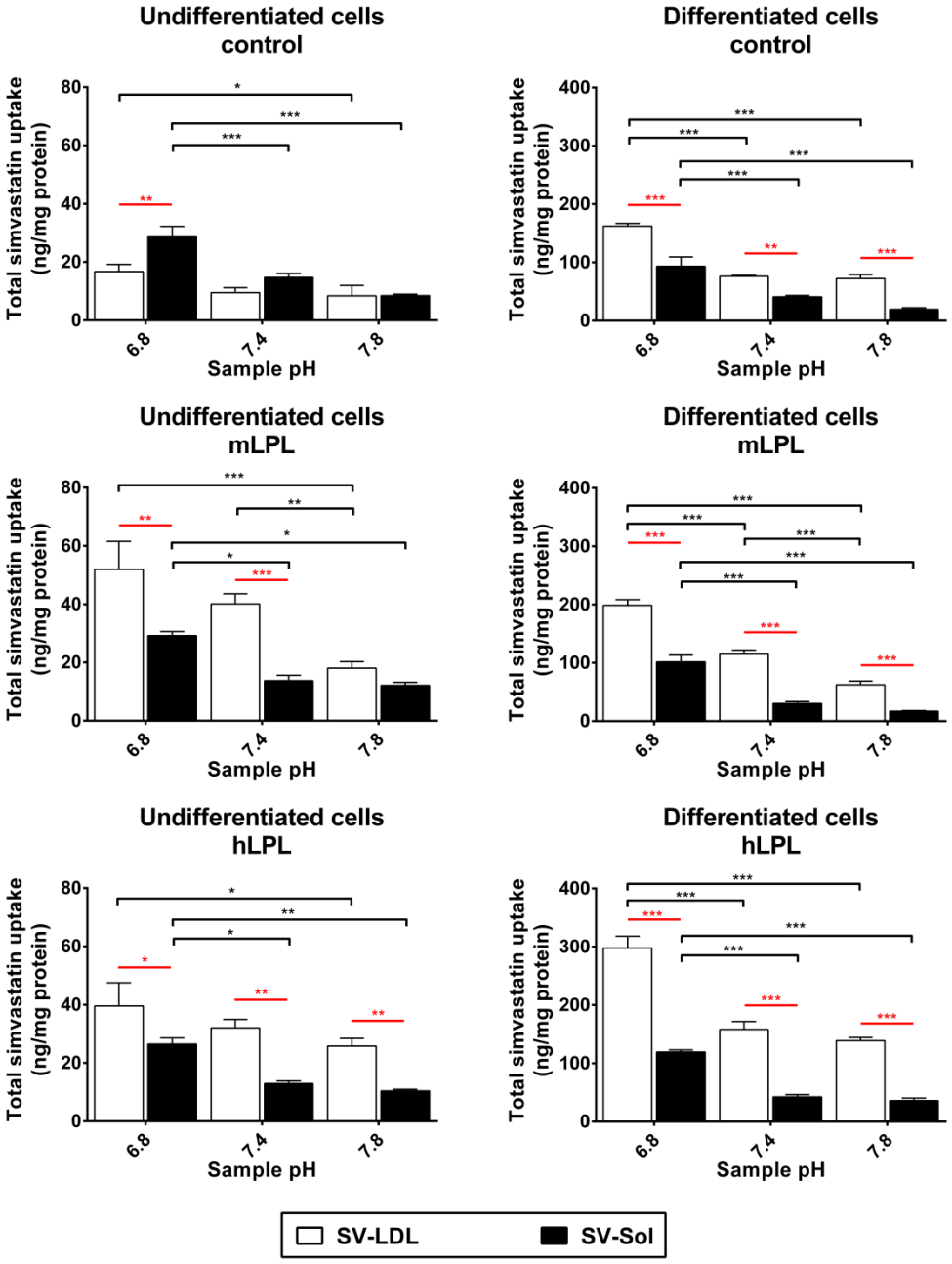


Figure 6.10: Total simvastatin uptake by undifferentiated and differentiated C2C12 after 3 hours of incubation with simvastatin either loaded into LDL fraction or applied directly in solution. Data are presented as mean ± SE, (n=5). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, *p < 0.05, **p < 001, ***p < 0.001. Red straight lines denote differences in simvastatin uptake between cells treated with SV-LDL and SV-Sol. Black arched lines denote differences in simvastatin uptake by cells maintained at different pH.

To illustrate the role of LPL in the cellular uptake of simvastatin-loaded lipoprotein particles, the activity of the enzyme was measured in the different cell types used in our studies. The enzyme activity was found to be almost two- and three- fold higher in undifferentiated C2C12 cells that overexpress hLPL or mLPL compared to control cells, respectively. On the other hand, differentiated C2C12 cells that overexpress mLPL showed higher enzyme activity compared to those expressing hLPL or control cells (Figure 6.11).

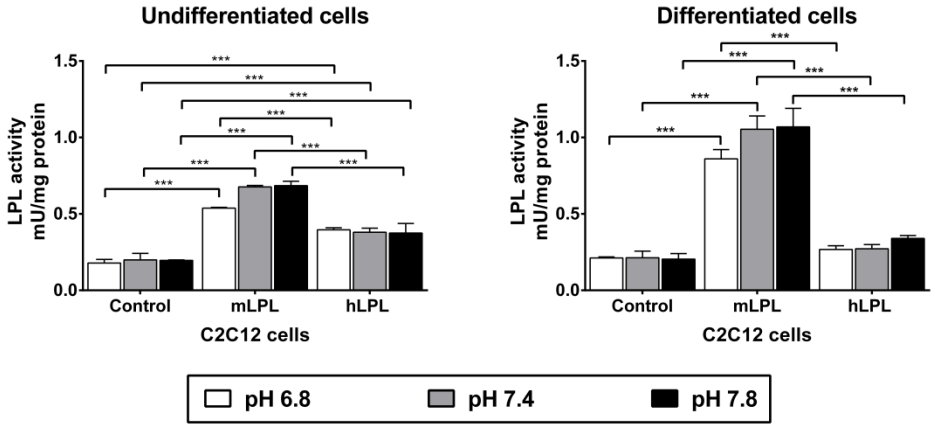


Figure 6.11: Lipoprotein lipase activity in undifferentiated and differentiated C2C12 cells overexpressing mLPL, hLPL and control cells transfected with empty vector. Data are presented as mean ± SE, (n=3). Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, *** $p < 0.001$.

6.3.5 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cytotoxicity of simvastatin

The effects of simvastatin association with plasma lipoproteins on the mitochondrial activity of C2C12 cells were assessed by MTT assay using simvastatin-loaded lipoprotein fractions. The incubations were performed at physiological pH, in addition to pH levels corresponding to acidosis and alkalosis, in order to further explore the combined effects hyperlipidaemia and pH alteration on the viability of muscle cells. Results from MTT assay over

different concentrations of simvastatin-loaded TRL and LDL fractions are shown in Figures 6.12 and 6.13, respectively. To provide a comparison between different treatment conditions, the IC_{50} were calculated, when applicable, for each growth inhibition-dose curve.

The results of cell viability assay showed higher cytotoxicity when C2C12 cells were treated with simvastatin-loaded lipoprotein fractions compared to cells treated with simvastatin solution. Furthermore, the inhibitory effect of simvastatin-loaded lipoprotein fractions on cell viability was more pronounced in cells that overexpress mLPL compared to those expressing hLPL or control cells. Cells that overexpress hLPL showed moderate response to simvastatin-loaded lipoprotein therapy and the IC_{50} values were in between those overexpressing mLPL and control cells. Both undifferentiated and differentiated cells were found to be more sensitive to simvastatin when applied loaded into LDL compared to simvastatin-loaded TRL fractions (Figures 6.12 and 6.13).

Maintaining undifferentiated and differentiated C2C12 cells in acidic culture medium resulted in significant reduction in cell viability regardless the cells being treated with simvastatin-loaded lipoprotein fractions or free simvastatin in solution. On the other hand, no significant reduction in cell viability was observed for cells maintained in alkaline culture medium following treatment with simvastatin-loaded lipoprotein fractions or simvastatin solution. At this alkaline pH (7.8), the viability of the cells remained above 50% even after treatment with the highest dose of simvastatin and therefore it was not possible to calculate the IC_{50} values under this treatment condition (Figures 6.12 and 6.13).

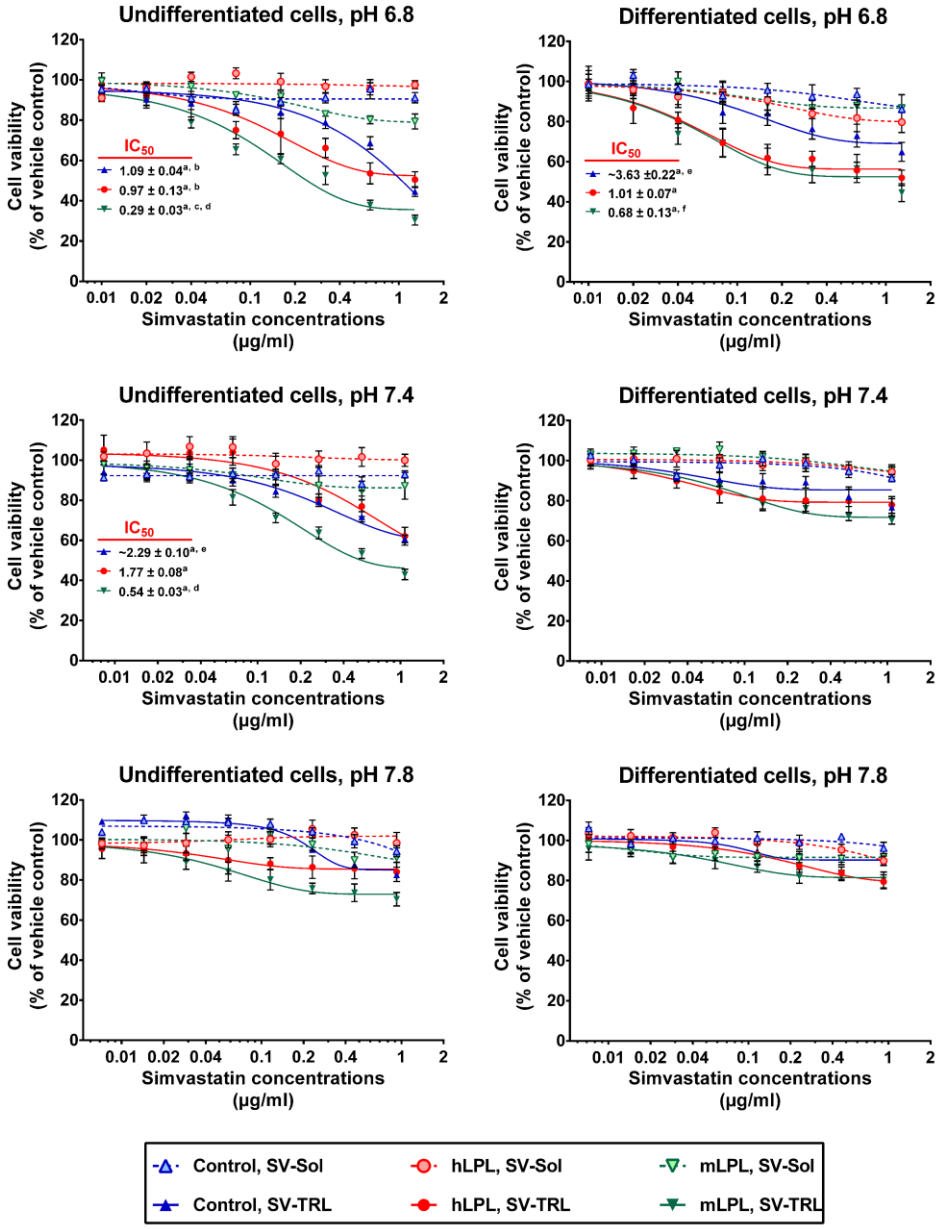


Figure 6.12: MTT assay of undifferentiated and differentiated C2C12 cells overexpressing mLPL, hLPL, and control cells transfected with empty vector following treatment with simvastatin either loaded into TRL fraction or applied directly in solution at different pH. Data are presented as mean ± SE of 3 experiments, 8 replicate per experiment. The IC_{50} values are listed on graphs, when applicable, and were derived by computing dose-response curves using a 4-parameter, variable slope fit in GraphPad Prism. Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, [a]Significant difference from SV- Sol ($p < 0.001$); [b]Significant difference from pH 7.4 ($p < 0.001$); [c]Significant difference from pH 7.4 ($p < 0.01$); [d]Significant difference from hLPL ($p < 0.001$); [e]Significant difference from mLPL and hLPL ($p < 0.001$); [f]Significant difference from hLPL ($p < 0.05$).

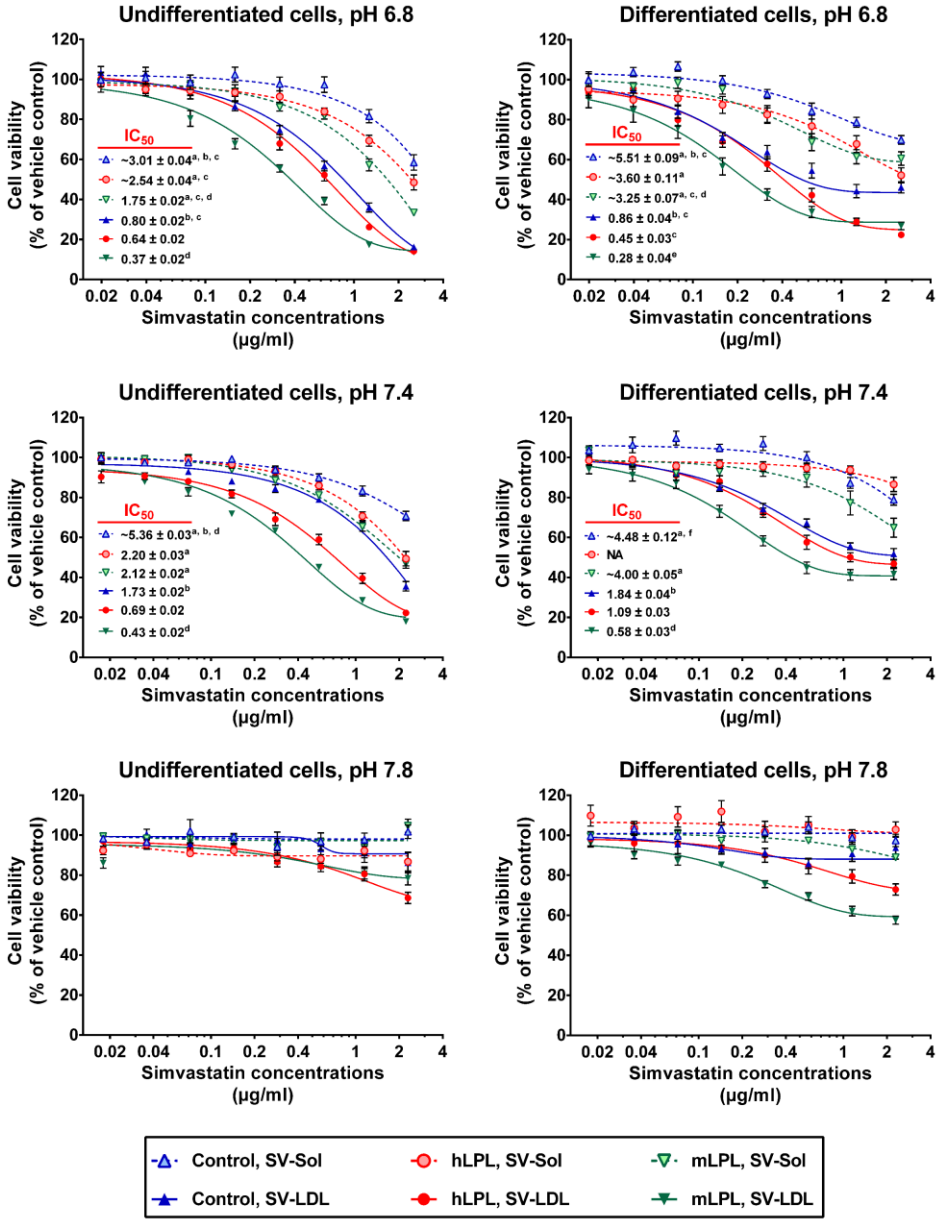


Figure 6.13: MTT assay of undifferentiated and differentiated C2C12 cells overexpressing mLPL, hLPL, and control cells transfected with empty vector following treatment with simvastatin either loaded into LDL fraction or applied directly in solution at different pH. Data are presented as mean ± SE of 3 experiments, 8 replicates per experiment. The IC_{50} values are listed on graphs, when applicable, and were derived by computing dose–response curves using a 4-parameter, variable slope fit in GraphPad Prism. Statistical analysis was done by 2-way ANOVA followed by Fisher's LSD post hoc test, [a]Significant difference from SV- LDL ($p < 0.001$); [b]Significant difference from mLPL and hLPL ($p < 0.001$); [c]Significant difference from pH 7.4 ($p < 0.001$); [d]Significant difference from hLPL ($p < 0.001$); [e]Significant difference from pH 7.4 ($p < 0.01$); [f]Significant difference from mLPL ($p < 0.001$).

Results of LDH release from undifferentiated and differentiated C2C12 cells that have different LPL activity are shown in Figure 6.14. Overall, more LDH was released from these cells when treated with simvastatin-loaded lipoprotein fractions compared to their treatment with simvastatin solution. The higher LPL enzyme activity in undifferentiated cells that overexpress mLPL or hLPL produced an additional increase in LDH release when treated with simvastatin-loaded TRL. Moreover, treating these cells with simvastatin-loaded LDL resulted in greater LDH release compared to cells that have low endogenous enzyme activity. Higher LDH release was observed with cells maintained in acidic culture medium compared to neutral and alkaline medium following exposure to simvastatin solution or simvastatin-loaded lipoprotein fractions.

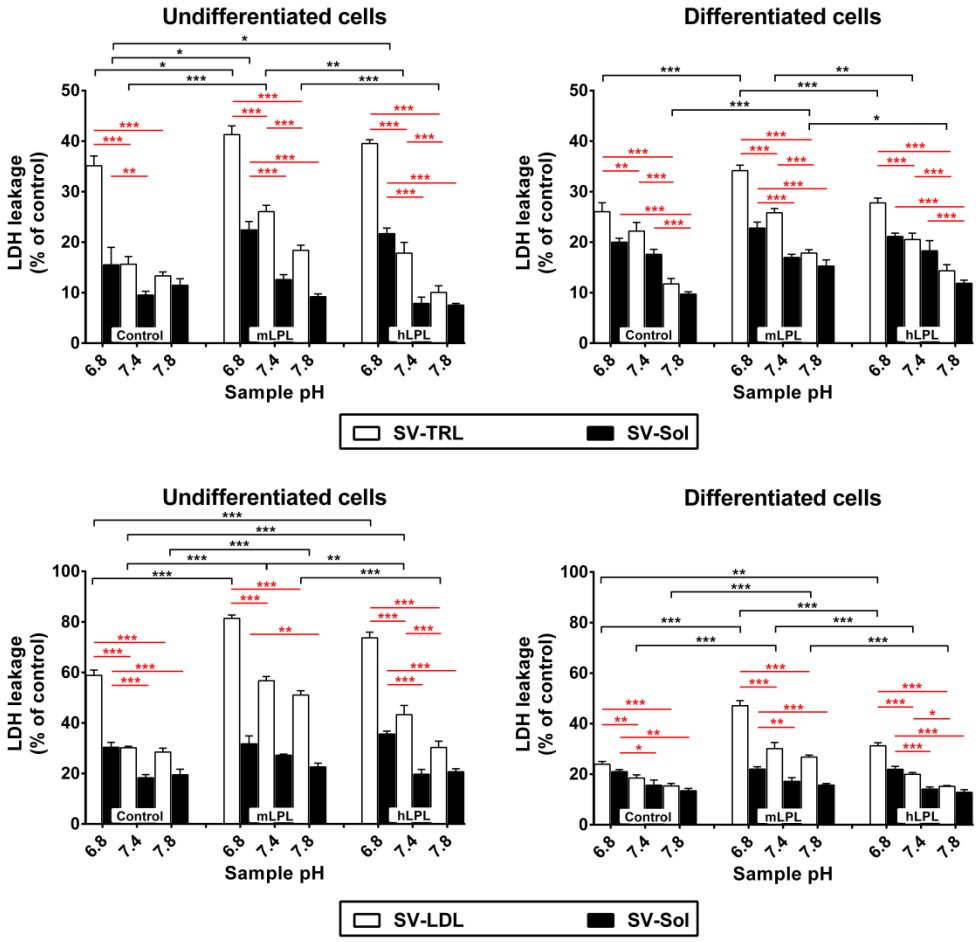


Figure 6.14: LDH release assay of undifferentiated and differentiated C2C12 cells overexpressing mLPL, hLPL, and control cells transfected with empty vector following treatment with simvastatin either loaded into lipoprotein fractions or applied directly in solution at different pH. Data are presented as mean ± SE, (n=6). Statistical analysis was done 2-way ANOVA followed by Fisher's LSD post hoc test, *$p < 0.05$, **$p < 001$, ***$p < 0.001$. Black arched lines denote differences in LDH release between different cell types. Red straight lines denote differences in LDH release from a single cell type maintained at different pH. SV-TRL, simvastatin-loaded triglyceride-rich lipoproteins; SV-LDL, simvastatin-loaded low-density lipoprotein; SV-Sol, simvastatin solution.

6.4 Discussion

Although the exact cause of statin-induced myotoxicity remains obscure, the presence of certain comorbidities such as hepatic or renal failure, hypothyroidism, and diabetes mellitus have been suggested to affect the occurrence and severity of statin-associated muscle symptoms.[64, 65] Acidosis, secondary to some of the above-mentioned comorbidities, has recently been shown to potentiate cytotoxicity of lipophilic statins *in vitro*.[156] The current study describes the role of hyperlipidaemia, a primary disorder for which statins is considered as first choice therapy, in development and potentiation of statin-induced myotoxicity. An increased association of lipophilic drugs with plasma lipoproteins in hyperlipidaemic patients can affect drug distribution and toxicity profile.[122, 216] Since acidosis is known to enhance the lipophilicity of statins,[156] it is possible that the coexistence of acidosis with hyperlipidaemia could further augment the risk of statin-induced myotoxicity. To the best of our knowledge, this is the first work that elucidates the influence of pathological or postprandial hyperlipidaemia alone or in combination with acidosis/alkalosis on the development and severity of simvastatin-induced myotoxicity.

A previous study has been conducted to investigate the influence of high-fat diet on statin-induced myotoxicity.[223] However, that study did not focus on the contribution of postprandial elevation in plasma lipoprotein levels and their role in modifying tissue distribution of statins. Instead, it has linked the increase in muscle damage observed in fluvastatin-treated rats to the elevation in plasma statin concentration as a result of hepatic steatosis which causes disturbance in hepatic statin uptake and clearance.[223]

6.4.1 *In silico* prediction of statins association with chylomicrons

Results from *in silico* model for prediction of drug uptake by plasma chylomicrons indicate that physicochemical properties of statins highly influence their uptake by plasma lipoproteins (Table 6.1). Simvastatin lactone (lipophilic statin) with a high Log$D_{7.4}$ value and low polar surface area showed the highest association with chylomicrons. On the other hand, less lipophilic statins (simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid) showed no predicted association with chylomicrons. Previous studies have utilized melting point and solubility of lipophilic drugs in long chain triglycerides as indicators of the extent of association with plasma lipoproteins.[118, 153, 224] In the present study, an *in silico* model that relies on a combination of physicochemical parameters was used to predict the degree of association of statins with chylomicrons. This *in silico* model was previously developed by plotting physicochemical parameters of 19 lipophilic compounds (obtained from ACD/I-Lab) against their experimental uptake by chylomicrons using SIMCA-P software package.[152] The plot yielded a list of correlation coefficients with a good predictive power of the degree of association with chylomicrons.

6.4.2 Association of statins with protein-free artificial lipid particles and plasma-derived isolated chylomicrons

Artificial fat emulsion (Intralipid®) has been used to assess the degree of association of lipophilic simvastatin with lipoprotein particles because of its structural resemblance to chylomicrons.[152] Intralipid® droplet composed of neutral lipid core (triglycerides from soya bean oil) stabilized by a monolayer

of egg phospholipid (lecithin).[225] Simvastatin lactone showed considerable association with Intralipid® particles at physiological pH and no significant differences were detected at different pH levels (Figure 6.1). This can, at least partially, be related to the nature of artificial lipid particles which can act as a barrier and protect the encapsulated simvastatin against pH-dependent chemical hydrolysis.[225]

The association of simvastatin lactone with Intralipid® emulsion was also assessed at pH 4.5. Although pH 4.5 is outside the range likely to be found in patients, this pH has been considered to be the optimum pH at which no inter-conversion of statins occurs.[175, 182, 187] Although working with such low pH might affect physical stability of the fat emulsion, similar proportions of simvastatin lactone were recovered in fat emulsion at this pH compared to that obtained from emulsions of other pH levels. Intralipid® emulsion remains stable at mild acidic or alkaline pH.[4-8] However, decreasing the pH below 5 is expected to reduce the electrostatic repulsion between lipid droplets, resulting in increased droplet size and coalescence.[225-227]

Ex vivo study was used to assess the degree of association of statins with natural chylomicrons derived from human plasma. Similar to *in silico* prediction, simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid showed no association with human chylomicrons *ex vivo*. When the association of simvastatin lactone was assessed with human chylomicrons, a significant pH-dependent trend was observed. Acidic pH resulted in the highest association (Figure 6.1), suggesting that in pathophysiological condition of acidosis more simvastatin would be able to associate with the lipid-rich plasma lipoprotein fractions.

In the present study, chylomicrons were isolated from the rest of plasma components in order to eliminate the possible interference from simvastatin associated with albumin and other lipoproteins. The isolated chylomicrons are expected to have low degree of albumin contamination (0.1-0.2%).[153] The Absence of passive diffusion of simvastatin through the density gradient was tested by running control experiment and no simvastatin was detected in the upper 1 mL layer. Therefore, all recovered simvastatin is entirely the result of its association with chylomicrons.

Although chylomicron fraction was produced from hypertriglyceridaemic human plasma samples, the emulsion was diluted to final triglyceride levels of 100 mg/dL (to achieve normal triglyceride levels that correspond to normolipidaemic condition). The use of standard experimental conditions in the *ex vivo* association study (constant triglyceride concentrations in the chylomicron emulsion and constant molar concentration of tested statins) allow the comparison with the *in silico* model that had been developed under similar conditions.[151] Higher percentage of simvastatin lactone is expected to associate with chylomicrons upon entrance into the blood stream especially in the presence of postprandial or pathological hypertriglyceridaemia which is often associated with elevated levels of TRL. Such association raises the possibility of altered therapeutic and toxic response of lipophilic statins.

A higher percentage of association of simvastatin lactone with chylomicrons and Intralipid® were obtained in *in vitro* and *ex vivo* experiments comparing to the *in silico* model. These results suggest that the *in silico*

prediction of drug association with chylomicrons underestimated the degree of association of lipophilic statins.

6.4.3 Effect of hyperlipidaemia alone or in combination with acidosis/ alkalosis on simvastatin association with plasma lipoproteins

Hyperlipidaemia can affect the pharmacokinetic and pharmacodynamic profiles of a number of lipophilic drugs.[228] This may occur through alteration in the pattern of drug binding to plasma proteins (albumin and α-acid glycoprotein) due to a shift of lipophilic drug toward the non-polar lipoprotein fractions such as TRL and LDL.[120]

The concentrations of plasma lipoproteins in the blood can vary significantly among individuals as a function of diet or disease states. This variability could have significant implications on the fate of drug molecules that associate with plasma lipoproteins. Most of the earlier studies on the effects of hyperlipidaemia on the biological activities of lipophilic drugs have been related to pathological hyperlipidaemia. This chronic condition is associated with alteration of VLDL and LDL levels.[116, 122, 126, 229] On the other hand, postprandial hyperlipidaemia is characterised by an acute and transient elevation in TRL, mostly chylomicrons.

In the present study, simvastatin has been shown to associate with different lipoprotein fractions in hyperlipidaemic and normolipidaemic plasmas. The association was more significant with the lipoprotein fractions obtained from hyperlipidaemic plasma (Figure 6.2). Several factors have been suggested to influence the extent of drug association with plasma lipoproteins. Some of these factors are linked to the molecular nature of the compounds

such as drug lipophilicity. Other factors are related to the nature of lipoproteins such as size, total lipid contents or apolipoprotein components of lipoprotein fractions.[230] In the present study, significant positive correlations were observed between the triglyceride and cholesterol contents of lipoprotein fractions (TRL and LDL) and the degree of simvastatin association with these fractions (Figures 6.5 and 6.6).

Our results provide a potential explanation for the observation of Bláha *et al*., 2010 who reported a significant drop in serum concentrations of simvastatin and atorvastatin in patients with severe familial hypercholesterolemia following extracorporeal LDL-cholesterol elimination therapy. Although statin binding to plasma proteins and subsequent adsorption to columns and filters of LDL-apheresis and hemorheopheresis has been suggested to be a possible mechanisms,[231] statins association with LDL appears to be the most likely mechanism that could explain the significant reduction in serum statin levels following this procedure.

More simvastatin has been shown to associate with plasma lipoproteins at acidic pH compared to physiological and alkaline pH. The higher association at acidic pH occurs as a result of simvastatin's increased lipophilicity since the majority of simvastatin molecules remain in the highly lipophilic lactone form at low pH (Figures 6.7 and 6.8). These results are consistent with previous studies that demonstrated pH dependency in drug binding to plasma lipoproteins.[130, 232, 233]

6.4.4 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cellular uptake of lipophilic statin

Drug binding with plasma lipoproteins has been suggested to affect the blood-tissue partitioning of lipophilic compounds, resulting in alterations in the pharmacological activity and/or potential toxicity of these compounds.[117] In this study, the association of simvastatin with plasma lipoproteins, particularly TRL and LDL, has been shown to enhance the cellular uptake of lipophilic statin by C2C12 cells (Figures 6.9 and 6.10). The partitioning of lipophilic statin from plasma lipoproteins to skeletal muscle cells seems to be mediated by LPL. This enzyme is responsible for the intravascular hydrolysis of triglycerides within lipoproteins into fatty acids and monoacylglycerol. It also regulates the cellular uptake of fatty acid and cholesterol and controls the transport and internalisation of lipoprotein particles by peripheral tissues via enhancing their binding to lipoprotein receptors.[132-134] Statin uptake from plasma lipoproteins could be of a particular importance when skeletal muscle tissue expresses high levels of LPL. Several conditions have been reported to increase LPL expression in human skeletal muscle cells, such as chronic exercise, fibrate therapy, and fasting.[234] These conditions are expected to increase the risk of statin-induced myopathy by increasing the muscle uptake of simvastatin via the above-mentioned mechanism. Indeed, the majority of professional athletes were found to be intolerant to most of the available statins.[219] This group of population is known to have increased muscle LPL expression and skeletal muscle acidosis.[235, 236]

The extent of simvastatin uptake by C2C12 cells was proportional to the activity of LPL in these cells. More simvastatin was taken up by cells that have

higher LPL activity (mLPL > hLPL > empty) (Figures 6.9 and 6.10). Our results are in accordance with previous studies that demonstrated an increase in the transfer of α-tocopherol (a highly lipophilic compound) from plasma lipoproteins and Intralipid® particles to human fibroblast cells and rat skeletal muscle myoblasts (L6 cells) in the presence of exogenous lipoprotein lipase.[237, 238] The results are also in agreement with Sattler *et al.,*1996 who reported an increase in skeletal muscle uptake of α-tocopherol in transgenic mice overexpressing lipoprotein lipase.[239] The enhancement of simvastatin uptake by C2C12 cells that have high LPL activity could be attributed to the higher triglyceride hydrolysis rates[237] and the bridging function of the enzyme between lipoprotein-drug complex and muscle cells.[239]

Although the mechanisms by which simvastatin is transported from plasma lipoprotein particles to the cells have not been investigated in this study, several assumptions can be proposed based on previously published literature.[237-242] The transfer of lipoprotein-associated simvastatin could happen independently from lipoprotein particle uptake.[241] Simvastatin is expected to be released from the lipoprotein particles by lipoprotein lipase-mediated hydrolysis of TRL and then transported across cell membranes by passive diffusion due to its lipophilicity.[239] Other possible mechanisms involve the internalisation of lipoprotein-drug complex via lipoprotein receptors mediated endocytosis which delivered it to the lysosomes as intact particle[240, 243]. Within the lysosome, the complex would be then hydrolyzed and simvastatin could be liberated. In addition, lipoprotein-drug complex uptake might occur via routes independent of lipoprotein receptors pathway.[242] Substantial evidence is available to support the role of lipoprotein lipase in enhancing lipoprotein-drug

complex uptake either by lipoprotein receptor-dependent or independent internalisation pathways.[242, 243]

More simvastatin was found to be taken up by C2C12 cells when treated with simvastatin-loaded LDL compared to simvastatin-loaded TRL. This process could be of clinical significance since statin therapy has been shown to induce upregulation of LDL receptors in the skeletal muscle cells,[234] resulting in an increased uptake of simvastatin-associated LDL by muscle cells via LPL-mediated mechanism.

The cellular uptake of simvastatin by C2C12 cells was found to be strongly affected by the pH levels of the culture medium. Higher simvastatin uptake was observed in acidic pH compared to physiological and alkaline pH. These results are in agreement with our previous observations that showed higher cellular uptake of statins at acidic condition compared to physiological and alkaline environment.[156]

6.4.5 Effect of hyperlipidaemia alone or in combination with acidosis/alkalosis on cytotoxicity of simvastatin

In the present study, the higher cytotoxicity of simvastatin observed in the presence of elevated plasma lipoprotein levels is linked to drug association with TRL and LDL. These lipoprotein fractions can serve as carriers for lipophilic statins and have been shown to increase simvastatin uptake by muscle cells (Figures 6.9 and 6.10). These results are consistent with previous studies that reported an increase in the antiproliferative effect of ciclosporin and α-tocopheryl succinate and enhanced renal cytotoxicity of amphotericin as a result of drug association with LDL fraction.[124, 240, 244]

In accordance with the uptake studies, the cytotoxicity profile of simvastatin-lipoprotein complex in C2C12 cells was found to be proportional to the activity of LPL in these cells. Higher cytotoxicity was observed in cells that have higher LPL enzyme activity (mLPL > hLPL > empty) (Figures 6.12 and 6.13). The effect of simvastatin association with plasma lipoproteins on the cytotoxicity was further augmented when the skeletal muscle cells were maintained at acidic pH compared to physiological and alkaline pH. The higher cytotoxicity of simvastatin under acidic conditions could be attributed to the greater proportions of the lactone form of simvastatin that are associated with plasma lipoprotein fractions. This lipophilic lactone from has higher ability to cross the cell membranes of muscle cells and achieve high intracellular concentrations. On the other hand, the low cytotoxicity observed under alkaline conditions is due to the low levels of the lactone form recovered within the lipoprotein fractions under these conditions, resulting in lower intracellular concentrations of simvastatin.[156]

6.5 Conclusions

In the present study, elevated plasma lipoprotein levels as a result of pathological or postprandial hyperlipidemia significantly affect tissue distribution and muscle toxicity of lipophilic statins. Enhanced cytotoxicity was observed with simvastatin lactone in response to drug association with the non-polar lipoprotein fractions (TRL and LDL). The affinity of statins to bind plasma lipoproteins is dependent on a number of factors including the physicochemical properties of statins and the pH dependent inter-conversion between the hydroxy acid and lactone forms. The lipophilicity of statins is one of the critical factor that affects the plasma lipoproteins binding and cellular uptake of these drugs. Lipoprotein binding appears to have a major impact on the efficacy and safety of lipophilic statins, particularly in the presence of acidosis. Acidosis enhances the lipophilicity of statin by minimizing the conversion of the lipophilic lactone form to the hydrophilic hydroxy acid form and thereby increases statin association with plasma lipoproteins. Our findings suggest more selective and conservative approach, as well as tighter monitoring of statin-related skeletal muscle symptoms when prescribing lipophilic statins to hyperlipidemic patients who are at risk of acidosis.

Chapter Seven

7 Conclusions, Implications, Limitations and Future Work

7.1 Conclusions

This work suggested that comorbidities such as acidosis or hyperlipidaemia, and especially a combination of hyperlipidaemia with acidosis, could contribute to enhanced muscle toxicity following administration of lipophilic statins.

Acid-base imbalance affects the inter-conversion of statins between the lactone and hydroxy acid forms. The conversion of lactone form of statins to the corresponding hydroxy acid form is strongly pH-dependent. At physiological and alkaline pH, the lactone form undergoes substantial conversion while this conversion occurs at lower extent under acidic conditions. On the contrary, the conversion of the hydroxy acid form to the corresponding lactone form is negligible at any pH levels except at the pH of gastric fluid.

Our data also demonstrated that lipophilic simvastatin lactone was more cytotoxic to undifferentiated and differentiated skeletal muscle cells than the more hydrophilic simvastatin hydroxy acid, pravastatin lactone, and pravastatin hydroxy acid. Furthermore, undifferentiated C2C12 cells were found to be more sensitive to statin-induced myotoxicity than differentiated muscle cells. Physiological pH and alkalosis can protect against statin-induced myotoxicity whereas acidosis enhances statin-induced myotoxicity as demonstrated by increased cellular uptake of statins under acidic conditions.

These findings provide novel insight into the mechanisms of statin-induced myotoxicity in the presence of acidosis and alkalosis. By minimizing

the chemical and enzymatic hydrolysis of the lactone form, acidosis can enhance statins uptake by the skeletal muscle cells and consequently potentiate their myotoxicity. On the other hand, alkalosis can potentiate the hydrolysis of the lactone ring rendering statins less lipophilic and therefore limits their penetration into the skeletal muscles and increases their uptake by the liver.

Our study also demonstrated that hyperlipidaemia enhanced the association of lipophilic statin with the non-polar plasma lipoprotein fractions such as TRL and LDL. This association contributed to increased cellular uptake of statins by skeletal muscle cells resulting in a high local intracellular concentration of statins through an LPL-mediated process. Moreover, the high intracellular concentrations of statins induced significantly higher myotoxicity compared to normolipidaemic conditions.

The affinity of statins to associate with plasma lipoproteins is dependent on a number of factors including the physicochemical properties of statins and the pH-dependent inter-conversion between the lactone and hydroxy acid forms. Indeed, the lipophilicity of statins is a critical factor affecting the plasma lipoproteins binding and the cellular uptake of these drugs. Alteration of lipophilicity of statins has been shown to affect the myotoxic potential of these drugs. The coincidence of acidosis with hyperlipidaemia further augmented the myotoxicity of lipophilic statin. Acidic pH enhanced the association of lipophilic statin with plasma lipoproteins by minimising the conversion of lipophilic lactone form to the hydrophilic hydroxy acid. As a result, the intracellular concentrations of statins in muscle cells, as well as myotoxicity were increased.

The potential clinical significance of these results is that hyperlipidaemia, especially when combined with acidosis, requires extra caution and close monitoring by clinicians who should follow a conservative approach when prescribing lipophilic statins to these patients. Hydrophilic rather than lipophilic statins could be a preferable choice in hyperlipidaemic patients who are prone to metabolic acidosis.

7.2 Implications of the Current Research

Statins are used for prevention and treatment of chronic conditions that require long-term drug administration. Therefore, drug safety and patients' compliance are prerequisites, especially among the elderly population who often have multiple comorbidities.

This work provides a novel insight into the role of disturbances in acid-base balance and elevated plasma lipoprotein levels in the development of statin-induced myotoxicity. The translational importance would be of great significance in recognising high-risk patients, improving the safety profile and minimising the chance of progression of muscular adverse effects to more severe and potentially fatal rhabdomyolysis. Prevention of adverse drug reactions can reduce the health care burden and costs with a substantial reduction in patient morbidity and mortality.

The effect of acidosis and hyperlipidaemia on statin-induced muscle toxicity is of a particular important in case of lipophilic statins, such as simvastatin. Acidic conditions enhance the cytotoxicity of statins as a result of increased cellular uptake of the more lipophilic lactone form or the unionised hydroxy acid form. On the other hand, alkaline conditions have a protective

effect against statin-induced myotoxicity because of the inability of statin to achieve adequate intracellular concentrations as a result of conversion to the more hydrophilic ionized hydroxy acid form. Therefore, one can assume that there could be a potential role for plasma pH alkalinization (for example by using sodium bicarbonate) in reducing the chance of development of severe and potentially fatal muscle side effects.

Understanding the mechanisms by which disease states such as acid-base imbalance and hyperlipidaemia affect the toxicity profile of lipid-lowering drugs is essential in drug selection. The coincidence of acidosis with hyperlipidaemia implies a detrimental impact on the health status of individuals who are prescribed lipophilic statins and have these conditions. This requires extra caution by clinicians who should follow a conservative approach and close monitoring when prescribing lipophilic statins to these patients. Hydrophilic instead of lipophilic statins could be a preferable choice in patients who have hyperlipidaemia with or without acidosis. Other measures should be considered for statin intolerant patients who are at high risk of cardiovascular diseases. These measures include lifestyle changes and the use of non-statin lipid lowering drugs such as natural and semisynthetic sterols, ezetimibe, bile acids sequestrants, fibrates, and proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitors.[245-248]

7.3 Limitations

The current studies have several limitations. First, a key question that we couldn’t provide a full answer is that to what extent does pH-mediated chemical reaction plays a role in statin conversion compared to the enzymatic

conversion. Although we have shown that the rate of both enzymatic and chemical conversion of simvastatin lactone is slower at acidic pH compared to physiological and alkaline pH, our results did not tell which conversion process is predominant over the other. To answer this question, *in vivo* studies need to be performed using animals of experimentally induced acidosis or alkalosis versus animals of physiological blood pH.

Another limitation of the current *in vitro* studies is that the concentrations of statins used in the cytotoxicity experiments were higher than those reported in the *in vivo* situation. Previous study has shown that a single oral dose of simvastatin lactone of 40 mg in healthy Caucasian volunteers produces a maximum plasma concentration (C_{max}) of 21-31 nM of simvastatin lactone and 5-15 nM of the corresponding hydroxy acid form.[249] The C_{max} of pravastatin hydroxy acid in haemodialysis patients was found to be 108 nM following a single 20-mg intravenous dose of pravastatin.[250] Higher concentrations of statins were required to induce cytotoxicity in our *in vitro* study. Possibly, this is linked to the fact that these drugs can induce myotoxicity *in vivo* in only minority of patients. The concentrations of statins used in cytotoxicity studies have been chosen based on a systemic review of literature that described the *in vitro* experiments of statin-induced myotoxicity in which similar or even higher concentrations of statins were used.[38, 71, 72, 198-200] These high ranges have been used to assess the trend of the effect of pH alteration and lipoprotein association on the myotoxic potential of statins.

Attempts were made to understand why undifferentiated C2C12 cells are more sensitive to statin-induced cytotoxicity compared to differentiated cells by examining the expression of a number of drug transporters. The results did

not provided a clear explanation of this phenomenon and further studies are required to clarify the bases of these differences.

Another limitation is that we were unable to determine whether simvastatin is incorporated to the core of plasma lipoproteins or associated with the surface of plasma lipoproteins. In the present study, cultured C2C12 cells that overexpress LPL were used to study the uptake of simvastatin from plasma lipoproteins without using endothelial cell layer. Results obtained using this system might not be strong enough to reflect what would happen *in vivo* since this endothelial cell layer can act as an additional barrier to the transport of free and lipoprotein-bound simvastatin. Further studies are therefore required to elucidate the mechanism of transfer of simvastatin from plasma lipoproteins to skeletal muscle cells.

7.4 Future Work

Exploration of the risk factors of statin-induced myotoxicity should receive substantial attention since the use of these drugs has become much more common in the last few years. At the present time, there is no *in vivo* study that explains the myotoxicity profile of different statins under condition of disturbed blood pH and/or elevated plasma lipoprotein levels. Therefore, the next step should focus on confirming the hypotheses of the roles of acidosis and hyperlipidaemia in the development and potentiation of statin-induced myotoxicity *in vivo*. This requires establishing animal models (probably rats) of statin-induced myotoxicity with low blood pH in order to mimic the clinically encountered cases of acidosis as previously described in the literature.[218, 251-253] In addition, animal models with postprandial and

pathological hyperlipidaemias need to be established in rats in order to examine the effect of elevated plasma lipoprotein levels on occurrence and severity of statin-induced myotoxicity. Based on our *in vitro* results, the effect of acidosis and hyperlipidaemia on statin-induced muscle toxicity is particularly important in case of lipophilic statins. Therefore, future *in vivo* studies should be focused on using lipophilic statins, such as simvastatin, lovastatin or atorvastatin.

The objectives of these *in vivo* studies would cover the changes in pharmacokinetic behaviour, biodistribution, tissue uptake and toxicity profiles of lipophilic statins in response to plasma pH alteration and/or hyperlipidaemia. The inter-conversion of statins between the lactone and hydroxy acid forms would also be examined in the plasma of the animal models since this inter-conversion has been shown, *in vitro,* to have a major impact on the cytotoxicity of statins. Short-term *in vivo* studies would be used to examine the effect of comorbidities on pharmacokinetics and tissue distribution of lipophilic statins. The effects of acid-base imbalance and hyperlipidaemia on the toxicity profile of statins will be studies using animals with long-standing induced comorbidities.

To elucidate the effects of the acidosis and hyperlipidaemia on the pharmacokinetics and tissue distribution of lipophilic statins, acute acidosis, and acute hyperlipidaemia will be induced in experimental animals. Acute acidosis will be induced in the animals by oral gavage of ammonium chloride, as previously widely described in the literature.[252, 253] Control animals will receive sodium chloride by oral gavage. On the other hand, acute hyperlipidaemia will be induced by oral gavage of long chain triglycerides

(sesame oil) three hours prior to the administration of statins. Control animals will receive oral gavages of water. Following induction of acute acidosis or hyperlipidaemia, simvastatin will be administered by single oral gavage or IV bolus. In pharmacokinetic studies, blood samples will be withdrawn at predetermined time points from the jugular vein cannula installed under general anaesthesia two days before the experiment. In biodistribution studies, animals will be sacrificed at predetermined time points, and biceps femoris muscle, liver, and other tissues will be obtained.

For elucidation of the role of acidosis, hyperlipidaemia or a combination of acidosis with hyperlipidemia in the development of statin-associated muscle toxicity, animals will be induced to develop chronic acidosis and/or chronic hyperlipidaemia. Simvastatin will be formulated as a suspension in water containing 0.5% w/v hydroxypropyl methylcellulose and 0.1% w/v polysorbate 80, and will be administered to rats by oral gavage (88 mg/kg) once daily for up to 12 days as previously described.[218, 251] Chronic acidosis will be induced by adding ammonium chloride to drinking water, as previously described.[254] Chronic hyperlipidaemia will be induced by animals feeding with high-fat diet starting one week before the first administration of simvastatin, as previously described.[223] Control animals will receive normal diet and drinking water. Rats will be sacrificed on days 8 and 12 following initiation of treatment, blood samples and skeletal muscle tissue of the biceps femoris muscle will be harvested, as previously described.[218]

The outcomes will be measured by determining concentrations of lactone and hydroxy acid forms of statins in plasma and tissues of rats. The concentrations will be then subjected to pharmacokinetic modelling and

simulation using software such as Phoenix WinNonlin. Advanced physiology-based PK/PD models will be used to correlate the co-morbidities and the progression of muscle toxicity with concentrations of statins obtained in plasma and tissues. The acidosis will be monitored by measuring blood and tissue pH using pH microelectrode. Hyperlipidaemia development will be monitored by measuring plasma triglyceride, total cholesterol, and LDL-cholesterol levels using commercially available kits. Muscle toxicity will be assessed by multiple markers that have been recently validated in a rat model of statin-induced myopathy.[218, 251] These markers include plasma CK levels, rat muscle atrophy F-box (MAFbx) mRNA, Cathepsin-L mRNA, light chain 3 (LC3) mRNA, rat muscle RING finger 1 (Murf) mRNA, pyruvate dehydrogenase kinase isozyme 4 (PDK4) mRNA, CK mRNA, as well as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) mRNA.

Several challenges are expected to be encountered while performing such *in vivo* studies. These might include the extremely low statin concentrations in the plasma and other tissues as a result of extensive hepatic first-pass metabolism and intravascular chemical conversion. This might result in statin concentrations below the lower limit of quantification of the applied analytical methods making the detection of the intracellular concentration of statins exceptionally difficult. Therefore, all drug concentrations should be analysed using LC-MS/MS system.

The ability to reduce animals' suffering while receiving myotoxic doses of statin in the presence of concomitant pathologies (acidosis and/or hyperlipidaemia) is another challenging process. Close monitoring is required

to ensure that the animals are not exceeding the moderate severity limit, especially with long-term studies.

Our findings also call for clinical researchers to further explore the contribution of the suggested risk factors in the development and potentiation of statin-induced myotoxicity in human. Clinical studies require selecting patients with hyperlipidaemia alone or together with acidosis/alkalosis while using statins. These patients groups might be encountered in the intensive care units and renal dialysis units. Both prospective and retrospective studies can be performed using non-invasive procedures. Conducting these studies requires obtaining full medical histories from the patients, carrying out detailed physical examinations by an authorized specialist and obtaining blood samples. Blood samples will be used to determine plasma statin concentrations (both hydroxy acid and lactone forms) and measure the proportion of statins that are associated with plasma lipoproteins in the presence of the above-mentioned comorbidities. Total plasma statin concentrations and the percentage associated with plasma lipoproteins will be used to explore the correlation between statin use and the incidence and severity of statin-induced myotoxicity in this patients group. Patients who develop myotoxicity from lipophilic statins will be switched to hydrophilic statins and the progression of the muscle symptoms can be followed prospectively.

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8 Appendix

8.1 Conversion of Pravastatin Hydroxy Acid in Simulated Gastric Fluid

The conversion of pravastatin hydroxy acid was also studied in acidic medium of the stomach using fasted state simulated gastric fluid (FSSGF). This fluid consisted of sodium taurocholate, lecithin and sodium chloride at final concentrations of 160 µmol/L, 40 µmol/L, and 68.4 mmol/L, respectively. The pH of FSSGF was adjusted to 1.6 using 0.1 N HCl. Pravastatin hydroxy acid was incubated with FSSGF at a concentration of 50 µg/mL and the mixture was shaken for 5 hours at 37°C, 250 rpm using Thermo Scientific MaxQ^{TM} 4000 orbital shaker. Samples of 100 µL volume were withdrawn from the reaction mixture at 30 minutes, 1, 2, 3, 4, 5 hours, and transferred into new tubes containing 50 µL ammonium acetate buffer (100 mmol/L, pH 4.5) to terminate the reaction. Vortex-mixing of 30 seconds was applied to each sample and then the samples were subjected to sample preparation for HPLC analysis using the method described in Section 2.2.

Results presented in Figure 8.1 shows that pravastatin hydroxy acid is susceptible to acid catalysed (nonenzymatic) conversion in the FSSGF. This acidic media of the stomach promotes the lactonisation and isomerisation of pravastatin hydroxy acid to pravastatin lactone and 3′ α-hydroxy pravastatin lactone, respectively. Approximately 15% of the administered dose is expected to convert to the more lipophilic lactone form by the time the drug transit the stomach. It is important to mention that 3′ α-hydroxy pravastatin lactone was not identified directly (using external analytical standard) but rather recognised based on previous literature.[255,256] Our results are in agreement with a recent study by Haandel *et al*., 2016 who reported variation in systemic exposure to

pravastatin hydroxy acid and its metabolites (pravastatin lactone and 3′ α-hydroxy pravastatin lactone) among paediatric patients following oral administration of pravastatin sodium. They attributed this variation to inter-individual variability in gastric pH in these patients.[255] The pre-systemic conversion of statins that administered in the hydroxy acid form in the stomach is expected to contribute to their muscle toxicity. This assumption is supported by the finding of Hermann *et al*., 2006 who reported an increase in systemic exposure to atorvastatin lactone metabolite by 2.4 fold in patients with atorvastatin-induced myopathy following daily administration of 10 mg atorvastatin hydroxy acid over a period of one week.[205]

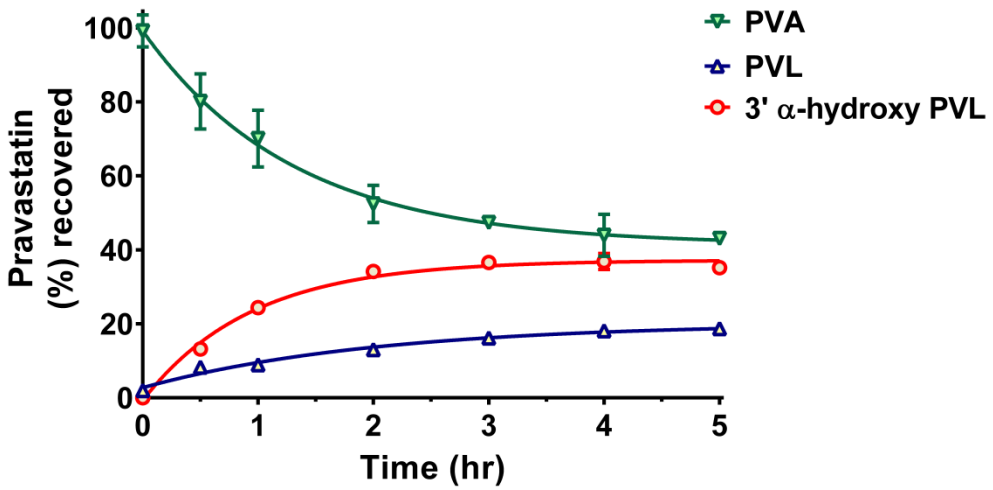


Figure 8.1: Conversion of pravastatin hydroxy acid (50 µg/mL) in simulated gastric fluid of pH 1.6. Green line demonstrates the disappearance of pravastatin hydroxy acid while the blue and red lines represent the formation of pravastatin lactone and its isomer 3′ α-hydroxy pravastatin lactone, respectively. Data are expressed as mean ± SD, (n = 3).

8.2 Inter-conversion of Statins in PBS, Human Plasma, and DMEM Culture Medium

The present section demonstrates the results of statin inter-conversion between the lactone and hydroxy acid form in PBS, human plasma, and cell culture medium at concentrations of 12.5, 25, and 100 µmol/L for simvastatin lactone and simvastatin hydroxy acid and 25, 100, and 200 µmol/L for pravastatin lactone and pravastatin hydroxy acid (Figures 8.2 – 8.7). These results are identical with those obtained in Section 4.3.1 in which statin inter-conversions were tested at a concentration of 50 µmol/L. Results demonstrated a pH dependency in the conversion of simvastatin and pravastatin from the lactone form to the corresponding hydroxy acid. Physiological and alkaline pH markedly enhances the conversion of the lactone form to the active hydroxy acid form whereas acidic pH significantly retards the conversion resulting in greater proportion of statins remaining in the lipophilic lactone form. On the other hand, pH alteration has not been shown to affect the conversion of the hydroxy acid form to the corresponding lactone form. These data also indicate that different concentrations of statins used in these studies have no effect on the rate and extent of statin inter-conversion between the lactone and the hydroxy acid forms.

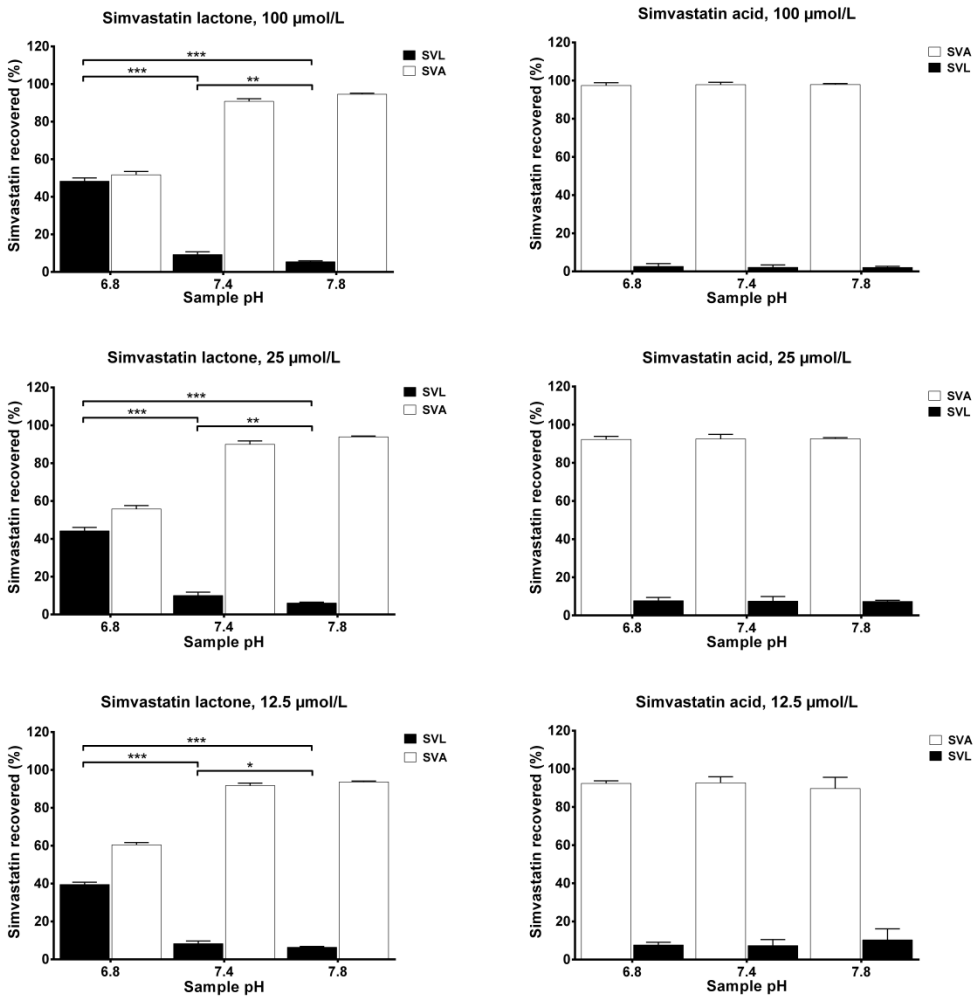


Figure 8.2: Inter-conversion of simvastatin between lactone and hydroxy acid forms in PBS of different pH levels. Simvastatin lactone and simvastatin hydroxy acid were incubated with PBS of different pH levels (6.8–7.8) at concentrations of 100, 25 and 12.5 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid.

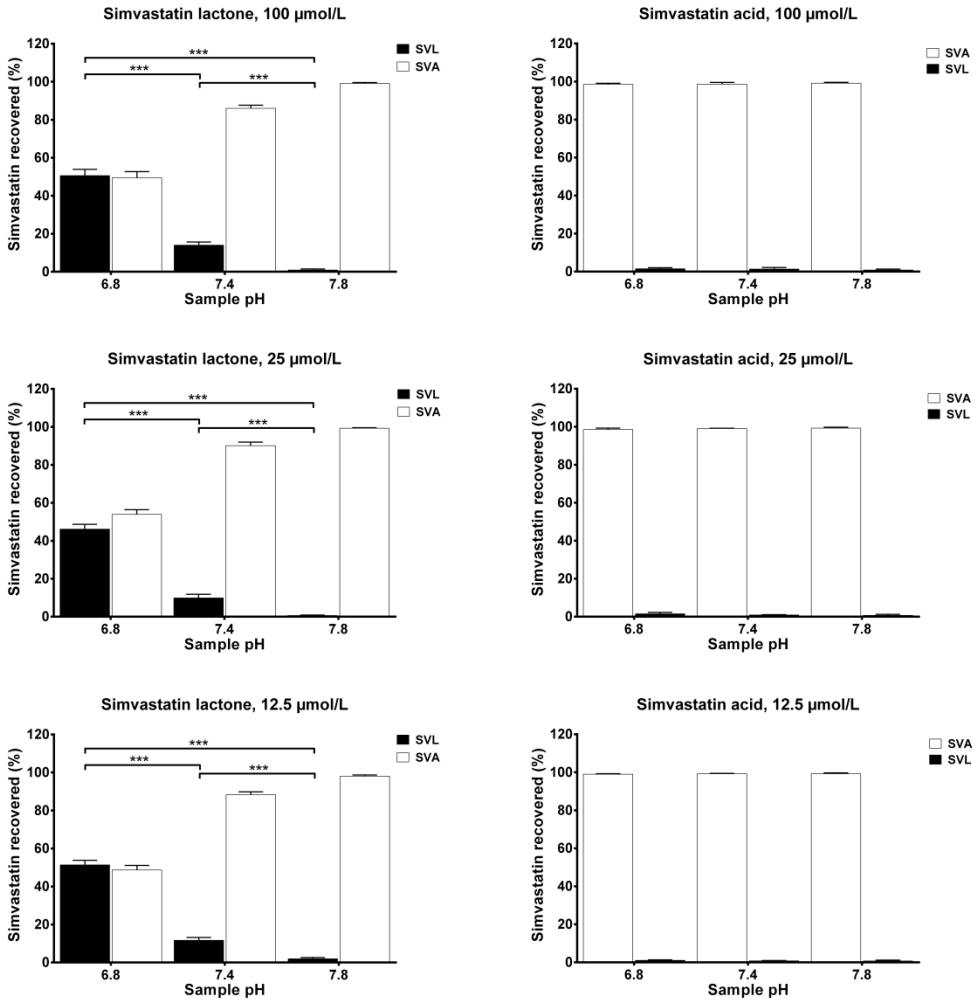


Figure 8.3: Inter-conversion of simvastatin between lactone and hydroxy acid forms in human plasma of different pH levels. Simvastatin lactone and simvastatin hydroxy acid were incubated with human plasma of different pH levels (6.8–7.8) at concentrations of 100, 25 and 12.5 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p < 0.001$). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid.

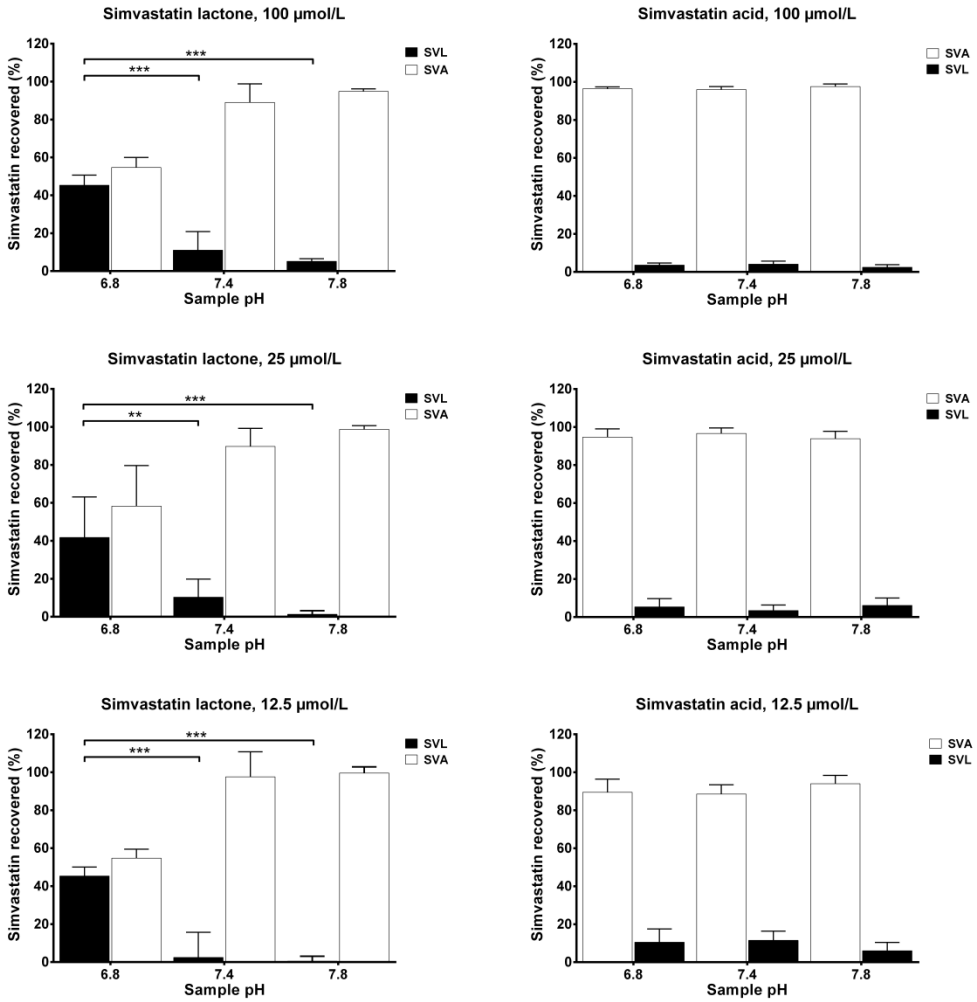


Figure 8.4: Inter-conversion of simvastatin between lactone and hydroxy acid forms in DMEM of different pH levels. Simvastatin lactone and simvastatin hydroxy acid were incubated with DMEM of different pH levels (6.8–7.8) at concentrations of 100, 25 and 12.5 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). SVL, simvastatin lactone; SVA, simvastatin hydroxy acid.

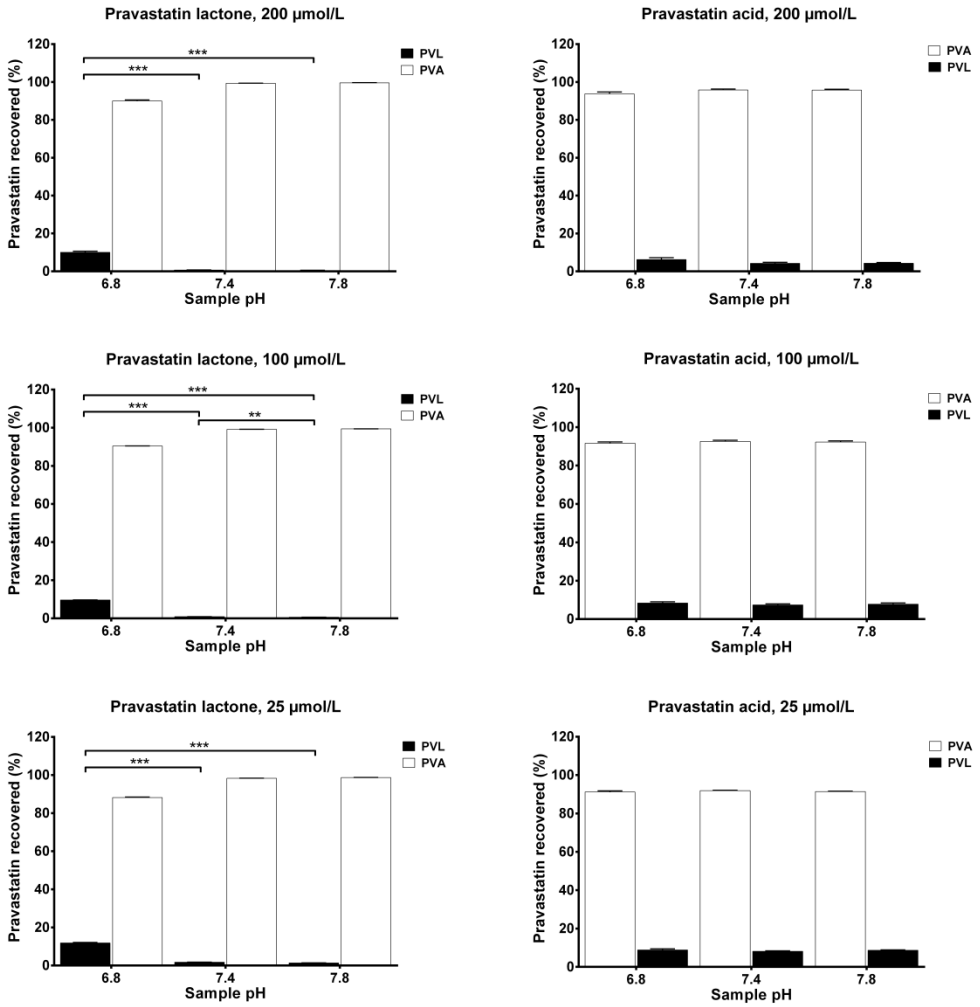


Figure 8.5: Inter-conversion of pravastatin between lactone and hydroxy acid forms in PBS of different pH levels. Pravastatin lactone and pravastatin hydroxy acid were incubated with PBS of different pH levels (6.8–7.8) at concentrations of 200, 50 and 25 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.

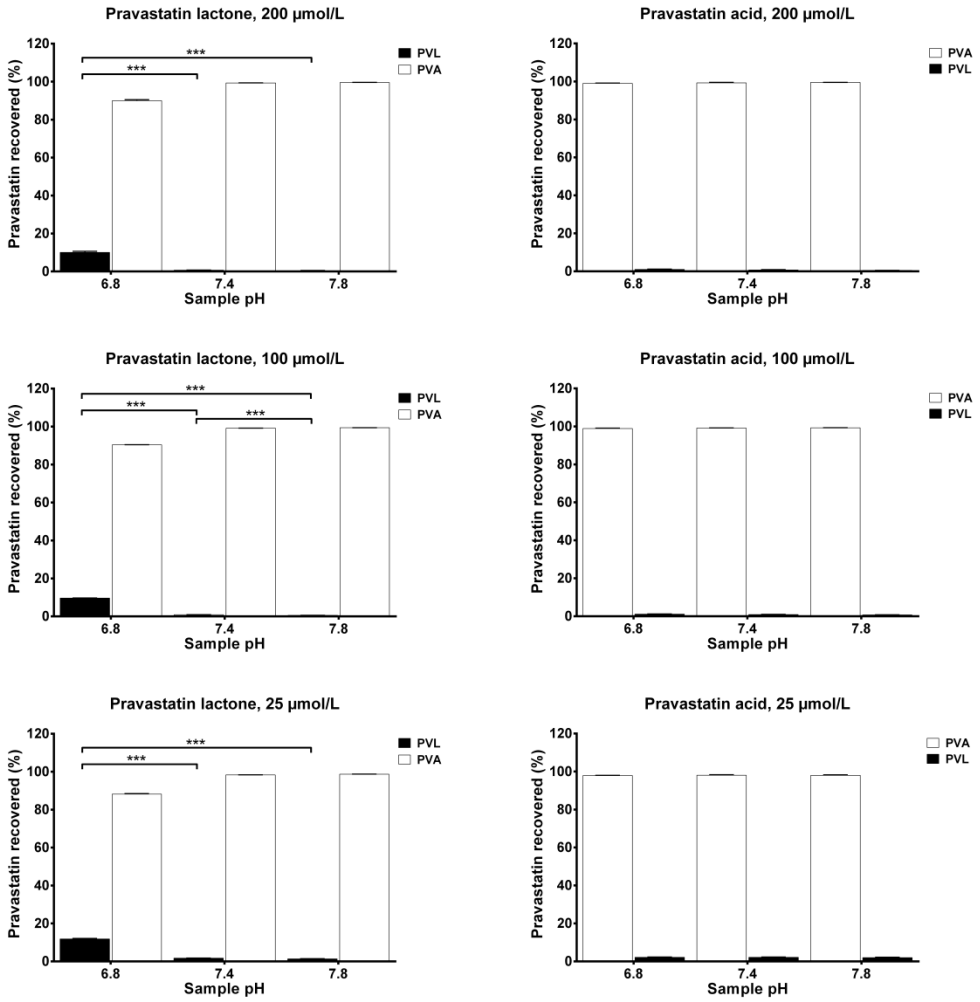


Figure 8.6: Inter-conversion of pravastatin between lactone and hydroxy acid forms in human plasma of different pH levels. Pravastatin lactone and pravastatin hydroxy acid were incubated with human plasma of different pH levels (6.8–7.8) at concentrations of 200, 100 and 25 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p < 0.001$). PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.

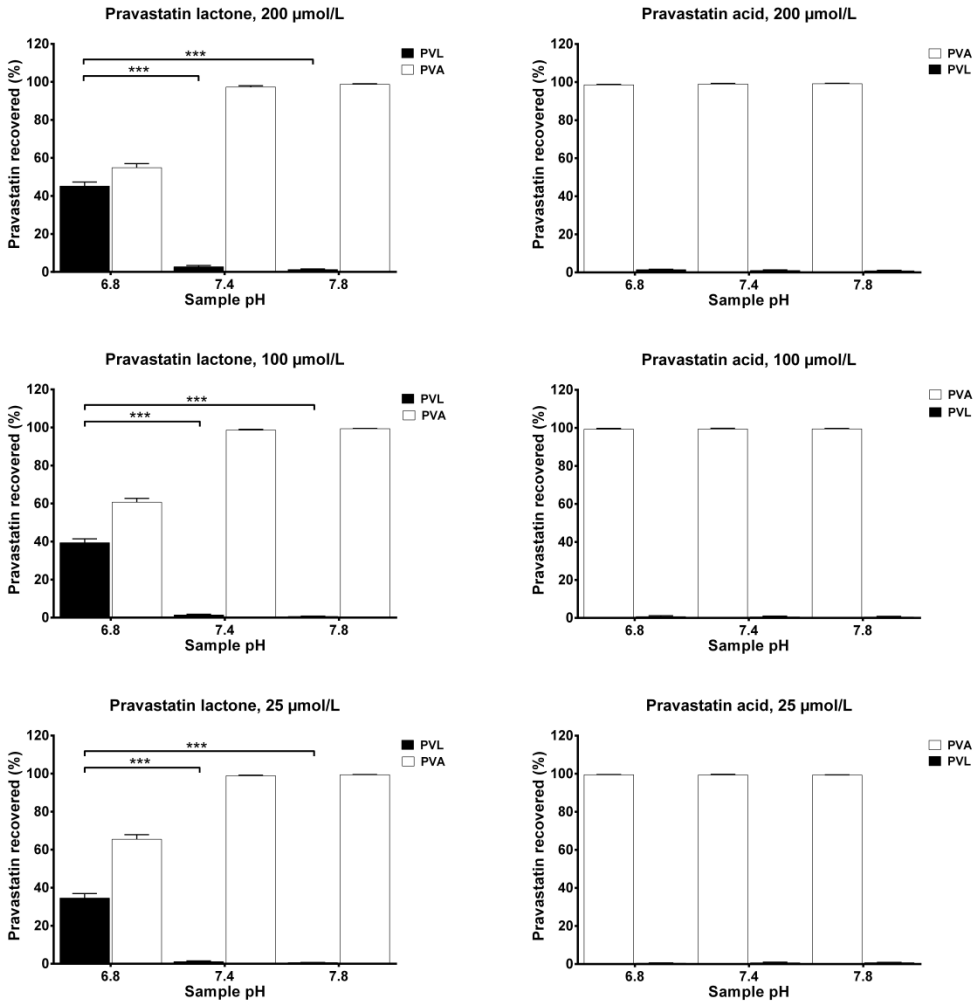


Figure 8.7: Inter-conversion of pravastatin between lactone and hydroxy acid forms in DMEM of different pH levels. Pravastatin lactone and pravastatin hydroxy acid were incubated with DMEM of different pH levels (6.8–7.8) at concentrations of 200, 100 and 25 µmol/L for 24 hours at 37°C. The percentages of the lactone and hydroxy acid forms recovered after 24 hours of incubation were determined by HPLC-UV system and the results are expressed as mean ± SD, (n = 6). Differences between samples of different pH were analysed by one-way ANOVA followed by Tukey's post hoc test (*** $p <$ 0.001). PVL, pravastatin lactone; PVA, pravastatin hydroxy acid.