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**MODEL-BASED APPROACHES TO CHARACTERIZE CLINICAL
PHARMACOKINETICS OF ATORVASTATIN AND ROSUVASTATIN
IN DISEASE STATE**

BY

JOYCE S. MACWAN

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOMEDICAL AND PHARMACEUTICAL SCIENCES**

UNIVERSITY OF RHODE ISLAND

2013

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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2013

ABSTRACT

Cardiovascular disorders are the leading cause of death in the United States. Members of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) class of lipid-lowering drugs are used worldwide for the prevention and treatment of cardiovascular disorders. Cardiovascular disorders are the primary cause of morbidity and mortality in patients with diabetes mellitus and obesity as well as renal and liver transplantation. The rising global burden of these chronic disorders has resulted in long-term use of statins to prevent and treat cardiovascular disorders in diverse patient populations. Several statins are commercially available; however, atorvastatin calcium (Lipitor®, Pfizer Pharmaceuticals, NY) is the world's top selling medication of all time; whereas, rosuvastatin calcium (Crestor®, AstraZeneca, DE) is the most efficacious member of statin family.

Although, statins are well-tolerated, approximately 7% of patients on statin therapy experience myotoxicity, which is ranging from a mild condition called myalgia to a rare but potentially fatal rhabdomyolysis requiring hospitalization. A meta analysis study reported by the United States FDA indicated three times higher incidence of rhabdomyolysis in patients with diabetes mellitus. Previously published *in vitro* and clinical studies identified the role of lactone metabolites in myopathy. Our group found significantly elevated plasma concentrations of atorvastatin lactone metabolites in the stable kidney transplant recipients with diabetes mellitus. Our study indicated that reduced clearance of lactone could be attributed to decreased activity of cytochrome P450 (CYP) 3A4, which is the main drug metabolizing enzyme. Prior

studies assessed the effect of genetic polymorphism in drug metabolizing enzymes and transporters on pharmacokinetics and toxicological properties of parent drug and lactone metabolite in healthy Finish and Korean populations.

Currently, limited information is available on the effect of concurrent diseases and genetic polymorphisms of drug metabolizing enzymes and transporters on pharmacokinetics of acid and lactone forms of atorvastatin. Altered pharmacokinetics of atorvastatin acid or lactone in concomitant diseases possibly influences the clinical outcome, resulting in unfavorable benefit/risk ratio. In this study, we have assessed the impact of inherent demographic characteristics in conjunction with coexisting diseases and genetic polymorphisms using a population pharmacokinetic analysis utilizing a nonlinear mixed effect model to identify potential covariates that explain the variability in pharmacokinetic properties of acid and lactone forms of atorvastatin.

A physiologically-based pharmacokinetic modeling approach was used for the prediction of pharmacokinetics of orally administered atorvastatin acid and rosuvastatin acid along with their major metabolites from *in vitro* data allowing mechanistic characterization of the observed concentration-time profile. A mechanistic modeling is needed to provide insights into the interplay of various phenomena involved in oral absorption and metabolism of drugs. Additionally, simulations through virtual patients using physiologically-based pharmacokinetic modeling will allow to design a population pharmacokinetic study and to determine significant covariates.

Manuscript I of this dissertation provides detailed information of pharmacokinetic and pharmacological properties of atorvastatin and rosuvastatin acid. Moreover, it describes the effect of various factors such as age, gender, race, food, liver and kidney diseases, time of drug administration, and genetic polymorphism of drug metabolizing enzymes and transporters on the pharmacokinetic properties of both statins. Atorvastatin acid is significantly metabolized by CYP3A4, and it is more likely to cause clinically important drug-drug interaction. Previously reported drug-drug interactions of atorvastatin are discussed in this manuscript. This manuscript will be submitted for publication to “Clinical Pharmacokinetics” as a review article.

Due to lack of simple and sensitive methods for simultaneous quantification of atorvastatin and its five metabolites in human plasma, a liquid chromatography tandem-mass spectrometry (LC-MS/MS) bioanalytical method was developed.

Manuscript II explains in detail about the development and validation of a sensitive, selective and simple LC-MS/MS assay for simultaneous quantitative determination of parent drug and its five metabolites (*published in Anal Bioanal Chem.* 2011 Apr;400(2):423-33).

Esterase activity is a key reason of instability of ester-containing drugs in biological matrices. The effect of several anticoagulants on lactone to acid interconversion was investigated by comparing different types of plasma (sodium heparin, K₂EDTA and sodium fluoride/potassium oxalate) and serum. No, statistically significant difference was found between serum and plasma with various anticoagulants; however, sodium fluoride (esterase inhibitor) plasma was preferred to ensure stability of lactone upon

long-term storage of clinical study samples. Comprehensive stability studies were conducted prior to the method validation to establish stability conditions for unstable lactone analytes during the extraction steps as well as storage duration of clinical samples. The method was validated according to recent FDA guidelines. The post-column infusion test was performed to assess matrix effect. *Ortho- and para*-hydroxy analytes have similar precursor ion-product ion transitions. Additionally, the acid form could possibly undergo in-source fragmentation and, following the loss of water, the resulting product would interfere with their respective lactone forms. For these combined reasons, chromatographic conditions that would assure baseline chromatographic separation of the respective analytes were determined. This goal was achieved using a narrow-bore Zorbax-SB phenyl column. Because this column provided excellent peak focusing a high S/N was obtained that enabled us to use a simple protein precipitation extraction procedure without performing pre-concentration steps for sample clean up. The extraction method contained a simple protein precipitation step requiring only 50 μ L of plasma and achieved a lower limit of quantification of 50 pg/mL for all six analytes.

To the best of our knowledge, no published bioanalytical method has described a fully validated LC-MS/MS assay for the quantification of rosuvastatin lactone metabolite in human plasma. A sensitive and simple LC-MS/MS assay was developed for the simultaneous quantification of parent drug and its two metabolites in human plasma. **Manuscript III** describes the development and validation of an LC-MS/MS method for the simultaneous quantification of rosuvastatin acid and its two metabolites; N-

desmethyl rosuvastatin and rosuvastatin lactone (*published in Anal Bioanal Chem.* 2012 Jan; 402(3):1217-27).

Like atorvastatin lactone, rosuvastatin lactone is also very unstable. Stability of all the three analytes was tested in various conditions such as non-buffered human plasma and buffered human plasma (human plasma diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer). For the proposed assay, plasma was diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer due to prevent the loss of lactone form (25%) that occurred in non-buffered plasma after 1 month storage at -80 °C. Furthermore, to ensure stability of rosuvastatin lactone metabolite during extraction of samples, 0.1% v/v glacial acetic acid in methanol was used as precipitating agent to minimize the interconversion of lactone to acid and vice versa. With the use of narrow-bore Zorbax-SB Phenyl column, lower limit of quantification of 0.1 ng/mL for acid and lactone forms of rosuvastatin, and 0.5 ng/mL for N-desmethyl rosuvastatin acid were achieved using 50 µL of buffered human plasma.

The impact of concurrent chronic disorders and polymorphisms in genes coding for drug metabolizing enzymes and transporters involved in the parent drug and the major metabolite elimination were investigated through a population pharmacokinetic modeling approach using NONMEM software (**Manuscript IV**). The plasma concentrations of parent drug and metabolite of one hundred and thirty two, male (n=77) or female (n=55) non-transplant (diabetic, n=46; non-diabetic, n=53) or the stable kidney transplant (diabetic, n=22; non-diabetic, n=11) recipients receiving a single oral dose or multiple oral doses of Lipitor (atorvastatin calcium) were included

in the study. The study samples were analyzed using an LC-MS/MS method described in Manuscript II.

A complex parent-metabolite combined population pharmacokinetic model was developed by analyzing a total of 639 concentrations including both acid (n=322) and lactone (n=317) forms of atorvastatin through a nonlinear mixed-effects modeling approach to identify and interpret the genetic, demographic, physiological and pathological factors that significantly affect the pharmacokinetic properties of atorvastatin acid and its major metabolite. Concentration-time profiles of atorvastatin acid and lactone metabolite were adequately described respectively, using a two-compartment model with first-order oral absorption and a one-compartment model with linear elimination, with some degree of interconversion between the two forms. Covariate model building was conducted to investigate and determine sources of variability (covariates) that elucidate differences in pharmacokinetic parameters between patients, through univariate analysis followed by stepwise forward addition and backward elimination. Covariate analysis identified the kidney transplantation status and lactate dehydrogenase (liver enzyme) as significant covariates affecting the apparent clearance of atorvastatin lactone metabolite. Renal transplant recipients had 50% lower metabolite clearance than non-transplant patients. However, polymorphisms in genes coding for enzymes and transporters as well as biomarkers of diabetes such as HbA1c and serum glucose levels did not significantly affect the pharmacokinetics of either parent drug or metabolite. The final model was validated using a visual predictive check method and nonparametric bootstrap analysis, which guaranteed robustness of the present population pharmacokinetic model. The finding

of the study indicated the need of careful monitoring while prescribing atorvastatin treatment to the kidney transplant population. This manuscript will be submitted for publication to “Clinical Pharmacology and Therapeutics” as a research article.

Manuscript V (to be submitted to “Molecular Pharmaceutics”) presents a whole-body physiologically-based pharmacokinetic (PBPK) model for atorvastatin and rosuvastatin acid with their respective metabolites allowing a mechanistic characterization of observed plasma concentration-time profiles of parent drug and its metabolites. Plasma samples obtained from the stable kidney transplant recipients with diabetes mellitus were analyzed using LC-MS/MS methods described in Manuscripts II and III.

The GastroPlus (Simulations Plus, Inc., USA) advanced compartmental absorption and transit (ACAT) model, generic PBPK module and population estimates for age-related physiology feature were used to predict systemic exposure of both statins following an oral administration in stable kidney transplant recipients with diabetes mellitus. The required number of input parameters was obtained experimentally, *in silico* and from the literature. Atorvastatin acid undergoes extensive gut and hepatic metabolism mainly by CYP3A4. *In vitro* K_m and V_{max} values of metabolic clearance of atorvastatin acid previously determined in our laboratory using diabetic human liver microsomal fractions were implemented in the model. The model used a built-in utility for conversion of *in vitro* K_m and V_{max} values to *in vivo* values. To predict observed large volume of distribution of both statins, the Berezhkovskiy algorithm was utilized to determine tissue distribution of perfusion-limited tissues. The observed mean

plasma concentration-time curves for both statins and their metabolites were adequately described by the proposed PBPK model. A parameter sensitivity analysis identified that systemic exposure of both statins is significantly affected by changes in intestinal transit time. Part of the validation process included virtual trial simulations, which allowed incorporation of inter-subject variability. The virtual trial simulation results showed that the observed mean plasma concentration-time curves of both statins lay between 90% confidence interval of simulated concentrations of ten virtual patients. The present whole-body PBPK model demonstrated that *in vitro* metabolic clearance data generated from a specific disease tissue were superior for adequate prediction of systemic exposure of an extensively metabolized drug that might have modified due to altered activity of drug metabolizing enzyme in a specific disease state

In summary, the work presented in this dissertation evaluate the effect of preexisting disease states including diabetes mellitus and renal transplant as well as genetic polymorphisms in drug metabolizing enzymes and transporters that predominantly contribute to overall disposition of atorvastatin acid and its lactone metabolite. The study indicated that the clearance of lactone, a myotoxic metabolite of atorvastatin acid, is significantly decreased by 50% in the stable kidney transplant recipients. However, the unbalanced sample size in this study restricts to delineate the individual influence of renal transplant and diabetes mellitus on clearance of parent drug and its metabolite. In addition, for the first time the disposition of atorvastatin acid and rosuvastatin acid as well as their respective metabolites was characterized using a mechanistic modeling approach, to predict observed plasma concentration-time

profiles in the kidney transplant recipients with diabetes mellitus. The PBPK model that integrated *in vitro* kinetic parameters for metabolic clearance of atorvastatin acid measured in human liver microsomal fractions of diabetic livers were superior for the prediction of observed plasma concentration-time curve of atorvastatin acid.

Overall, this study demonstrated a significant reduction of clearance of atorvastatin lactone metabolite in patients with the kidney transplant and thus they might be at higher risk of developing myotoxicity. This finding indicated the need of careful monitoring of atorvastatin acid therapy in the kidney transplant recipients who are on multiple medications and also have lifetime co-morbidities. Moreover, mechanistic modeling suggested that the systemic exposure of both statins is very sensitive to change in intestinal transit time. It would be beneficial to study the activity of intestinal drug metabolizing enzymes and transporters in diabetes mellitus and its impact on pharmacokinetics of statins.

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DEDICATION

To My Family

PREFACE

This dissertation was prepared according to the University of Rhode Island ‘Guidelines for the Format of Theses and Dissertations’ standards for Manuscript format. This dissertation consists of five manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT I: Clinical Pharmacokinetics of Atorvastatin and Rosuvastatin

This manuscript has been prepared for publication and will be submitted to “Clinical Pharmacokinetics” as a review article.

MANUSCRIPT II: Development and Validation of a Sensitive, Simple and Rapid Method for Simultaneous Quantitation of Atorvastatin and its Acid and Lactone Metabolites by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

This manuscript has been published in a peer-reviewed journal “Analytical and Bioanalytical Chemistry”, April 2011.

MANUSCRIPT III: A Simple Assay for Simultaneous Determination of Rosuvastatin acid, Rosuvastatin-5s-lactone and N-desmethyl rosuvastatin in Human Plasma using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

This manuscript has been published in a peer-reviewed journal “Analytical and Bioanalytical Chemistry”, January 2012.

MANUSCRIPT IV: Development of a Combined Parent-Metabolite Population Pharmacokinetic Model for Atorvastatin Acid and its Lactone Metabolite: Implication of Renal Transplantation

This manuscript has been prepared for publication and will be submitted to “Clinical Pharmacokinetics” as a research article.

MANUSCRIPT V: Development of Physiologically-Based Pharmacokinetic Model for Atorvastatin Acid and Rosuvastatin Acid with their Metabolites: *In vitro and in vivo studies*

This manuscript has been prepared for publication and will be submitted to “Molecular Pharmaceutics” as a research article.

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MANUSCRIPT I

To be submitted as review article to Clinical Pharmacokinetics

Clinical Pharmacokinetics of Atorvastatin and Rosuvastatin

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Running title: Pharmacokinetic properties of atorvastatin and rosuvastatin acid

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Keywords: | atorvastatin | rosuvastatin | statins | pharmacokinetics | genetic polymorphism | drug interaction | lactone | metabolite

Abbreviations: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), Area under the plasma-concentration time curve (AUC), Atorvastatin acid (ATV), Atorvastatin lactone (ATV-LAC), Breast cancer resistant protein (BCRP), Cytochrome P450 (CYP), Low density lipoprotein-cholesterol (LDL-C), Maximum plasma concentration ($C_{\max}$), Organic anion-transporting polypeptide (OATP), Paraoxonases (PON), P-glycoprotein (P-gp), Pharmacokinetics (PK), Rosuvastatin acid (RST), Rosuvastatin-5S-lactone (RST-LAC), Time to reach maximum plasma concentration ($T_{\max}$), Single nucleotide polymorphism (SNP), UDP-glucuronosyltransferase (UGT)

Abstract

Dyslipidemia is the main risk factor for the development of atherosclerosis and coronary artery diseases. Cardiovascular disorders are the first leading cause of death in the United States and a major cause of morbidity and mortality in patients with diabetes mellitus, obesity, renal or liver transplantation. 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) class of lipid-lowering drugs are the first choice for prevention and treatment of cardiovascular diseases. The increasing global burden of heart diseases resulted in extensive use of statin therapy in a diverse patient population. Statin-associated skeletal muscle toxicity is the most common side effect with statin therapy that sometimes results in life threatening rhabdomyolysis.

Among commercially available statins, worldwide, atorvastatin acid is the top-selling prescribed medication; however, rosuvastatin acid is considered the most efficacious statin. The present, review article summarizes the clinical pharmacokinetics properties of two widely prescribed antihyperlipidemic agents, atorvastatin and rosuvastatin acid.

Atorvastatin acid is a biopharmaceutics classification system (BCS) and biopharmaceutical drug disposition classification system (BDDCS) class II drug and, therefore, it is highly soluble and permeable and exhibits complete absorption following an oral dosing. It is >98% bound to plasma proteins and display extensive peripheral tissue distribution. Significant metabolism, both in the gut and liver, primarily by cytochrome P450 (CYP) 3A4, plays a key role in the first-pass effect that explains its remarkably low oral bioavailability (14%). It is also biotransformed into glucuronide metabolites mediated by uridinediphosphoglucuronyl-transferase (UGT) enzymes and

undergoes lactonization. The parent drug and its metabolites are mainly excreted into bile. The drug is a substrate of several hepatic uptake transporters of organic anion-transporting polypeptide family. Moreover, it is believed to be a substrate of efflux transporters including p-glycoprotein (MDR1) and breast cancer resistant protein (BCRP), which may govern intestinal absorption and biliary excretion. Single nucleotide polymorphism of drug metabolizing enzymes and transporters modulates pharmacokinetics and toxicological properties of the parent drug and metabolites. It is more likely to cause CYP 3A4 mediated clinically relevant drug-drug interaction.

Rosuvastatin acid, a BCS and BDDCS class III drug is highly soluble and less permeable and, therefore, demonstrate selective hepatic uptake. It is one of the most effective statins due to its unique characteristics such as superior binding affinity, and tight binding interactions at the enzyme active site. It is highly bound to plasma proteins mainly to albumin. It undergoes minimal metabolism and hence eliminated unchanged in the feces. Moreover, multiple hepatic organic anion-transporting polypeptide (OATP) uptake and BCRP efflux transporters significantly contribute to hepatobiliary disposition of rosuvastatin. Genetic polymorphisms in these transport proteins may significantly alter systemic exposure of rosuvastatin acid. Because of insignificant metabolism, it is less likely to cause clinically important metabolic drug-drug interactions.

Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels. The epidemic of diabetes has increased because of obesity and lifestyle changes. In 2010, diabetes mellitus was ranked as the seventh leading cause of death in the United States [1]. Moreover, worldwide the number of people with diabetes is projected to increase to 366 million by 2030 in all-age groups [2].

Diabetes mellitus is the primary risk factor for coronary artery disease and stroke [3], which are ranked as the leading causes of death in the United States [4, 5]. An update to the guidelines of Adult Treatment Panel III, issued by the National Cholesterol Education Program, indicates that a larger number of diabetic patients than non-diabetic individuals were administered 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) for the treatment of hypercholesterolemia [6]. Several statins including simvastatin, atorvastatin acid (ATV), rosuvastatin acid (RST) and pravastatin acid are the commonly prescribed lipid-lowering agents. Atorvastatin calcium (Lipitor®, Pfizer Pharmaceuticals, NY, USA) is the world's best selling medication of all time [1]. A clinical trial conducted in patients with type 2 diabetes mellitus concluded that ATV is effective in the primary prevention of serious cardiovascular events including stroke, irrespective of low density lipoprotein-cholesterol (LDL-C) level before treatment [7] ; therefore, ATV is widely prescribed in the diabetic population [6].

Atorvastatin is extensively metabolized by cytochrome P450 (CYP) 3A4/5 and biotransformed into pharmacologically active *ortho*- and *para*- hydroxylated metabolites [8] that remain in equilibrium with their respective pharmacologically inactive lactone

forms [9], which have 83-fold higher affinity for CYP3A4 [8, 10]. Although, statins are well-tolerated, approximately 7% of patients on statins therapy experience mild or severe skeletal muscle complaints. The incidence of statin-associated adverse effects is dependent on the dose or plasma concentration of statins. Moreover, the risk is higher in old age, metabolic disorders, renal and hepatic disorders as well as patients receiving inhibitors of CYP enzymes and/or transporters [11]. Because of elevated incidence of statin-associated rhabdomyolysis, cerivastatin was deliberately withdrawn from the United States market in 2001 [12]. A meta-analysis study conducted by the United States FDA, using data from 252,460 patients treated with lipid-lowering agents found in average 0.44 incidences of rhabdomyolysis per 10,000 person-years with statin monotherapy and the incidence is increased when statins are co-administered with fibrates. Moreover, diabetic patients on statin monotherapy exhibited approximately 2.9 times higher relative risk of rhabdomyolysis requiring hospitalization [13].

Hermann et al. [14] showed significantly higher levels of concentration of atorvastatin lactone metabolite in patients experiencing statin-associated myopathy. Moreover, an *in vitro* study, using primary human skeletal muscle cells, showed that atorvastatin lactone (ATV-LAC) had a 14-fold higher potency to induce myotoxicity as compared to the acid form [15]. The lactone/acid concentration ratio could potentially be used as a specific diagnostic tool for statin-induced muscle toxicity [16]. Recently, *in vitro* study published by our group has shown a significant reduction in the expression and activity of CYP3A4 in human livers from donors with diabetes mellitus [17]. Moreover, CYP3A4 is the main enzyme involved in metabolic clearance of ATV-LAC [17]. The oxidative biotransformation of ATV-LAC is considered to be the primary pathway for ATV

elimination [8]. A previously published clinical study showed significantly lowered clearance of ATV-LAC in stable kidney transplant recipients with diabetes mellitus and indicated clinical significance of this finding while prescribing ATV treatment in this population who have additional co-morbidities and are on multiple medications [17].

Rosuvastatin acid, a synthetic HMG-CoA inhibitor is also widely prescribed medication due to its unique properties including selective liver uptake and increased potency. It exhibits minimal metabolism and thus is less likely to cause CYP3A4 mediated metabolic drug-drug interactions. However, it is a substrate of multiple hepatic OATP uptake transporters and may cause transporter mediated drug-drug interactions with oral antidiabetic drugs [18].

Increased plasma levels of statins and their metabolites may elevate the risk of clinically significant side effects especially in a diabetic population receiving concomitant medications [17]. Therefore, it is essential to better characterize the pharmacokinetics (PK) of the most commonly prescribed statins and the factors that possibly affect PK properties as well as clinically significant drug-drug interactions.

Atorvastatin calcium (Lipitor®)

1.1. Physicochemical properties of atorvastatin calcium

Currently, ATV is used as calcium salt of an active acid. Atorvastatin calcium [(3*R*,5*R*)-7-[3-(phenylcarbamoyl)-5-(4-fluorophenyl)-2-isopropyl-4-phenyl-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium salt], C₆₆H₆₈CaF₂N₄O₁₀ has a molecular weight of 558.62 and pK_a of 4.46. It is white to off-white crystalline powder; soluble in methanol, dimethyl sulfoxide and slightly soluble in water. It is insoluble in aqueous solution of pH 4 and below and it is very slightly soluble in pH 7.4 phosphate buffer and water [19].

1.2. Clinical uses

Atorvastatin acid is a synthetic drug from the most efficient statin class of lipid lowering agents and is widely used to treat dyslipidemia and cardiovascular disorders. Atorvastatin acid blocks cholesterol biosynthesis by reversible, competitive inhibition of the rate-limiting enzyme, HMG-CoA reductase. It is used as a monotherapy and/or fixed dose combination therapy along with exercise and diet to reduce the risk of heart attacks and stroke. It is contraindicated in pregnant and nursing females, and patients with liver diseases [20].

1.3. Mechanism of action

Atorvastatin acid is a competitive inhibitor of HMG-CoA, the main enzyme, catalyzing the rate limiting step of endogenous cholesterol biosynthesis and thus reduces the cholesterol content of the hepatocytes. This results in an up-regulation of LDL-C-receptors, which subsequently increases the uptake and catabolism of LDL-C and lowers

its plasma levels. The pleiotropic effects of ATV include anti-inflammatory actions, reduction of platelet aggregation, improvement of endothelial function and antiproliferative activity on smooth muscle [21].

1.4. Side effects

Skeletal muscle related problems are the common side effects that develop with statin treatment. Myotoxicity ranging from a mild condition called myalgia (muscle ache or weakness without creatine kinase elevation) to rare but potentially fatal rhabdomyolysis (severe muscle breakdown with marked creatine kinase elevation [>10 times above the upper limit of normal] resulting in hospitalization [11]. Liver injury is another serious side effect associated with the use of ATV. Patients are generally monitored especially when they experience skeletal muscle or liver related problems. In this situation, to minimize the risk of side effects, dose adjustment or discontinuation of therapy is initiated by clinicians. The other common side effects of ATV are diarrhea, nausea, runny or stuffy nose, mild sore throat, and stomach upset [20].

1.5. Pharmacokinetics properties

A. Absorption

Oral absorption of a drug reflects the movement of drug from the gastrointestinal tract into the blood stream. It is significantly affected by the physicochemical properties of the drug and formulation including dissolution rate, stability in the gastrointestinal tract and permeability. Moreover, physiological factors including gastric emptying time, peristaltic movement and gastric pH also affect the oral bioavailability of drug. Complete

absorption of ATV is expected due to its high solubility and permeability at intestinal pH (pH=6).

Upon oral administration, ATV undergoes extensive first pass metabolism through the gut wall and liver and exhibits very low absolute bioavailability (14%). The extensive gut wall extraction of ATV is the result of intestinal CYP3A4, UDP-glucuronosyltransferase (UGT) enzymes and P-glycoprotein (P-gp) efflux transporter interplay [22].

Atorvastatin acid reaches maximum plasma concentration ($C_{\max}$) of 3.61 $\mu\text{g/L}$ after 10 mg of an oral dose and time to $C_{\max}$ ($T_{\max}$) is 1.5 hr. It exhibits the linear PK for both $C_{\max}$ and area under plasma-concentration time curve (AUC) over the dose range of 5-40 mg. The net transport of drug across the membrane is governed mainly by intestinal P-gp efflux transporter, the active uptake transporter H^+ monocarboxylic carrier and to a lesser extent by passive diffusion. However, the role of organic anion-transporting polypeptide (OATP) uptake transporters for intestinal absorption is not known yet [22]. A secondary peak in an individual plasma concentration-time profile indicates enterohepatic recirculation of ATV [23].

B. Distribution

The volume of distribution of a drug reflects the extent of extravascular tissue binding. It is affected most importantly by binding affinity of the drug to plasma proteins, blood elements and by permeability into tissue and membrane. The plasma protein binding of ATV is >98%, and its volume of distribution is 381L, which is higher than the total volume of body water indicating extensive tissue binding [22].

C. Metabolism

Atorvastatin acid undergoes complete metabolism and the liver is the major site for its elimination despite the significant first-pass effect through the gut wall. The detailed metabolic pathways for phase I and II are shown in **Figure I-1** and **I-2**, respectively. It is extensively metabolized through Phase I enzymes primarily by CYP3A4/5 in the intestine and liver and it forms pharmacologically active *ortho*- and *para*-hydroxylated metabolites [8]. The parent drug and its active acid metabolites remain in equilibrium with their respective inactive lactone forms [9], which have 83-fold higher affinity for CYP3A4 and exhibit higher metabolic clearance [8, 10]. The results of *in vitro* study conducted using human liver microsomes indicated a predominant role of CYP3A4 in the biotransformation of ATV-LAC [24].

The study published by Prueksaritanont et al. demonstrated the role of phase II enzymes in the clearance of ATV through glucuronidation as a novel route. The formation of ATV-LAC is also attributed to glucuronidation metabolic pathway, which is mediated mainly through UGT1A1 and 1A3 enzymes [25, 26].

Lactones are hydrolyzed either chemically or enzymatically mainly by paraoxonase (PON1 and PON3) enzymes and esterases [27, 28]. Lactonization of an acid form is also mediated by Coenzyme A-dependent pathway through the formation of acyl-CoA thioester [29]. Moreover, glucuronidation and a low pH environment are also responsible for lactonization [26].

Hepatic uptake transporters, OATP1B1 and OATP1B3 play a significant role in the active uptake of ATV and its metabolites. Atorvastatin acid and its metabolites are also

substrates of efflux transporters including P-gp and BCRP, which govern their intestinal absorption and liver elimination [30].

D. Elimination

Biliary route is the major elimination pathway of the parent drug and its metabolites and approximately 1% of the administered dose is excreted through renal route. This indicates that the liver is the primary site for ATV metabolism and elimination. The clearance of ATV is 625 mL/min in human [22].

1.6. Effect of age and gender

The clinical PK of ATV is affected by age and gender; however, dose adjustment is not required. The $C_{\max}$ and AUC of ATV were 42.5% and 27.3 % higher, respectively in an elderly population aged between 66-92 years compared to young individuals (age 19-35 years). Similarly, $C_{\max}$ and AUC of ATV were 17.6 % higher and 11.3% lower respectively, in women and male subjects. The terminal half-life is 36.2% longer, and $T_{\max}$ is 5.5% shorter in elder participants than young subjects. The terminal half-life and $T_{\max}$ were 19.9% and 39.1 % shorter, respectively in women as compared to men. The differences in the PK of ATV could be age-related effects on intestinal and liver enzymatic activity [31].

1.7. Effect of race

No difference in the PK of ATV was observed between Asian and Caucasian population, therefore modifications of dosing recommendations are not required for either race [32].

1.8. Effect of food

In a clinical study assessing the influence of food on the rate and extent of ATV absorption found that $C_{\max}$ and AUC of ATV decreased by 47.9 % and 12.7%, respectively when administered with food. The $T_{\max}$ of ATV increased from 2.6 h to 5.9 h when administered with food. Moreover, the mean elimination half-life decreased from 37.5 h to 32 h with food. Food with medium fat content significantly decreased the rate of absorption ($T_{\max}$) but had a minor effect on the extent of absorption [33]. Therefore, food does not appear to affect ATV therapeutic efficacy.

1.9. Effect of renal and hepatic impairment

Urinary route is a minor route of excretion and thus renal impairment had no effect on the PK of ATV. Moreover, haemodialysis did not have any effect on the PK of ATV. However, $C_{\max}$ and AUC were 5 fold and ≥ 11 fold greater, respectively in patients with Child-Pugh Class A and B liver impairment, respectively. Therefore, dose adjustment is not required in renal impairment, but the dosing schedule should be carefully determined for patients with liver dysfunctions [34].

1.10. Effect of time of administration

The rate and extent of absorption of ATV were lowered when it was administered in the evening as compared to the morning administration to patients with hypercholesterolemia. No difference was noted for mean elimination half-life between times of administration. Moreover, time of administration also had no effect on the efficacy of ATV [23].

1.11. Effect of genetic polymorphism

Genetic polymorphism of various transporters and drug metabolizing enzymes involved in the disposition of ATV significantly affects its PK. The effect of single-nucleotide polymorphism (SNP) of OATP1B1 (*SLCO1B1*), P-gp (*ABCB1*), BCRP (breast cancer resistant protein, *ABCG2*) and UGT1A3 enzyme on the PK of ATV and ATV-LAC are summarized in **Table I-1**. Moreover, a genome-wide association study has provided compelling evidence for strong association of myopathy with SNP located within *SLCO1B1* [35]. In addition, Riedmaier et al. have demonstrated that polymorphisms of PON1 and PON3 esterase enzymes are associated with changes in ATV-LAC hydrolysis and increased the expression of PON1 mRNA in human liver tissues [28].

1.12. Drug-drug interaction

The U.S. FDA database reviewed by Thompson et al. reported approximately 58% cases of rhabdomyolysis related to statins are associated with the concomitant medication affecting statin metabolism [11]. The potential of a serious clinical drug interaction is highest when concomitant medications are metabolized by the same CYP enzyme [36]. Metabolism of 60% of marketed medications is dependent on the activity of CYP3A. Concomitant drugs that are inhibitors of CYP3A4 or UGT enzymes increase plasma levels of ATV and its metabolites therefore may enhance the risk of statin-induced rhabdomyolysis [13, 37-40]. Moreover, several cases reported severe statin-related rhabdomyolysis with concurrent use of drugs including cyclosporine, colchicine, fusidic acid, delavirdine, diltiazem, esomeprazole, clarithromycin, antiretroviral drugs,

gemfibrozil and fluconazole that inhibit enzymes or transporter systems, which are involved in the disposition of ATV [41-49].

2. Rosuvastatin calcium (Crestor®)

2.1. Physicochemical properties of rosuvastatin calcium

Rosuvastatin is a synthetic drug and is available as white to off-white crystalline calcium salt of an active acid. Chemical formula of rosuvastatin calcium is (E,3R,5S)-7-[4-(4-fluorophenyl)-2-[methyl(methylsulfonyl)amino]-6-propan-2-ylpyrimidin-5-yl]-3,5-dihydroxyhept-6-enoate), C₄₄H₅₄CaF₂N₆O₁₂S₂. It is sparingly soluble in water, having logP and pKa values of 2.52 and 4.44 respectively [50]. The molecular weight of RST is 481.54. The chemical structure of rosuvastatin calcium is shown in **Figure I-3**.

2.2. Clinical uses

Rosuvastatin calcium (Crestor®) is widely used as an adjunct therapy to diet for lowering elevated plasma levels of total cholesterol, LDL-C and triglycerides for the treatment of various cardiovascular related disorders including primary hyperlipidemia, mixed dyslipidemia, hypertriglyceridemia and homozygous familial hypercholesterolemia [51].

2.3. Mechanism of action

Rosuvastatin acid is relatively hydrophilic and therefore exhibits selective hepatic uptake with limited access to non hepatic tissues. It competitively inhibits HMG-CoA reductase enzyme, a rate-limiting enzyme of the mevalonate pathway for cholesterol biosynthesis. It thereby decreases intracellular levels of cholesterol, which results in increased LDL-C receptors in the liver facilitating the plasma clearance of LDL-C. Rosuvastatin acid also exerts non-lipid lowering action like vasculoprotective effects possibly by reducing inflammation of endothelial cells [52].

2.4. Side effects

Skeletal muscle-related complaints, nausea, headache, abdominal pain and asthenia are the most common adverse effects that develop with RST treatment [51].

2.5. Pharmacokinetic properties

A. Absorption

Upon oral administration, RST undergoes moderately rapid absorption that is estimated to be approximately 50%. The modest absolute bioavailability (approximately 20%) and a high hepatic extraction ratio (0.63) suggest significant first pass metabolism [53, 54]. After a single 20 mg post-dose, a $C_{\max}$ of 6.1 ng/mL was measured at approximately 5 h ($T_{\max}$). The $C_{\max}$ and AUC are linear over the dosage range of 5 to 80 mg after both single and seven daily doses; moreover, it does not accumulate following repeated dosing. It also exhibits enterohepatic recirculation suggested by secondary peak in plasma concentration time profile [53, 54].

B. Distribution

Rosuvastatin acid is reversibly bound to plasma proteins mainly albumin with a bound fraction of 88%. The large mean volume of distribution at steady state (134L) demonstrated extensive peripheral tissue distribution primarily in the liver [53].

C. Metabolism

Rosuvastatin acid undergoes limited metabolism mainly by CYP2C9 isoenzyme and to a lesser extent through CYP2C19 and CYP3A4 [55]. It is biotransformed into its

pharmacologically active main metabolite, an N-desmethyl derivative which has seven-fold lower inhibitory effect on HMG-CoA [54]. A previously published *in vitro* study has shown that upon incubation of RST with human liver microsomes, RST underwent significant glucuronidation through UGT1A1 and 1A3 to form acyl glucuronide conjugates and lactonization to pharmacologically inactive 5S-lactone (RST-LAC) metabolite [55]. The chemical structures of both the metabolites are shown in **Figure I-4**. The average $C_{\max}$ values for RST-LAC and N-desmethyl rosuvastatin were 12–24% and <10% of the parent RST $C_{\max}$, respectively [54].

D. Elimination

Fecal (approximately 90% of the dose) and renal (approximately 10% of the dose) are the major and minor routes of excretion of RST, respectively. Rosuvastatin acid mainly excretes unchanged (76.8% of the dose) in feces with elimination half-life of 20 h [54].

2.6. Effect of age and gender

The effect of age and gender on the PK of RST was studied in young (18-35 years) and elderly (>65 years) healthy volunteers, with an average age of 24 years and 64 years, respectively. An average $AUC_{(0-t)}$ was 6% and of $C_{\max}$ was 12% greater in the young subjects as compared to the older subjects. Similarly, the mean of $AUC_{(0-t)}$ was 9% and of $C_{\max}$ was 18% lower in male as compared to female groups. These minor differences are not considered clinically important and therefore no dose adjustment is recommended [56].

2.7. Effect of race

Systemic exposure of RST and the two metabolites were significantly higher in white subjects as compared with Chinese, Malay and Asian-Indian living in the same environment. The PK differences could be due to genetic polymorphisms of *SLCO1B1* gene resulting in altered transport activity of RST by OATP1B1 hepatic uptake transporter [57].

2.8. Effect of food

The extent of absorption of RST is not affected by food; while the rate of absorption is decreased by 20%, the efficacy of the drug remains unchanged. Therefore, RST can be administered with or without food [58].

2.9. Effect of hepatic and renal impairment

The clinical PK of RST at steady state were similar between patients with mild (Child-Pugh class A) to moderate (Child-Pugh class B) hepatic impairment and subjects with normal hepatic functions. However, severe hepatic impairment may increase plasma systemic exposure of RST [59]. The systemic exposure of RST was similar between patients with mild-to-moderate renal impairment (creatinine clearance ≥ 30 ml/min/1.73 m²) and healthy volunteers at steady state. Nevertheless, the exposure was three fold higher in patients with severe renal impairment (creatinine clearance < 30 ml/min/1.73 m²) as compared to healthy subjects [51]. Rosuvastatin acid plasma concentrations were approximately 50% higher in patients undergoing haemodialysis therapy as compared with healthy controls [60].

2.10. Effect of time of administration

The PK and pharmacodynamic properties of RST were not different between the morning and evening time of oral administration in healthy volunteers who received a daily dose of 10 mg for 14 days [61].

2.11. Effect of genetic polymorphism

Various clinical studies conducted in healthy Finish and Korean population have described the impact of SNP in OATP1B1 and BCRP transporters on the PK of RST, which is summarized in **Table I-2**.

2.12. Drug interactions

Rosuvastatin acid has a limited metabolism and is less likely to interact with other clinically used drugs. Administration of azole antifungal drugs including itraconazole [62], fluconazole [63] and ketoconazole [64] had no or very small effect on the systemic exposure of RST and thus these interactions are not considered clinically relevant. These clinical drug interactions studies supported the previous findings regarding limited metabolism of RST through CYP2C9, CYP2C19 and CYP3A4. Similar results were obtained when RST was administered with the macrolide antibiotic, erythromycin, which is a potent CYP3A4 inhibitor and demonstrated the minor role of CYP3A4 in RST metabolism [65].

Upon oral co-administration of RST with HIV protease inhibitors including atazanavir/ritonavir or fosamprenavir/ritonavir [66] and liponavir/ritonavir, [67] several fold increase of AUC and C_{max} may occur following the inhibition of BCRP mediated intestinal uptake and/or biliary efflux and OATP1B1 mediated hepatic uptake. A

combined therapy of RST and HIV protease inhibitors warrants careful administration. Gemfibrozil [68, 69] and cyclosporine [70] significantly increased plasma concentrations of RST by inhibiting OATP mediated hepatic uptake. A minimal change in the systemic exposure of RST was observed with simultaneous administration of fenofibrate [71], rifampicin [72], dalcetrapib [73] and silymarin supplements [74] and therefore these interactions are not considered clinically relevant. The herbal medicine baicalin decreased AUC of RST due to induction of OATP1B1 mediated hepatic uptake [75]. Simultaneous administration of RST with antacid preparation containing aluminum hydroxide and magnesium hydroxide reduced systemic exposure of RST by approximately 50%, and the effect was decreased upon administration of antacid 2 hrs after RST dosing [76]. Rosuvastatin acid can increase anticoagulant activity of warfarin, and thus careful monitoring is required upon co-administration of both drugs. The exact mechanism of the PD interaction between these two drugs is not known yet [77]. Coadministration of ezetimibe with RST significantly reduces LDL-C and triglyceride levels in serum however; no significant PK interaction was noticed [78].

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Figure I-1. Proposed oxidative metabolic (Phase I) pathway of atorvastatin acid.

Figure I-2. Proposed glucuronidation (Phase II) metabolic pathway of atorvastatin acid.

Figure I-3. Chemical structure of rosuvastatin calcium.

a)

b)

Figure I-4. Chemical structures of metabolites of rosuvastatin acid a) N-desmethyl rosuvastatin b) Rosuvastatin-5S- lactone.

Table I-1. Major polymorphisms in genes coding drug metabolizing enzymes and transporters affecting the pharmacokinetics of atorvastatin acid (ATV) and atorvastatin lactone (ATV-LAC).

Gene name	Enzyme or transporter	SNP	dbSNP ID	Population	Effect	Analytes	Ref
ABCB1	P-gp	c.1236 C>T	rs1128503	Healthy finish	AUC:TTT/TTT>CGC/CGC	ATV	[79]
		c.2677 G>T/A	rs2032582	volunteers	t _{1/2} : TTT/TTT>CGC/CGC		
		c.3435C>T	rs1045642	Koreans	t _{1/2} : TTTT>GGCC/GTCT	ATV ATV-LAC	[80]
ABCG2	BCRP	c.421C>A	rs2231142	Healthy finish volunteers	AUC:AA>(C/C) AUC:AA>(C/C and C/A)	ATV ATV-LAC	[81]
SLCO1B1	OATP1B1	OATP1B1*5 (521T>C)	rs4149056	Healthy white	AUC:CC>(T/T and T/C)	ATV	[82]
				volunteers	AUC:TC>(T/T)		
				Koreans	AUC: *15/*15>*1a/*1a, *1b/*1b , *1b/*15,*1a/*15, *1a/*1b	ATV	[80]
UGT1A3	UGT1A3	UGT1A3*1	NA	Healthy finish	t _{1/2} : *1/*6< *1/*1,*1/*2,*2/*2,*1/*3	ATV	[83]
		UGT1A3*2		volunteers	t _{1/2} : *2/*3< *1/*1,*1/*2,*2/*2	ATV-LAC	
		UGT1A3*3			AUC ratio (L/A):*1/*1<*1/*2,*2/*2		
		UGT1A3*6					

Table I-2. Major polymorphisms of drug metabolizing enzymes and transporters affecting the pharmacokinetics of rosuvastatin acid.

Gene name	Enzyme or transporter	SNP	dbSNP ID	Population	Effect	Ref
ABCG2	BCRP	c.421 C>A	rs2231142	Healthy finish volunteers	AUC:AA>(C/C and C/A) C _{max} :AA>(C/C and C/A)	[81]
				Koreans	AUC: CC< (C/A and A/A) C _{max} : CC>(C/A and A/A) CL/F: CC>(C/A and A/A)	[84]
SLCO1B1	OATP1B1	OATP1B1*5 (521T>C)	rs4149056	Healthy white volunteers	AUC:CC>(T/T) C _{max} :CC>(T/T)	[82]
				Koreans	AUC: *15/*15,*1a/*15> *1a/*1a C _{max} : *15/*15,*1a/*15> *1a/*1a AUC: *15/*15> *1b/*15 C _{max} :*15/*15> *1b/*15 or *1b/*1b	[57, 85]

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MANUSCRIPT II

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Development and Validation of a Sensitive, Simple and Rapid Method for Simultaneous Quantitation of Atorvastatin and its Acid and Lactone Metabolites by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Running title: A LC-MS/MS assay for quantification of atorvastatin and metabolites

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Abbreviations: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), Atorvastatin acid (ATV), Cytochrome P450 3A4 (CYP3A4), High level quality control (HQC), High-performance liquid chromatography (HPLC), Internal standard (IS), Liquid chromatography-tandem mass spectrometry (LC-MS/MS), Low level quality control (LQC), Lower limit of quantitation (LLOQ), Multiple reaction monitoring (MRM), Peak plasma concentration ($C_{\max}$), Pharmacokinetics (PK), Quality control samples (QCs), Signal-to-noise (S/N), UDP-glucuronosyltransferases (UGTs)

Abstract

The aim of the proposed work was to develop and validate a simple and sensitive assay for the analysis of atorvastatin (ATV) acid, *ortho*- and *para*-hydroxy-ATV, ATV lactone, *ortho*- and *para*-hydroxy-ATV lactone in human plasma using liquid chromatography-tandem mass spectrometry.

All six analytes and corresponding deuterium (d5) labeled internal standards were extracted from 50 μ L of human plasma by protein precipitation. The chromatographic separation of analytes was achieved using a Zorbax-SB Phenyl column (2.1 mm $\times$ 100 mm, 3.5 μ m). Mobile phase consisted of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B). All analytes including *ortho*- and *para*-hydroxy metabolites were baseline separated within 7.0 min using a flow rate of 0.35 mL/min. Mass spectrometry detection was carried out in positive electrospray ionization mode, with multiple reaction monitoring scan.

The calibration curves for all analytes were linear ($R^2 \geq 0.9975$, $n=3$) over the concentration range of 0.05-100 ng/mL and with LLOQ of 0.05 ng/mL. Mean extraction recoveries ranged between 88.6-111%. Intra- and inter-run mean %accuracy were between 85-115% and %imprecision was $\leq 15\%$. Stability studies revealed that ATV acid and lactone forms were stable in plasma during bench top (six hours on ice-water slurry), at the end of 3 successive freeze and thaw cycles, and at -80 $^{\circ}$ C for 3 months. The method was successfully applied in a clinical study to determine levels of ATV and its metabolites over 12-hour post dose in patients receiving atorvastatin.

Introduction

According to the American Heart Association and Centers for Disease Control and Prevention, cardiovascular diseases are the leading cause of death in the United States [1,2]. Hypercholesterolemia is a major risk factor for the progression of atherosclerosis, the principal cause of the development of coronary heart diseases. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used for the treatment of hypercholesterolemia. Statins induce a significant reduction in total plasma cholesterol concentration by reversible inhibition of the rate limiting enzyme, HMG-CoA reductase of the mevalonate pathway, which is responsible for the endogenous biosynthesis of cholesterol [3].

Worldwide, atorvastatin (ATV) calcium (Lipitor®, Pfizer Pharmaceuticals, NY) is the top selling prescribed medication [4]. Upon oral administration, the pharmacologically active calcium salt of ATV undergoes mainly cytochrome P450 3A4 (CYP3A4) mediated oxidative metabolism and forms two active metabolites, *ortho*-hydroxy-ATV and *para*-hydroxy-ATV [5]. The parent drug and its acid hydroxy metabolites are in equilibrium with their corresponding inactive lactones forms, ATV lactone, *ortho*-hydroxy-ATV lactone and *para*-hydroxy-ATV lactone [6]. The metabolism pathway of atorvastatin, as described by Kantola et al. is shown in **Figure II-1** [7]. Atorvastatin is the only statin with active metabolites since 70% of the ATV HMG-CoA reductase inhibition has been attributed to its hydroxy acid (*ortho* and *para*) metabolites [8].

Atorvastatin is clinically administered with a once daily dose of 10 to 80 mg [3]. The drug undergoes extensive first-pass metabolism in the gastrointestinal tract and/or liver,

with ~14% absolute oral bioavailability after a 10 mg oral dose. The peak plasma concentrations ($C_{\max}$) are reached within 1 to 3 hour after oral administration of ATV. Food intake decreases ATV area under the concentration-time curve and $C_{\max}$ by ~25% and 9 %, respectively. Atorvastatin is highly bound to plasma protein ($\geq 98\%$) with an average volume of distribution of 381 L and total clearance of 625 mL/min. After 40 mg oral dose, $C_{\max}$ (mean $\pm$ SD) of ATV, ATV lactone, *ortho*-hydroxy-ATV, *ortho*-hydroxy-ATV lactone and *para*-hydroxy-ATV lactone were 13.4 ± 9.5 , 3.8 ± 2.6 , 9.8 ± 6.1 , 4.5 ± 6.0 and 1.8 ± 1.0 ng/mL, respectively [7].

Although statins are well tolerated, skeletal muscle toxicity is the main adverse effect associated with statin treatment that results in decreased adherence to the therapeutic regimen [9]. Several hypotheses based on depletion of the products of the mevalonate pathway have been proposed to explain the molecular mechanism of statin-related myopathy; however, the underlying mechanism for statin-induced myopathy has not been fully elucidated [10]. Hermann et al. observed significantly higher levels of ATV lactone, *para*-hydroxy-ATV, *ortho*-hydroxy-ATV, and *para*-hydroxy-ATV lactone metabolites in patients experiencing ATV-induced myopathy. The authors suggested potential clinical utility of metabolite to parent concentration ratio as a new diagnostic marker to assess the risk of ATV-associated myopathy [11]. Moreover, an *in vitro* study using primary human skeletal muscle cells found that ATV lactone has a 14-fold higher potency to induce myotoxicity as compared with its acid form further corroborating the usefulness of ATV lactone concentration as a marker for statin-induced myopathy [12]. The plasma levels of *para*-hydroxy-ATV is almost 10-fold lower as compared with *ortho*-hydroxy-ATV [7]; thus, it is necessary to achieve an adequate LLOQ to be able to

quantify the concentration of *para* metabolites in plasma, especially when ATV is administered at low dose.

To date, several methods have been published for quantification of ATV and its metabolites in a biological matrix (plasma or serum), bulk drug, pharmaceuticals products, and aqueous samples. Various techniques employed include an electrochemical method [13], high-performance liquid chromatography with ultra violet detection [14-17], enzyme inhibition [18,19], liquid chromatography-mass spectrometry [20], microbore liquid chromatography/electrospray ionization-tandem mass spectrometry [21], and liquid chromatography-tandem mass spectrometry [22-30].

High-performance liquid chromatography with ultra violet or electrochemical detection methods typically have a higher limit of quantification and are more time consuming. Gas chromatography meets the required LLOQ for most analytes; however, it needs complex derivatization steps. Enzyme inhibition assays are sensitive and easy to implement but are non-specific and do not provide any information on the metabolite concentrations. Undeniably, mass spectrometry has become the method of choice for quantification of metabolite concentration in biological matrices due to its superior selectivity. However, few methods [20,25,26] have been reported for simultaneous determination of ATV and the five ATV metabolites (*ortho*- and *para*-hydroxy-ATV, ATV lactone, *ortho*- and *para*-hydroxy-ATV lactone). Moreover, most reported methods require time-consuming extraction procedures, and they did not provide the sensitivity required to quantify *para*-hydroxy-ATV, that is present at ~10% of the total concentration, after administration of low dose ATV [20,25,26]. Furthermore, most methods require 500-1000 μ L plasma volume [25,26]. In this manuscript, we describe

the development and validation of a highly sensitive method for determination of ATV and its five metabolites using 50 μ L of plasma.

Experimental

Reagents and chemicals

ATV calcium hydroxy acid (*ortho*-hydroxy-ATV and *para*-hydroxy-ATV) and lactone metabolites (ATV lactone, *ortho*-hydroxy-ATV lactone and *para*-hydroxy-ATV lactone) and corresponding deuterium (d5) labeled internal standards (IS) (d5-ATV, d5-ATV lactone, d5-*ortho*-hydroxy-ATV, d5-*para*-hydroxy-ATV, d5-*ortho*-hydroxy-ATV lactone and d5-*para*-hydroxy-ATV lactone) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). HPLC grade acetonitrile and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade methanol was from Pharmco Products Inc. (Brookfield, CT, USA). A Milli Q50 (Millipore, Bedford, MA, USA) water purification system was used to generate HPLC grade water. Human drug-free plasma with sodium fluoride/potassium oxalate as an anticoagulant was purchased from Bioreclamation Inc (Westbury, NY, USA). Human plasma from individuals on ATV therapy was obtained by venipuncture in sodium heparin, fluoride/potassium oxalate or K₂EDTA vacutainers (BD, Franklin lakes, NJ, USA) after obtaining informed consent.

Chromatographic conditions

The separation of analytes was performed on a Zorbax-SB Phenyl, Rapid Resolution HT (2.1 mm × 100 mm) column with 3.5 µm particle size from Agilent Technologies (Wilmington, DE, USA), preceded by a 0.5 µm filter (Supelco, Bellefonte, PA). The analytical column was maintained at 40 °C temperature. Mobile phase, consisting of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B), was used at a flow rate of 0.35 mL/min

for separation and rapid elution of analytes from the extracted matrix within 7.0 min. During the first 0.3 min of the gradient, %B increased from 10% to 60% where it was kept until 0.8 min and then gradually increased to 75% until 4.6 min. At 4.61 min, %B was decreased to 10% and kept to re-equilibrate the column until 7.0 min.

Mass spectrometric conditions

The LC-MS/MS system consisted of an Agilent Technologies 1200 series HPLC system with binary pumps, autosampler, thermostatted column compartment and micro vacuum degasser (Santa Clara, CA) coupled to an API 4000 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada), equipped with Turbo V^{TM} ion source. Mass spectrometric detection and quantitation of all analytes were carried out using multiple reaction monitoring (MRM) scan in electrospray positive ion mode. Q1 and product ion scans were obtained by infusing solutions of individual analytes and their respective IS using an infusion pump.

The following MRM transitions were selected: (m/z, Q1→Q3) of ATV (m/z, 559.2→440.2), d5-ATV (m/z, 564.2→445.2), ATV lactone (m/z, 541.2→448.2), d5-ATV lactone (m/z, 546.2→453.2), *ortho*-hydroxy-ATV (m/z, 575.2→440.2), d5-*ortho*-hydroxy-ATV (m/z, 580.2→445.2), *para*-hydroxy-ATV (m/z, 575.2→440.2), d5-*para*-hydroxy-ATV (m/z, 580.2→445.2), *ortho*-hydroxy-ATV lactone (m/z, 557.2→448.2) and d5-*ortho*-hydroxy-ATV lactone (m/z, 562.2→453.2), *para*-hydroxy-ATV lactone (m/z, 557.2→448.2), d5-*para*-hydroxy-ATV lactone (m/z, 562.2→453.2). Source temperature and gas parameters were optimized after the chromatographic conditions were finalized by infusing a solution of *para*-hydroxy-ATV in T junction with the mobile

phase consisting of 70% of solvent B. All peak areas were obtained using Sciex Analyst 1.5.1 data processing software.

Standards/QCs preparation

Separate stock solutions of ATV (0.4 mg/mL), *ortho*-hydroxy-ATV (1.0 mg/mL) and *para*-hydroxy-ATV (1.0 mg/mL) were prepared in acetonitrile:water mixture (90:10, v/v). Similarly, 1.0 mg/mL separate stock solutions of ATV lactone, *ortho*-hydroxy-ATV lactone and *para*-hydroxy-ATV lactone were prepared in acetonitrile. Intermediary stock solutions of composite mixtures of acids only and lactones only (100 µg/mL for each compound) were prepared in acetonitrile:water mixture (90:10 v/v) and acetonitrile, respectively, and were used to spike calibrators and quality control samples (QCs) in drug-free human plasma kept on an ice-water slurry.

Individual deuterated (d5) IS of acid and lactone stock solutions of 1.0 mg/mL were prepared in acetonitrile:water (90:10 v/v) and acetonitrile, respectively. Intermediary internal standard solutions containing 50 ng/mL of either acids or lactones were prepared in acetonitrile. All stock and working standard solutions were stored at -20 °C until use. Eight calibration standards with concentrations ranging from 0.05 ng/mL to 100 ng/mL and quality control samples at four concentration levels (0.05, 0.15, 5.00 and 75.0 ng/mL) were prepared in drug-free human plasma by using respective spiking solutions and were stored at -80 °C.

Sample extraction

Calibrators, QCs, control blank, double blank, and patient samples were thawed on ice-water slurry and vortex-mixed thoroughly for 10 seconds. A 50 μ L aliquot of each plasma sample was aliquoted into 1.5 mL polypropylene tube; all samples were treated with 200 μ L of 0.1% acetic acid in acetonitrile (as a protein precipitation reagent) containing 0.8 ng/mL of the combined acids and combined lactone IS, except for double blank. All tubes were vortex-mixed for 10 seconds and thereafter all precipitated proteins were separated by centrifugation for 15 min at 14,000 g and 4°C temperature. The supernatant was transferred to a fresh glass vial and 10 μ L was injected onto LC-MS/MS.

Validation of the assay

The validation of the method was performed according to general recommendation guidelines for bioanalytical methods by U.S. Food and Drug Administration (FDA) [31]. Validation parameters including selectivity, sensitivity, accuracy, precision, recovery and stability were determined.

Stability

The stability studies aimed to establish the conditions in which the analytes are stable and the degree of inter-conversion of acid to lactone or lactone to acid forms. The first set of QC samples contained composite mixture of all six analytes. In the second set, QC samples contained only ATV and its two hydroxy acid metabolites. The third set comprised lactone-only QC samples containing only ATV lactone and its two hydroxy metabolites. All three sets of QC samples were prepared at low level quality control (LQC) (0.15 ng/mL) and high level quality control (HQC) (75.0 ng/mL) concentration levels. The following stability studies were carried out:

- I. Post preparative stability (autosampler stability): The stability of the analytes in processed samples, kept in the autosampler at 4°C, was tested by reinjecting one of the three validation runs 24 hours after extraction.
- II. Bench-top stability: Triplicate LQC and HQC kept on ice-water slurry for six hours were extracted along with freshly spiked calibrators.
- III. Long-term stability: Long-term stability at -80 °C was established by analyzing six replicates of LQC and HQC for all three sets of QC samples, after two weeks, one month and three months.
- IV. Freeze and thaw stability: Freshly spiked triplicates of LQC and HQC of all three sets of QC samples were stored at -80 °C for at least 12 hours and were thawed on ice water slurry. At the end of three successive freeze and thaw cycles, QC samples were extracted along with freshly spiked calibrators and QCs.

Linearity, accuracy and precision

Eight-point calibration curves were obtained using 0.05, 0.10, 0.50, 2.00, 10.0, 50.0, 90.0 and 100 ng/mL calibrators. All calibrators, six replicates of the first set of QC samples at four concentration levels, double blank (without IS) and control blank (with IS) were tested in three runs to evaluate the intra- and inter-run imprecision and accuracy of the method.

Limit of quantitation

Lower limit of quantitation (LLOQ) is defined as the lowest concentration of an analyte, which has at least five times higher response (signal-to-noise, $S/N=5/1$) compared to blank response and can be analyzed with an acceptable accuracy and imprecision (accuracy within 80- 120% and imprecision $\leq 20\%$). Five serial dilutions of plasma sample containing 0.200 ng/mL of each analyte were made by mixing equal volumes of spiked plasma with blank plasma. Six replicates of each spiked sample were extracted and S/N of peak and %CV of the analyte/IS ratio were calculated. The sample with S/N at least five and %CV below 20% for each analyte was selected as potential LLOQ for the validation.

Selectivity

Drug-free human plasma samples from six different donors were evaluated for the presence of endogenous matrix components that may interfere with quantitation of the analytes or the IS. Area of the peaks eluting at the same retention time as the analytes and internal standards of interest, in extracted selectivity samples, were compared with those in chromatograms obtained from LLOQ samples.

Recovery and matrix effect

Triplicates of LQC and HQC from the first set of QC samples along with blank plasma samples were extracted and were used to evaluate recovery and matrix effect (enhancement or suppression of ionization). Solutions with concentrations equivalent to 100% recovery of the extracted LQC (0.03 ng/mL) and HQC (15 ng/mL) containing all analytes and IS were prepared in water: 0.1% acetic acid in acetonitrile, 1:4 (v/v) (reference for matrix effect) and in extracted blank plasma (reference for recovery). In

addition, matrix effect was also evaluated using a post-column infusion method. This test was performed by infusing a solution containing 20 ng/mL of analytes and IS prepared in solvent B:solvent A (70:30 v/v) at 10 μ L/min along with the injections of the blank solvent, double blank matrix extract and extracted HQC to identify the region of ionization suppression.

To investigate the potential of interference and matrix effect from co-administered medications and their metabolites, specific to transplanted population, a pool plasma sample was prepared from samples collected from the three stable kidney transplant patients treated with sirolimus (2, 2, or 4 mg/day), mycophenolic acid (2000 mg/day) and prednisone (5 mg/day), but not with atorvastatin. The pool sample was obtained by mixing equal volumes of plasma from samples collected at 1, 2, 4, 8 and 12 hours after the morning dose of the co-administered medications. The pooled plasma samples were spiked with LQC and six replicates were extracted along with calibration STD, QCs, and a non-spiked pool.

Application of the method in a clinical pharmacokinetic study

The proposed method was successfully applied to determine the levels of acid and lactone forms of ATV and hydroxy metabolites in human plasma samples obtained from a pharmacokinetic study conducted to investigate ATV disposition in the stable kidney transplant recipients who received ATV.

Results and discussion

Sample collection and preparation

Lactonization of an acid form and hydrolysis of lactone to an open-acid form of ATV is mediated through UDP-glucuronosyltransferase (UGTs) [32] and esterases (paraoxonases) [33], respectively. Esterase activity is the most important cause of instability of ester-containing drugs in biological matrices. We tested the effect of several anticoagulants on lactone to acid interconversion by comparing different types of plasma (sodium heparin, K₂EDTA and sodium fluoride/potassium oxalate) and serum. In addition, one blood sample was collected for each anticoagulant and serum from five representative patients who were on 10-40 mg daily dose of ATV. The plasma and serum were separated by centrifugation at 1500 g and concentrations of all analytes were determined using the validated assay. Moreover, freshly spiked HQC containing lactone only analytes that was freshly prepared in serum or plasma with different anti-coagulants were analyzed. We could not find statistically significant difference (One-way ANOVA, $p>0.9$) between serum and various anticoagulants (**Table II-1**); however, we preferred to use sodium fluoride (esterase inhibitor) plasma to ensure stability of lactone upon long-term storage [34].

Liquid-liquid and solid phase extractions usually involve tedious and time-consuming extraction steps such as drying and the addition of pH modifiers, which in this case may lead to undesirable acid-lactone interconversion. A previously reported LC-MS/MS assay for ATV and metabolites (27) extensively studied the known stability issues of highly unstable lactone and showed that the possible interconversion between lactone and

acid forms can be minimized by lowering the working temperature to 4 °C and lowering the plasma pH to 4-6. Non-acidified acetonitrile as a precipitating agent resulted in significant interconversion of lactone to acid form during the resident time of the extracted samples in an autosampler. Therefore, 0.1% glacial acetic acid in acetonitrile was used to minimize the interconversion of lactone to acid.

LC-MS/MS detection

Ortho- and *para-* analytes have the same precursor ion-product ion transitions. Additionally, the acids could potentially undergo in-source fragmentation and, following the loss of water, the resulting product would interfere with their respective lactones. For these combined reasons, we sought chromatographic conditions that would assure baseline chromatographic separation of the respective analytes. To achieve this goal, various reverse phase analytical columns were tested and the required selectivity, as well as the excellent peak shape (in terms of sharpness and symmetry), were achieved using a narrow bore Zorbax-SB phenyl column. Because this column provided excellent peak focusing, a high S/N was obtained that enabled us to use a simple protein precipitation extraction without performing pre-concentration steps for sample clean up. Total elution of analytes using a gradient mixture of mobile phase resulted in a run time of 7.0 min, including the re-equilibration of the column. The retention times of ATV, ATV lactone, *ortho*-hydroxy-ATV, *para*-hydroxy-ATV, *ortho*-hydroxy-ATV lactone and *para*-hydroxy-ATV lactone were 3.9, 4.4, 3.8, 3.2, 4.2 and 3.5 min, respectively (**Figure II-2**).

The MS was operated using electrospray ionization probe in positive ion mode to obtain high signal intensity. For each analyte, [M+H]⁺ was the major precursor ion used to

obtain the product ion spectra. The major product ions are formed by the neutral loss of the (phenylamino)carbonyl group and phenylamino group, from acid and lactone compounds, respectively. Temperature and gas parameters of the source were optimized based on *para*-hydroxy-ATV, since the plasma concentration of this analyte is typically lower than other analytes.

The compound specific parameters i.e. declustering potential (DP), entrance potential (EP), collision cell exit potential (EXP) and collision energy (CE) and similarly, gas parameters including curtain gas (CUR=30 psi), gas 1 (GS1=60 psi), gas 2 (GS2=30 psi), and collision gas (CAD=10 psi), ionspray voltage (IS=5500 V), and temperature (TEM=550 °C), were optimized to achieve maximum signal. No significant in-source inter-conversion was observed from acid to lactone form.

Method validation

The analytes were stable in the conditions described above. The post preparative stability assessment proved that extracted samples can be injected after being kept at 4°C for 24 hours post extraction, the anticipated resident time in autosampler; furthermore, analytes were stable after three cycles of freeze and thaw and during bench top stability carried out on ice-water slurry. The data obtained also revealed that the samples were stable in the matrix after 3 months storage at -80 °C. No interference was observed from endogenous components with analytes and IS in blank plasma from six different donors, demonstrating the specificity of the method.

The calibration curves obtained from analyte/IS peak area ratios vs. nominal concentration were linear using weighted ($1/x^2$) linear regression over the concentration

range, with correlation coefficients (R^2) ≥ 0.9975 . According to FDA guidelines, the accuracy of calibration standards and quality control samples ($n > 5$) should be between 85-115%, and imprecision below 15%, except at the LLOQ level, for which accuracy may be between 80-120% and imprecision below 20%. For the current assay, the measured mean values ($n=3$) were within 91.6-106% of their nominal values for calibrators (Table II-2), 89.2-110% for intra-run QCs (results not presented) and 91.7-107% for inter-run QCs (Table II-3), which indicates acceptable accuracy of the proposed method.

A lower limit of quantitation of 0.05 ng/mL was achieved for all analytes and a chromatogram of an extracted LLOQ is shown in **Figure II-2**. The extraction recovery for all analytes was within 88.6-111%. No significant matrix effect was observed for each analyte upon calculating the ratio of average area response of reference for recovery to reference for the matrix effect at LQC and HQC concentration levels.

Evaluation of the matrix effect is essential to assure the reliability of quantitative assays using HPLC-MS/MS and the integrity of the resulting pharmacokinetics data. We used stable isotope labeled internal standards for each of the analytes, as they compensate reasonably well during variations in sample analysis. However, due to their slightly different elution times compared to their respective non-labeled species, deuterated internal standards may not always compensate well for ionization enhancement or suppression due to coelution of matrix endogenous components, such as phospholipids [35]. Phosphatidylcholine and lyso-phosphatidylcholine are major human plasma phospholipids that produce matrix effect. Zhang and colleagues reported the key phospholipids in human serum extracts that can cause matrix effects by identifying the

precursor ions generating fragment with m/z 184 [36]. Following the same technique, we determined the retention time of phospholipids with MRM transitions: Q1 m/z 496, 520, 522, 524, 544, 758 and 782 and Q3 m/z 184. We have then adjusted the chromatographic conditions to ensure that the analytes do not co-elute with these phospholipids. To study matrix effect, post-column infusion of a solution containing the analytes and their respective IS, concomitantly with injecting extracted blank plasma were carried out. We monitored retention times of the major phospholipids as shown in **Figure II-3A**, and found that no significant ion suppression occurred at the retention time of the analytes or IS.

The post-column infusion chromatogram shown in **Figure II-3B** indicates that ATV and metabolites were eluted at retention times different from the retention time of key phospholipids. Post-column infusion of analytes and IS, concomitantly with injecting an extracted blank plasma, shows that the signal intensity of each analyte of interest does not change in the region of their respective elution period (**Figure II- 3C**). The mean of the calculated concentrations of six LQC samples prepared in pooled plasma from patients treated with an immunosuppressive regimen and who did not receive atorvastatin was between 92–97% of the expected nominal concentration. No interfering peaks were eluting at the retention times of the analytes of interest. This demonstrates that sirolimus, mycophenolic acid, prednisone, and their metabolites, do not interfere with the quantitation of atorvastatin and its metabolites.

Application of the method

The method was successfully applied to measure the concentration of ATV and its metabolites in the stable kidney transplant recipients who received atorvastatin for the treatment of hypercholesterolemia. The study protocol was approved by the Institutional Review Board of Rhode Island Hospital, Providence RI, USA. Written informed consent was obtained from all subjects after verbal explanation of the study protocol prior to enrolling in the study.

In **Figure II- 4A**, ATV and metabolites steady-state 12-hour concentration-time profile, obtained from the four kidney transplant recipients is shown. All patients received a 10 mg dose of ATV in the morning along with a triple immunosuppressive regimen that included oral sirolimus, mycophenolic acid and prednisone. The concentration-time profiles for ATV and metabolites show that the method has an acceptable LLOQ for quantitation of all analytes including the *para*-hydroxy metabolites. Moreover, in **Figure II-4B**, metabolite to parent ATV concentration ratio obtained from the nine kidney transplant recipients treated with 10, 20 and 40 mg of ATV dose is shown. The concentration of ATV and metabolites were measured in nine plasma samples over a 12-hour post dose period and the average ratio for each metabolite is presented. As shown in **Figure II-4B**, in our patient population, the concentration of ATV, *ortho*-hydroxy-ATV-lactone and *para*-hydroxy-ATV-lactone were greater than the parent ATV, whereas, the concentration of *ortho*-hydroxy-ATV and *para*-hydroxy-ATV was lower than the parent ATV concentration

Conclusion

We describe a reproducible, reliable, sensitive and simple bioanalytical method. The major advantages of the present method, as compared with previous reports, are low LLOQ, 50 μ L plasma volume and simple extraction method. The proposed validated bioanalytical technique accomplishes the required selectivity, sensitivity, accuracy, and imprecision to be applied to various studies involving pharmacokinetics, drug metabolism, clinical pharmacology/toxicology, bioavailability/bioequivalence, and drug-drug interactions for accurate quantitative analysis of ATV and its five metabolites. This method offers an easy and convenient way to conduct routine clinical monitoring of toxic metabolites, therefore, it can be used by toxicologists and clinical chemists who wish to implement the metabolite/parent drug ratio as a new diagnostic marker for identifying patients at risk of developing ATV-induced myotoxicity.

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Adapted from Kantola et al. (1998)

Figure II-1. Proposed metabolism pathway of atorvastatin to atorvastatin lactone, ortho-hydroxy-atorvastatin, para-hydroxy-atorvastatin, ortho-hydroxy-atorvastatin lactone, and para-hydroxy-atorvastatin lactone.

Figure II-2. Chromatograms showing the integrated peaks of atorvastatin acid (A), atorvastatin lactone (B), ortho-hydroxy-atorvastatin (C), para-hydroxy-atorvastatin (D), ortho-hydroxy- atorvastatin lactone (E), and para-hydroxy-atorvastatin lactone (F) from extracted calibration standard at the lower limit of quantitation (0.05 ng/mL).

Figure II-3. Injection of matrix spiked with analyte and IS without post-column infusion along with MRM transitions of key phospholipids. Chromatograms showing the peaks of analytes and corresponding IS along with key phospholipids (A). Chromatogram obtained with post-column infusion shows no matrix effect at the retention times of analytes and IS. Arrow indicates region where the signal of compounds infused post-column is suppressed during the elution of endogenous matrix components (B). Chromatogram of a blank sample injected while the analytes and IS are infused post column. Arrow indicates region where the signals of analytes and IS infused post-column are suppressed during the elution of endogenous matrix components (C).

A

B

Figure II-4. Plasma concentration (ng/mL) versus time profiles of atorvastatin and metabolites, in acid and lactone forms, from the four stable kidney transplant recipients who received a 10 mg atorvastatin dose; data are expressed as mean and error bars represent standard deviation (A) Metabolite/parent atorvastatin concentration from the stable kidney transplant recipients (n=9) on a steady-state dose of 10-40 mg atorvastatin (B).

Table II-1. Effect of Various Anticoagulants on Interconversion of Atorvastatin (ATV) lactones; the first part represents ATV lactone %accuracy and %CV for blank samples of serum or plasma with different anti-coagulants freshly spiked with ATV lactones at high quality control level. The second part of the table represents the mean and standard deviation of ATV and metabolite concentration from 5 patients at steady-state ATV with samples obtained as serum or plasma with different anti-coagulants.

Analytes added		Type of anticoagulants			
		Sodium heparin	K ₂ EDTA	Serum	Sodium fluoride
ATV lactone	% Accuracy	98.8	102	101	104
	% CV	3.3	4.3	3.5	4.5
<i>Para</i> -hydroxy-ATV lactone	% Accuracy	101	102	102	105
	% CV	4.1	3.2	3.2	4.8
<i>Ortho</i> -hydroxy-ATV lactone	% Accuracy	98.0	101	99.4	102
	% CV	3.5	3.7	6.2	5.0
Concentration of analytes in representative patients	Concentration (ng/mL)				
ATV	Mean	2.94	2.51	2.80	2.20
	Std dev	3.7	3.2	3.5	2.7
ATV lactone	Mean	2.43	2.32	2.38	2.16
	Std dev	2.2	2.1	2.2	2.0
<i>Para</i> -hydroxy-ATV	Mean	0.33	0.32	0.34	0.22
	Std dev	0.3	0.3	0.3	0.2
<i>Para</i> -hydroxy-ATV lactone	Mean	0.35	0.34	0.35	0.32
	Std dev	0.3	0.3	0.3	0.3
<i>Ortho</i> -hydroxy-ATV	Mean	2.91	2.57	2.78	2.25
	Std dev	4.0	3.5	3.8	2.9
<i>Ortho</i> -hydroxy-ATV lactone	Mean	4.87	4.46	4.87	4.24
	Std dev	5.5	5.2	5.8	4.7

n = 3

% accuracy=100- [(mean-nominal)/nominal]*100

%CV calculated as (standard deviation/mean)*100

Table II-2. Summary of Standards and Calibration Curve Parameters from Three Individual Runs.

Analyte		STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	R²
	STD (ng/mL)	0.05	0.10	0.50	2.00	10.0	50.0	90.0	100	
ATV	%Accuracy	100	100	98.7	97.0	104	98.5	99.0	103	0.9983
	%CV	3.2	7.7	9.6	0.1	0.1	4.1	4.3	6.2	
ATV lactone	% Accuracy	97.6	104	102	100	98.0	103	93.1	102	0.9975
	%CV	5.1	8.7	7.5	1.4	2.0	8.2	5.6	6.4	
Para-hydroxy-ATV	% Accuracy	97.7	106	94.2	95.5	104	103	95.8	104	0.9987
	%CV	2.2	3.9	1.2	2.4	3.1	5.2	6.0	4.4	
Para-hydroxy-ATV lactone	% Accuracy	100	99.4	103	98.5	102	104	94.4	98.6	0.9981
	%CV	3.4	8.3	7.5	3.2	2.7	4.5	2.6	0.6	
Ortho-hydroxy-ATV	% Accuracy	98.6	103	97.8	95.0	103	99.5	97.1	105	0.9980
	%CV	1.2	2.3	1.5	4.0	2.1	5.7	4.4	4.1	
Ortho-hydroxy-ATV lactone	% Accuracy	98.2	104	98.3	101	100	105	91.6	102	0.9984
	%CV	4.6	9.1	3.2	0.6	3.2	1.9	1.8	3.0	

n = 3 (1 replicate for each of the three validation runs)

% accuracy=100- [(mean-nominal)/nominal]*100

%CV calculated as (standard deviation/mean)*100

Table II-3. Summary of Quality Control Samples from Three Individual Runs.

Analyte	QC (ng/mL)	QCs			
		0.05	0.15	5.00	75.0
ATV	%Accuracy	100	105	97.6	91.7
	%CV	9.2	6.2	4.0	4.6
ATV lactone	%Accuracy	105	100	99.5	96.4
	%CV	9.4	8.1	4.9	4.4
Para-hydroxy-ATV	%Accuracy	97.4	107	95.6	94.0
	%CV	7.4	5.5	6.6	3.2
Para-hydroxy-ATV lactone	%Accuracy	103	98.5	101	97.0
	%CV	8.6	6.5	4.8	3.8
Ortho-hydroxy-ATV	%Accuracy	98.1	106	95.8	93.9
	%CV	13	7.9	4.4	4.7
Ortho-hydroxy-ATV lactone	%Accuracy	98.0	98.2	100	97.4
	%CV	12	7.8	4.5	3.8

n = 18 (6 replicates for each validation run).

% accuracy=100- [(mean-nominal)/nominal]*100

%CV calculated as (standard deviation/mean)*100

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MANUSCRIPT III

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A Simple Assay for Simultaneous Determination of Rosuvastatin acid, Rosuvastatin-5s-lactone and N-desmethyl rosuvastatin in Human Plasma using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Running title: LC-MS/MS assay of rosuvastatin acid and its two metabolites.

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Abbreviations: Cytochrome P450 (CYP), High- level quality control (HQC), High-performance liquid chromatography (HPLC), Internal standards (IS), Liquid chromatography-tandem mass spectrometry (LC-MS/MS), Lower limit of the quantification (LLOQ), Low- level quality control (LQC), Multiple reaction monitoring (MRM), N-desmethyl rosuvastatin (DM-RST), Peak plasma concentration ($C_{\max}$), Pharmacokinetic (PK), Quality control samples (QCs), Rosuvastatin acid (RST), Rosuvastatin-5S-lactone (RST-LAC), Signal-to-noise (S/N), Tetrabutyl ammonium hydroxide (TAH)

Abstract

A simple and sensitive assay was developed and validated for the simultaneous quantification of rosuvastatin acid (RST), rosuvastatin-5S-lactone (RST-LAC), and N-desmethyl rosuvastatin (DM-RST), in buffered human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

All the three analytes and the corresponding deuterium-labeled internal standards were extracted from 50 μ L of buffered human plasma by protein precipitation. The chromatographic separation of the analytes was achieved using a Zorbax-SB Phenyl column (2.1 mm $\times$ 100 mm, 3.5 μ m). The mobile phase consisted of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B). All the analytes were baseline-separated within 6.0 min using a flow rate of 0.35 mL/min. Mass spectrometry detection was carried out in positive electrospray ionization mode.

The calibration curves for all the analytes were linear ($R \geq 0.9964$, $n=3$) over the concentration range of 0.1-100 ng/mL for RST and RST-LAC, and 0.5-100 ng/mL for DM-RST. Mean extraction recoveries ranged within 88.0-106%. Intra- and inter-run mean percent accuracy were within 91.8-111% and percent imprecision was $\leq 15\%$. Stability studies revealed that all the analytes were stable in matrix during bench top (6 h on ice-water slurry), at the end of three successive freeze and thaw cycles and at -80 $^{\circ}$ C for 1 month. The method was successfully applied in a clinical study to determine the concentrations of RST and the two metabolites over 12-h post-dose in patients receiving rosuvastatin.

Introduction

Rosuvastatin acid (RST) is a synthetic and relatively hydrophilic lipid lowering agent [1]. It is widely used to treat hypercholesterolemia and to prevent progression of coronary artery diseases. Rosuvastatin decreases the concentration of low-density lipoprotein-cholesterol by reversible, competitive inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A, the rate-limiting reductase enzyme that is responsible for cholesterol biosynthesis [1]. It is one of the most effective and potent statins due to its unique characteristics such as selective uptake into hepatocytes, superior binding affinity, and tight binding interaction at the enzyme active site [2].

Rosuvastatin (Crestor®, AstraZeneca, Wilmington, DE, USA) is available as 5-40 mg tablets. It is orally administered as a calcium salt of the active hydroxy acid form with an absolute oral bioavailability of ~20%. Parent RST is biotransformed to two metabolites: rosuvastatin-5S-lactone (RST-LAC, inactive metabolite) and N-desmethyl rosuvastatin (DM-RST, active metabolite) [3] primarily by cytochrome P450 (CYP) 2C9, and to the lesser extent, by CYP 2C19, and CYP 3A4 isoenzymes [1]. Up to 50% of HMG-CoA-reductase inhibitor activity of RST has been attributed to DM-RST [3]. The chemical structures of RST and its two metabolites are shown in **Figure III-1**. After a single oral dose of RST, 90% of the dose was recovered in feces and 77% of the dose was excreted unchanged as the parent drug [3]. After an oral dose of 20 mg, the average peak plasma concentrations ($C_{\max}$) of RST was ~6 ng/mL [3]. The average $C_{\max}$ values for RST-LAC and DM-RST were 12–24% and <10% of the parent RST $C_{\max}$, respectively [3].

Statins are generally well tolerated. However, skeletal muscle toxicity is the major adverse effect associated with statin treatment that results in decreased adherence to the

therapeutic regimen. In addition, a few cases of RST-induced fatal rhabdomyolysis have been reported to date [4]. Several hypotheses based on depletion of the products of the mevalonate pathway have been proposed to explain the molecular mechanism of statin-related myopathy; though, the underlying mechanism for statin-induced myopathy has not been fully elucidated [5]. A clinical pharmacokinetic (PK) study of atorvastatin [6] proposed higher concentration of atorvastatin metabolites, as one of the possible mechanisms for statin-associated myopathy. In addition, an *in vitro* study [7] indicated that the lactone forms of statins are more myotoxic as compared to their respective acid forms. No information is currently available on the association between RST metabolite concentrations and drug related adverse effects.

To date, several methods have been reported for the quantification of parent RST. These methods have utilized high-performance liquid chromatography with ultra-violet detection (HPLC-UV) [8,9] or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [10-18] techniques. The maximum plasma concentration of RST is usually below 10 ng/mL [19]; thus, a sensitive analytical method is required for the quantification of RST in human plasma. Previously reported HPLC-UV methods are less sensitive with a lower limit of the quantification (LLOQ) in $\mu\text{g/mL}$ levels and also are more time consuming [8,9]. Quantification of RST and its metabolites in biological fluids is important with respect to understanding either the PK characteristics or pharmacological/toxicological properties. Specifically, the quantification of statin lactone may prove to have some diagnostic value as a possible marker for the development of myopathy. However, only two methods were previously reported for the quantification of DM-RST metabolite in human plasma, including individual estimation of DM-RST

[11] and simultaneous estimation of RST and DM-RST [15]. To the best of our knowledge, currently no published bioanalytical method has described a fully validated LC-MS/MS assay for the quantification of RST-LAC metabolite in human plasma.

Rosuvastatin lactone is highly unstable and the possible interconversion between lactone and acid forms can be minimized by lowering the working temperature to 4 °C and plasma pH to 4-6. Liquid-liquid and solid phase extractions usually involve time-consuming extraction steps such as drying and the addition of pH modifiers, which in this case may lead to undesirable acid-lactone interconversion. Several methods employed solid phase extraction for sample purification utilizing expensive automated [11,12,15,20] or manual manifold [16]. Plasma samples were concentrated more than two times in these methods to achieve adequate LLOQ [11,12,20,16]. Some methods have utilized traditional one or multi step liquid-liquid phase extraction for sample clean up using solvents such as ethyl-ether [10,18], methyl-tertiary-butyl ether [13] and ethyl acetate [17] and typically report low recovery. Lan et al. used tetrabutyl ammonium hydroxide (TAH) for ion pair liquid-liquid extraction to improve lipophilicity of RST; however, reported mean recoveries ranged within 47.5-62.2 % [21]. Moreover, addition of TAH to plasma may change its relatively neutral pH to a more alkaline pH; this condition may favor interconversion between lactone and acid forms in clinical samples. Most of the methods require a large sample volume (500 µL-1,700 µL) [10-14,8,21]. We report, for the first time, a fully validated LC-MS/MS assay for the simultaneous quantification of RST and its two metabolites, RST-LAC and DM-RST in a small volume (50 µL) of buffered human plasma.

Experimental

Reagents and chemicals

RST, RST-LAC, DM-RST and corresponding deuterium labeled internal standards (IS), d6-RST, d6-RST-5S-lactone and d6-DM-RST were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). HPLC-grade acetonitrile and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade deionized water was purified with a Milli Q50 (Millipore, Bedford, MA, USA) water purification system. Sodium acetate trihydrate (99%) and methanol d1 (CH₃OD) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was purchased from Pharmco Products Inc. (Brookfield, CT, USA). Drug-free human plasma, with heparin as an anticoagulant, was obtained from Bioreclamation Inc. (Westbury, NY, USA).

Chromatographic conditions

The analytical column was a Zorbax-SB Phenyl, Rapid-Resolution HT (2.1 mm×100 mm) with 3.5-μm particle size from Agilent Technologies (Wilmington, DE, USA), preceded by a 0.5 μm filter (Supelco, Bellefonte, PA). The column was maintained at 40 °C. Mobile phase, consisting of gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A), and 40% v/v methanol in acetonitrile (solvent B), was used at a flow rate of 0.35 mL/min for separation and rapid elution of the analytes from the extracted matrix within 6.0 min. The following mobile phase gradient scheme was used:

Total Time (min)	A (%)	B (%)
0.00	90	10
0.30	40	60
0.80	40	60
4.50	25	75
4.51	90	10
6.00	90	10

Mass spectrometric conditions

The LC-MS/MS system consisted of an Agilent Technologies 1200 series HPLC system comprising of a binary pump, an autosampler, a thermostatted column compartment, and a micro-vacuum degasser (Santa Clara, CA). The LC was coupled to an API 4000™ triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada), equipped with Turbo V™ source.

Triple-quadrupole MS/MS detection and the quantification of all the analytes were carried out in positive electrospray ionization mode using multiple-reaction monitoring (MRM) scan. Q1 and product ion scans were obtained by infusion of the individual analytes and the IS solutions using an infusion pump. The following MRM transitions (m/z , Q1 → Q3) were selected: RST (m/z , 482.2 → 258.2), d6-RST (m/z , 488.2 → 264.2), RST-LAC (m/z , 464.2 → 270.2), d6-RST-LAC (m/z , 470.2 → 276.2), DM-RST (m/z , 468.2 → 258.2), and d6-DM-RST (m/z , 474.2 → 264.2) for the best sensitivity and minimum interference from matrix components. The compound specific parameters i.e. declustering potential (DP), entrance potential (EP), collision cell exit potential (EXP) and collision energy (CE) were optimized to achieve maximum signal. Source parameters were set at curtain gas (CUR=20 psi), gas 1 (GS1=45 psi), gas 2 (GS2=20 psi), and collision gas (CAD=12 psi), ionspray voltage (IS=5500 V), and temperature

(TEM=450 °C) to achieve the optimal signal. Peak areas were obtained using AB SCIEX Analyst® 1.5.1 data processing software.

Preparation of standards and quality control solutions

Separate stock solutions of RST (1.00 mg/mL), and DM-RST (1.00 mg/mL) were prepared in methanol, while RST-LAC (0.50 mg/mL) was prepared in acetonitrile. Lactone forms ester by reaction with alcohols such as methanol. Thus, methanol was avoided in the preparation of the stock solution of the lactone compounds [22,23].

Intermediary individual stock solutions containing 100 µg/mL concentration of RST, DM-RST or RST-LAC were prepared in respective solvents and were used to spike calibrators and quality control (QC) samples (QCs) in buffered plasma kept on ice-water slurry. Drug-free heparin human plasma diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer was used to prepare calibrators and QCs samples.

Individual stock solutions of 1.0 mg/mL of d6-RST and d6-DM-RST were prepared in CH₃OD to avoid deuterium-hydrogen exchange. Stock solution of 1.0 mg/mL of d6-RST-LAC was prepared in acetonitrile. An intermediary IS solution containing 1 µg/mL of each IS was prepared in CH₃OD.

All stock and working standard solutions were stored at -20 °C until use. Eight calibration standards with concentrations ranging from 0.1 ng/mL to 100 ng/mL for RST and RST-LAC and 0.50 ng/mL to 100 ng/mL for DM-RST and QC samples at four concentration levels (0.10, 0.50, 7.50, and 76.0 ng/mL for RST and RST-LAC and 0.50, 2.50, 10.0 and 76.0 ng/mL for DM-RST) were prepared in drug-free buffered human plasma by using respective spiking solutions and were stored at -80 °C.

Sample extraction

Calibrators, QCs, zero standard, double blank and buffered patient samples were thawed on ice-water slurry and vortex-mixed thoroughly for 10 seconds. A 50- μ L aliquot of each buffered plasma sample was aliquoted into 1.5 mL polypropylene tube; all samples were treated with 200 μ L of 0.1% acetic acid in methanol (as a protein precipitation solvent) containing 2.00 ng/mL of d6-RST, 2.00 ng/mL of d6-RST-LAC and 20.0 ng/mL of d6-DM-RST except double blank. All tubes were vortex-mixed for 10 seconds and thereafter, centrifuged for 15 min at 14000xg and 4 °C. The supernatant was transferred to a clean glass vial and 15 μ L was injected onto LC-MS/MS.

Validation of the assay

The validation of the method was performed according to general recommendation guidelines for bioanalytical methods by the United States Food and Drug Administration [24]. Validation parameters including selectivity, sensitivity, accuracy, precision, recovery, matrix effect and stability were determined. The accuracy of calibration standards and QC samples ($n \geq 5$) should be within 85-115%, and imprecision should not exceed 15%, except at the LLOQ level, for which accuracy may be within 80-120% and imprecision should not exceed 20%.

Stability. Stability studies aimed to establish the conditions in which the degree of interconversion between acid and lactone was minimal. The first set of QC samples contained composite mixture of all the three analytes. In the second set, QC samples contained only RST and N-desmethyl RST. The third set comprised only RST-LAC. All three sets of QC samples were prepared at low-level quality control (LQC), 0.50 ng/mL for RST and RST-LAC and 2.50 ng/mL for DM-RST and high- level quality control (HQC), 76.0 ng/mL for all the three analytes concentration levels.

1. Post-preparative stability (autosampler stability): The capability of injecting processed samples after being kept in the autosampler at 4°C, was tested by reinjecting one of the three validation runs 24 h after extraction.
2. Bench-top stability: Triplicate LQC and HQC kept on ice-water slurry for 6 h were extracted along with freshly spiked calibrators.
3. Long-term stability: It was established for up to 1 month at -80 °C by analyzing six replicates of LQC and HQC, after 1 week and 1 month.
4. Freeze and thaw stability: Freshly spiked triplicates of LQC and HQC were prepared and stored at -80 °C for 24 h and were thawed on ice-water slurry. At the end of three successive freeze and thaw cycles, QC samples were extracted along with freshly spiked calibrators and QCs samples.

Linearity, accuracy, and precision. Eight-point calibration curves were obtained using 0.10, 0.20, 2.00, 10.0, 25.0, 50.0, 90.0, and 100 ng/mL for RST and RST-LAC and 0.50, 1.00, 5.00, 15.0, 30.0, 50.0, 90.0 and 100 ng/mL for DM-RST calibrators. All calibrators, six replicates of first set of QC samples at four concentration levels, double blank (without the IS), and zero standard (with the IS) were tested in three runs to evaluate intra-run and inter-run precision and accuracy of the method.

Limit of detection and quantification. LLOQ is defined as the lowest concentration of an analyte, which has at least five times higher response (signal-to-noise, S/N=5/1) compared with blank response and can be analyzed with an acceptable accuracy and imprecision (accuracy within 80-120% and imprecision $\leq 20\%$). Five serial dilutions of buffered plasma samples containing 0.500 ng/mL of RST and RST-LAC and 2.50 ng/mL of DM-RST were made by mixing equal volumes of spiked buffered plasma with

buffered blank plasma. Six replicates of each spiked sample were extracted, and S/N of peak and percent CV of the analyte/IS ratio were calculated. The sample with a S/N of at least five and percent CV below 20% for each analyte was selected as potential LLOQ for the validation.

Selectivity. The presence of endogenous matrix components that may interfere with the quantification of the analytes or the IS was evaluated using drug-free human buffered plasma containing heparin as anticoagulant (from six donors). The analytes and the IS responses in extracted selectivity samples were compared with the chromatograms obtained from LLOQ samples containing all the three analytes.

Recovery and matrix effect. Triplicates of LQC and HQC from first set of QC samples along with blank buffered plasma samples were extracted and were used to evaluate recovery and matrix effect (enhancement or suppression of ionization). Solutions with concentrations equivalent to 100% recovery of extracted LQC (0.5 ng/mL for RST and RST-LAC and 2.5 ng/mL for N-desmethyl RST) and HQC (15.2 ng/mL) containing all the analytes and the IS were prepared in water / 0.1% acetic acid in methanol, 1:4 (v/v; reference for matrix effect) and in extracted blank buffered plasma (reference for recovery).

In addition, matrix effect was also evaluated using a post-column infusion method. This test was performed by infusing a solution containing 20 ng/mL of the analytes and the IS prepared in solvent A/solvent B (30:70 v/v) at 10 μ L/min along with injections of the blank solvent, double blank matrix extract, and extracted HQC to identify the regions of ionization suppression. To investigate the potential interference from co-administered medications and their metabolites specific to the kidney transplant population, a pooled

buffered plasma sample was prepared from the three kidney transplant patients treated with tacrolimus, mycophenolic acid, and prednisone but not with RST. The pooled sample was obtained by mixing equal volumes of plasma (diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer) from samples collected 1, 2, 4, 8, and 12 h after the morning dose of the co-administered medications. The pooled buffered plasma sample was spiked with working standard solution of analytes at LQC level, and six replicates were extracted along with calibration calibrators, QCs samples, and a non-spiked pool.

Application of the method in a pharmacokinetic study

The assay was utilized in a preliminary PK study aiming to study disposition of RST in the kidney transplant recipients. The study protocol was approved by the Institutional Review Board of Rhode Island Hospital, Providence, RI, USA. Written informed consent was obtained from all subjects after verbal explanation of the study protocol. The blood samples were collected at various time points during a period of 12 h post-dose from the four kidney transplant recipients treated with 20 mg of a single oral dose of Crestor® along with a triple immunosuppressive regimen including oral tablets of tacrolimus, mycophenolate mofetil and prednisone as well as several other medications including proton pump inhibitors, antidiabetic agents, antibacterial agents, levothyroxin and cardiovascular drugs. Plasma sample was separated from whole blood with heparin as an anticoagulant at 1,500xg for 15 minutes and were immediately buffered with an equal volume of 0.1 M sodium acetate buffer (pH 4.0).

Results and discussion

Sample preparation, chromatography and MS detection

Various reverse phase analytical columns were tested. The required selectivity, as well as excellent peak shape (in terms of sharpness and symmetry), were achieved using a narrow bore Zorbax-SB phenyl column. This column exhibited good stability under a low pH mobile phase, and capability to decrease run times. Due to good peak focusing, the high S/N ratio obtained using this column enabled us to use a simple protein precipitation extraction without performing pre-concentration steps during sample clean up.

Methanol was chosen over acetonitrile as a precipitating agent because it provides excellent peak shape and resulted in an optimal signal of the analytes. Non-acidified methanol as a precipitating agent led to significant interconversion of lactone to acid form during the resident time of the extracted samples in the autosampler. Therefore, 0.1% glacial acetic acid in methanol was used to minimize the interconversion of lactone to acid and vice versa. Rapid elution of the analytes using a gradient mixture of mobile phase was obtained within a 6.0 min run time, including the re-equilibration time of the column. The retention times of RST, RST-LAC, and DM-RST were 3.3, 2.8 and 3.8 min, respectively (**Figure III-2**).

The MS/MS detection for RST can be achieved both in positive and negative ion mode, as it contains carboxyl and tertiary amine groups. Few of the published methods [10,15] suggested the use of a negative ion mode for the determination of RST to achieve a better LLOQ. We observed high signal intensity when using electrospray in positive ion mode, therefore, all analytes and the IS were monitored in positive ion mode. Rosuvastatin acid

gave its precursor ion at m/z 482.2. The principal product ion for RST was at m/z 258.2, with two minor fragments at m/z 300 and m/z 272. The most abundant product ion for DM-RST was at m/z 258.2 therefore, m/z 468.2→258.2 was chosen for the MRM transition. The structures of major product ions of RST and DM-RST monitored are shown in **Figure III-1b** [11,12]. For RST-LAC, the most intense product ion was at m/z 270.2 and minor was at m/z 282.2 and thus transition of m/z , 464.2→270.2 was selected. The proposed structure of major product ion of RST-LAC monitored is illustrated in **Figure III-1b**.

Method validation

The analytes were stable in the conditions described above. The post-preparative stability assessment proved the stability of extracted samples at 4°C for 24 h post-extraction, the anticipated resident time in the autosampler. No interconversion of analytes had occurred when they were monitored by re-injecting the HQCs spiked with each compound individually after 24 h. The analytes were found stable after three cycles of freeze and thaw and during bench top stability carried out on ice-water slurry (**Table III-1a**). Stability of all the three analytes was tested in various conditions such as non-buffered human plasma and buffered human plasma (human plasma diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer). Rosuvastatin acid and DM-RST were found stable in both kinds of plasma but approximately 25% loss of RST-LAC occurred in non-buffered plasma after 1 month storage at -80 °C (**Table III-1b**). Interconversion of acid to lactone or vice versa was also negligible in the buffered plasma. For the proposed assay validation, plasma was diluted 1:1 with 0.1 M, pH 4.0 sodium acetate buffer. No

interference was observed from endogenous components with the analytes and the IS in blank buffered plasma from six different donors, proving the specificity of the method.

The calibration curves obtained from the analyte/IS peak area ratios vs. nominal concentration were linear using weighted ($1/x^2$) linear least squares regression over the concentration range, with correlation coefficients (R) ≥ 0.9964 . The measured mean values ($n=3$) were within 92.4-111% of their nominal values for calibrators (**Table III-2**), 88.7-109% for intra-run QCs samples (results not presented), and 91.8-105% for inter-run QCs samples (**Table III-3**), which proves acceptable accuracy of the proposed method.

A LLOQ of 0.1 ng/mL was achieved for RST and RST-LAC, and 0.5 ng/mL for DM-RST. Chromatograms of an extracted LLOQ and blank plasma are shown in **Figure III-2a** and **2b**, respectively. The mean extraction recoveries for all the analytes were within 88.0-106%, as shown in **Table III-1a**. No significant matrix effect was observed for each analyte upon calculating the ratio of average area response of reference for recovery to reference for the matrix effect at LQC and HQC concentration levels.

Matrix effect is defined as the effect of co-eluting matrix components on ionization efficiency of the target analyte. Hence, suppression or enhancement of analyte response may have deleterious effects both on sensitivity and on the reproducibility of a particular assay. The integrity of resulting data could be adversely affected by lack of specificity, selectivity, accuracy and precision and may not be absolute [25]. Evaluation of the matrix effect is essential to insure reliability of quantitative assays using HPLC-MS/MS, and the integrity of PK data. Hence, the matrix effect was thoroughly studied as required by the FDA guidelines. We used the stable isotope labeled IS for each of the analytes, as they compensate reasonably well during variations in sample analysis. However, due to

their slightly different elution times compared with their respective non-labeled species, the deuterated internal standards IS may not always compensate for ionization enhancement or suppression due to co-elution of matrix endogenous components, such as phospholipids [26]. Phosphatidylcholine and lyso-phosphatidylcholine are the major phospholipids in human plasma causing significant matrix effect. Zhang et al. reported the key phospholipids in human serum extracts that can cause matrix effects by identifying the precursor ions generating fragment with m/z 184 [27]. Following the same technique, we determined the retention times of phospholipids with MRM transitions: Q1 m/z 496, 520, 522, 524, 544, 758 and 782 and Q3 m/z 184. We adjusted the chromatographic conditions to ensure that the analytes do not co-elute with the phospholipids.

To study the matrix effect, a post-column infusion of a solution containing the analytes and the IS, concomitantly injected with extracted blank matrix, was carried out. We monitored retention times of the major phospholipids, and we found that no significant ion suppression occurred at the retention times of the each analyte and the IS (data are not shown). The post-column infusion chromatogram shown in **Figure III-3a** indicates that RST and metabolites were eluted at retention times different from the retention times of key phospholipids. This shows that the signal intensity of each analyte of interest does not change in the region of their respective elution period (**Figure III-3b**). No significant matrix effect was observed for each analyte upon calculating the ratio of average area response of reference for recovery to reference for the matrix effect at LQC and HQC concentration levels.

The mean of the calculated concentrations of six LLQC samples prepared in pooled buffered plasma from patients treated with an immunosuppressive regimen and who did not receive RST was between 91–96% of the expected nominal concentration. No interfering peaks were eluting at the retention times of the analytes of interest. This demonstrates that tacrolimus, mycophenolic acid, prednisone and their metabolites do not affect the quantification of RST and its metabolites.

Application of the method

The present method was successfully applied in a clinical study for quantitative determination of RST and lactone metabolite over 12 h post-dose in the stable kidney transplant recipients after receiving a single dose of rosuvastatin. Individual plasma concentration-time profiles of RST and RST-LAC (12 h post-dose) are shown in **Figure III-4a and 4b**, respectively. The plasma concentrations for DM-RST are not shown in the figure as these concentrations were below the LLOQ for most of the time points.

Conclusion

In summary, we describe a reproducible, reliable, sensitive, and simple bioanalytical technique for the simultaneous determination of RST and metabolites in human plasma. The major advantages of the present method are the requirement for small plasma volume (50 μ L) and simple sample preparation.

The proposed validated bioanalytical technique accomplishes the required LLOQ, accuracy, and precision to be applied in various studies involving PK, drug metabolism, clinical pharmacology/clinical toxicology, bioavailability/bioequivalence and drug-drug interactions for accurate quantitative analysis of RST and its metabolites.

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Figure III-1a Structures of (i) rosuvastatin acid, RST (ii) N-desmethyl rosuvastatin, DM-RST and (iii) rosuvastatin-5S-lactone, RST-LAC.

Figure III-1b Structures of major product ions (i) rosuvastatin acid, RST and N-desmethyl rosuvastatin, DM-RST and (ii) rosuvastatin-5S-lactone, RST-LAC.

Figure III-2a Chromatograms showing the integrated peaks of rosuvastatin acid (i), N-desmethyl rosuvastatin (ii), and rosuvastatin-5S-lactone (iii) from extracted buffered calibration standard at lower limit of the quantification (0.1 ng/mL for rosuvastatin acid and rosuvastatin-5S-lactone and 0.5 ng/mL for N-desmethyl rosuvastatin).

Figure III-2b Chromatograms of extracted double blank buffered human plasma (RT represents retention time), rosuvastatin acid channel (i), N-desmethyl rosuvastatin channel (ii), and rosuvastatin-5S-lactone channel (iii).

Figure III-3a Chromatogram obtained with post-column infusion shows no matrix effect at the retention times of the analytes and the IS. Arrow indicates region where the signal of compounds infused post-column is suppressed during the elution of endogenous matrix components.

Figure III-3b Chromatogram of a blank sample injected while the analytes and the IS are infused post column. Arrow indicates region where the signals of the analytes and the IS infused post-column are suppressed during the elution of endogenous matrix components. From top to bottom, rosuvastatin-5S-lactone, d6-rosuvastatin acid, d6-rosuvastatin-5S-lactone, rosuvastatin acid, N-desmethyl rosuvastatin, d6-N-desmethyl rosuvastatin.

Figure III-4a Individual plasma concentration-time profiles of rosuvastatin acid of the stable kidney transplant recipients (n=4) who received a single oral dose of 20 mg of rosuvastatin.

Figure III-4b Individual plasma concentration-time profiles of rosuvastatin-5S-lactone of the stable kidney transplant recipients (n=4) who received a single oral dose of 20 mg of rosuvastatin.

Table III-1a. Results of stability studies and recovery (mean±%CV, n=3).

Analyte	Sample ID	Freeze & Thaw	Bench top	Autosampler stability	Recovery
Rosuvastatin (RST)	LQC	107±3.8	99.0±12	111±7.0	106±5.5
	HQC	98.7±2.1	105±3.0	107±0.7	88.0±4.1
Rosuvastatin-5S-lactone (RST-LAC)	LQC	101±14	98.3±12	95.6±3.4	98.8±10
	HQC	106±7.4	109±5.6	105±2.9	89.0±3.1
N-desmethyl rosuvastatin (DM-RST)	LQC	103±7.4	105±3.6	114±8.1	99.3±1.5
	HQC	106±3.0	108±3.9	107±2.5	90.2±4.5

Table III-1b. Stability studies after 1 week and 1 month at -80 °C (mean±%CV, n=3).

Analyte	Sample ID	after 1 week at -80 °C		after 1 month at -80 °C	
		Buffered plasma	Non-buffered plasma	Buffered plasma	Non-buffered plasma
Rosuvastatin (RST)	LQC	94.4±3.7	95.8±12	98.8±5.4	100 ±5.2
	HQC	95.4±5.9	97.6±5.5	103±3.0	93.4±4.5
Rosuvastatin-5S-lactone (RST-LAC)	LQC	99.4±3.1	81.5 ±12	105±1.9	74.8±6.1
	HQC	104 ±12	84.2 ±5.6	95.6±8.9	75.2±3.1
N-desmethyl rosuvastatin (DM-RST)	LQC	93.0±1.9	95.9 ±11	108 ±1.6	109±5.6
	HQC	92.7±5.7	95.9 ±5.7	94.6±0.6	92.0±8.9

% accuracy= (mean concentration/nominal concentration) x100

%CV calculated as (standard deviation/mean) x100

Table III-2. Summary of standards and calibration curve parameters from three individual runs.

Analyte		STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	R
Rosuvastatin (RST)	STD (ng/mL)	0.10	0.20	2.00	10.0	25.0	50.0	90.0	100	0.9964
	%Accuracy	102	96.6	93.0	101	97.3	111	102	97.8	
	%CV	4.8	10	2.7	5.5	9.7	3.6	2.1	9.6	
Rosuvastatin-5S-lactone (RST-LAC)	STD (ng/mL)	0.10	0.20	2.00	10.0	25.0	50.0	90.0	100	0.9980
	%Accuracy	102	96.3	101	102	98.2	101	98.5	102	
	%CV	0.6	0.9	5.9	3.9	4.6	5.8	1.2	9.6	
N-desmethyl rosuvasatin (DM-RST)	STD (ng/mL)	0.50	1.00	5.00	15.0	30.0	50.0	90.0	100	0.9980
	%Accuracy	100	101	92.4	99.4	100	101	107	99.2	
	%CV	1.4	1.8	6.2	4.0	4.5	5.2	6.3	5.6	

n = 3 (one replicate for each of the three validation runs)

% accuracy= (mean concentration/nominal concentration) x100

%CV calculated as (standard deviation/mean) x100

Table III-3. Summary of quality control samples from three individual runs.

Analyte	Quality control samples				
	QC (ng/mL)	0.10	0.50	7.50	76.0
Rosuvastatin (RST)	%Accuracy	96.8	105	97.5	99.0
	%CV	13	11	9.9	6.8
	QC (ng/mL)	0.10	0.50	7.50	76.0
Rosuvastatin-5S-lactone (RST-LAC)	%Accuracy	97.0	91.8	93.6	101
	%CV	10	12	8.1	6.3
	QC (ng/mL)	0.50	2.50	10.0	76.0
N-desmethyl rosuvasatin (DM-RST)	%Accuracy	102	101	95.3	93.1
	%CV	15	12	9.2	8.2

n = 18 (six replicates for each validation run)

% accuracy= (mean concentration/nominal concentration) x100

%CV calculated as (standard deviation/mean) x100

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MANUSCRIPT IV

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Development of a Combined Parent-Metabolite Population Pharmacokinetic Model for Atorvastatin Acid and its Lactone Metabolite: Implication of Renal Transplantation

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Running title: Population pharmacokinetic model for atorvastatin acid and its lactone metabolite

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Abbreviations: 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), Aspartate aminotransferase (AST), Atorvastatin acid (ATV), Atorvastatin lactone (ATV-LAC), Breast cancer resistant protein (BCRP), Chronic kidney disease (CKD), Conditional weighted residuals with interactions (CWRESI), Cytochrome P450 (CYP), Lactate dehydrogenase (LDH), Organic anion-transporting polypeptide (OATP), Paraoxonases (PON), P-glycoprotein (P-gp), Pharmacokinetics (PK), Single nucleotide polymorphism (SNP), UDP-glucuronosyltransferase (UGT), Visual predictive check (VPC)

Abstract

Aim: Atorvastatin calcium (Lipitor®, Pfizer Pharmaceuticals, NY) is an antihyperlipidemic agent from the statins class of drugs is the top-selling prescribed medication in the prevention and treatment of cardiovascular disorders. The aim of the present study was to develop a combined parent-metabolite population pharmacokinetic (PK) model of atorvastatin acid (ATV) and to investigate potential associations between clinical and demographic covariates on the population PK parameters.

Subjects and methods: Atorvastatin parent and metabolite plasma concentrations (1-11 per patient) of one hundred and thirty two, male or female non-transplant (diabetic, n=46; non-diabetic, n=53) or the stable kidney transplant recipients (diabetic, n=22; non-diabetic, n=11) who administered single or multiple oral doses of atorvastatin calcium were included in the study. Plasma concentrations of ATV and atorvastatin lactone (ATV-LAC) were quantified using previously validated liquid chromatography-tandem mass spectrometry assay. A total of 639 concentrations including both an acid (n=322) and lactone (n=317) forms of atorvastatin were analyzed by nonlinear mixed-effects modeling approach (NONMEM®, version 7.2.0) to identify the influence of patients' specific characteristics on PK properties of both the parent drug and its lactone metabolite. The first-order conditional estimation with interaction method was used to fit the data. The inter-subject variability was assessed using additive, exponential and proportional models. Likewise, the residual variability was evaluated using additive, exponential, proportional and combined additive-proportional error models. The influential covariates affecting pharmacokinetic parameters of both the parent and major metabolite were examined thorough PLT Tools (PLTsoft, San Francisco, CA, USA). A

stepwise covariate model building approach, forward addition (<3.84 , $p<0.05$, $df=1$) followed by backward elimination (≥ 7.9 , $p<0.005$) was used. The final model was validated using visual predictive check and nonparametric bootstrap analysis ($n=1000$).

Results: Pharmacokinetic characteristics of ATV and ATV-LAC were well described using a two-compartment model with first-order oral absorption and a one-compartment with linear elimination, respectively with some degree of interconversion between the two forms.

The inter-individual and the residual variability of pharmacokinetic parameters for both the parent drug and metabolite were modeled using an exponential and proportional error model, respectively. The population mean estimates of the final model parameters including absorption rate constant (K_a), apparent volume of distribution of ATV in the central compartment (V_2/F), apparent oral clearance of ATV to ATV-LAC (CL/F), apparent volume of distribution of ATV in the peripheral compartment (V_3/F), apparent inter-compartmental clearance of ATV (Q/F), apparent oral clearance of ATV-LAC to ATV (CL_M/F), apparent volume of distribution of ATV-LAC in the central compartment (V_M/F) and apparent inter-compartmental clearance of ATV-LAC (Q_M/F) were 0.771 h^{-1} , 481 L, 1126 L/h, 5462 L, 343 L/h, 506 L/h, 2349 L and 748 L/h, respectively.

The goodness-of-fit plots indicated good agreement between observed and individual as well as population predicted plasma concentrations of the parent and metabolite. In this study, we found renal transplantation, lactate dehydrogenase (LDH) liver enzyme and gender as the significant covariates, respectively for clearance and volume of distribution of ATV-LAC. Renal transplant recipients had 50% lower metabolite clearance compared

to non-transplant patients. The bootstrap analysis and visual predictive check demonstrated robustness of the present population pharmacokinetic model.

Conclusion: In summary, a combined parent-metabolite population pharmacokinetic model of ATV was developed. The pharmacokinetic analysis indicated significantly reduced clearance of lactone metabolite in the stable kidney transplant recipients. Greater risk of statin-related skeletal muscle toxicity is possibly because of decreased clearance of lactone metabolite. This finding should be taken into account while prescribing ATV treatment in the kidney transplant population who have additional co-morbidities and are on multiple interacting medications.

Introduction

Chronic kidney disease (CKD) is a major health issue that is common among the adult population in the United States. It is an irreversible and progressive disease, and if left untreated, chronic renal failure can advance to end stage renal disease. Chronic kidney disease is ranked as the eighth leading cause of death in the United States. At present, more than 20 million people are suffering from CKD in the United States [1]. Moreover, more than 35% of adults with diabetes, another growing global burden of diseases, have CKD [1].

Patients with CKD are at significantly greater risk of cardiovascular diseases mainly because of higher prevalence of diabetes mellitus, oxidative stress [2] and lipid abnormalities [3]. Statins [3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors] class of lipid lowering drugs are the choice of treatment for cardiovascular disorders in patients with or at a risk of CKD due to potential benefits attributed to its cardioprotective and nephroprotective properties [4]. Statins block cholesterol biosynthesis by reversible and competitive inhibition of the rate limiting enzyme, HMG-CoA reductase. A calcium salt of atorvastatin acid (ATV) (Lipitor®, Pfizer Pharmaceuticals, NY) is the best-selling statin of all time. It is administered as an active acid form, which undergoes extensive first-pass effect after oral administration [5]. Atorvastatin acid is extensively metabolized mainly by intestinal and liver cytochrome P450 (CYP) 3A4/5 and generates pharmacologically active *ortho* and *para* hydroxylated metabolites [6]. The parent drug and its active acid metabolites remain in equilibrium with their respective inactive lactone forms [7]. Atorvastatin lactone (ATV-LAC) has 83 fold greater affinity for CYP3A4 and exhibits higher metabolic clearance [6]. The

formation of lactone form is attributed to the glucuronidation metabolic pathway, which is mediated through UDP-glucuronosyltransferases (UGT) 1A1 and predominantly through 1A3 [8]. The enzymatic interconversion between acid and lactone forms is governed by esterases, paraoxonase (PON), UGT1A1 and 1A3 enzymes [8, 9].

Organic anion-transporting polypeptide (OATP) uptake transporters, OATP1B1 and OATP1B3, play a major role in the hepatic uptake of ATV and its metabolites [5]. Atorvastatin acid and its metabolites are also substrates of efflux transporters including P-glycoprotein (P-gp) [10] and breast cancer resistant protein (BCRP), which govern their intestinal absorption and liver elimination [11].

Statins are well tolerated; however, statin-related minor or severe skeletal muscle toxicity is the common adverse effect associated with statin therapy [12]. Several factors such as concomitant medications, metabolic disorders and hepatic or renal function aggravate the risk of statin-induced muscle complaints [13]. Hermann and colleagues [14], revealed significantly higher levels of ATV-LAC metabolite in patients experiencing statin-associated myopathy. Moreover, an *in vitro* study, using primary human skeletal muscle cells showed that ATV-LAC had a 14-fold higher potency to induce myotoxicity as compared to its acid form [15]. Additionally, a previous study has indicated lactone/acid ratio measurement as a specific diagnostic tool for statin-induced muscle toxicity [16].

Genetic propensity of an individual is a likely factor in the altered pharmacokinetics (PK) that may result in statin-associated myopathy [13]. Genetic polymorphism associated with OATP1B1 and OATP1B3 (*SLCO1B1* and *SLCO1B3*), P-gp (*ABCB1*), BCRP (*ABCG2*), bile salt export pump (*ABCB11*) and drug metabolizing enzymes (CYP and UGT) significantly affect ATV PK [17]. The extent of ATV-LAC formation is

significantly associated with UGT1A genetic polymorphisms that affect UGT1A3 expression [18] while the reverse reaction, ATV-LAC hydrolysis is influenced by PON1 and PON3 gene polymorphism [19].

Occurrences of severe rhabdomyolysis have been reported in patients with renal transplant because of concomitant use of statins with immunosuppressants and other medications [20-26] and associated co-morbidities. It is essential to investigate and interpret patients' demographic characteristics, genetic polymorphism as well as physiological and pathological characteristics that significantly alter pharmacokinetic properties of parent drug and its major metabolite in the kidney transplant recipients who require lifetime treatment with several concomitant drugs including immunosuppressants and who are at greater risk of developing adverse effects. Assessment of such factors will allow clinicians to select the right dose of ATV for optimum therapy.

The objective of this study was to develop a combined population PK model of ATV and ATV-LAC metabolite to allow the evaluation of chronic diseases, genetic polymorphisms as well as other patient specific characteristics.

Materials and methods

Study design

The open-label study was conducted in the kidney transplant and non-transplant recipients with and without diabetes mellitus at several study locations. The study protocol was approved by the Institutional Review Board of Rhode Island Hospital, Providence, RI, University of Rhode Island, Kingston, RI and South County Hospital, Wakefield, RI. The study procedures were explained verbally to the study participants, and thereafter their signed informed consent was obtained. All participants underwent normal physical examination on the day of the study and medical histories were documented for all study subjects. Except ten participants all were on steady state treatment with ATV in 5-80 mg dose range.

Patient population

The study population contained non-transplant (diabetic, n=46; non-diabetic, n=53) and the stable kidney transplant (diabetic, n=22; non-diabetic, n=11) recipients. Male (n=77) and female (n=55) participants over 18 years of age were included in the study. The subjects with congestive heart failure as defined by the New York heart association (NYHA) grades III and IV, liver dysfunction, pregnancy, undergoing active bacterial, fungal or viral infections, receiving CYP3A4/5 inhibitors or inducers were excluded from the study. The subjects with the kidney transplant were on a triple immunosuppressive drug regimen comprised of tacrolimus or sirolimus oral tablets, prednisone and mycophenolic acid either from mycophenolate mofetil (Cellcept™, Roche, Nutley, NJ) or mycophenolate sodium (Myfortic, Novartis, East Hanover, NJ).

Pharmacokinetic study

Blood samples (6 mL) were collected at various time points over 24 h post-dose. The blood was collected by venipuncture in Vacutainer® tube containing fluoride/potassium oxalate anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated by centrifugation at 1500xg and stored at -80°C until analysis. In addition to the PK study, additional blood samples were drawn to genotype each subject in EDTA Vacutainer® tube (Becton Dickinson, Franklin Lakes, NJ, USA) tube and stored in -80 °C until DNA extraction.

Quantitative analysis of ATV and ATV-LAC in plasma

Plasma levels of ATV and ATV-LAC were determined using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [27]. Briefly, both the analytes and their corresponding deuterium (*d*5) labeled internal standards were extracted from 50 µL of human plasma using 200 µL of 0.1% v/v glacial acetic acid in acetonitrile as protein precipitating solvent. The chromatographic separation of analytes was achieved within 7.0 min using a Zorbax-SB Phenyl column (2.1 mm × 100 mm, 3.5 µm) with a flow rate of 0.35 mL/min. The mobile phase consisted of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B). Mass spectrometry detection was carried out in positive electrospray ionization mode, with multiple reaction monitoring scan. The calibration curves for both analytes were linear ($R^2 \geq 0.9975$, n=3) over the concentration range of 0.05-100 ng/mL.

Genetic polymorphism study

Genomic DNA was extracted according to the procedure described in the manual using QIAGEN kit (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany). The subjects were genotyped for the following single nucleotide polymorphism (SNP):

CYP3A4 In6 C>T (rs35599367),

CYP3A5*3 219-237A>G (rs776746),

ABCB1 1236C>T (rs1128503), 2677G>T, A (rs2032582), 3435C>T (rs1045642),

ABCB11 1331C>T (rs2287622),

ABCC2 -24C>T (rs717620), 1249G>A (rs2273697), 3972C>T (rs3740066),

ABCG2 421C>A (rs2231142),

SLCO1B1 388A>G (rs2306283), 521C>T (rs4149056),

SLCO1B3 334T>G (rs4149117), 699G>A (rs7311358), 767G>C (rs60140950),

PON1 -108T>C (rs705379), -832G>A (rs854571), -1741G>A (rs757158) and

PON3 63C>T (rs13226149)

by allelic discrimination with a TaqMan Drug Metabolism Genotyping Assay on an Applied Biosystems 7300 Real-Time PCR (Applied Biosystems, Foster City, CA). *CYP3A4**1B -285A>G (rs2740574) and *UGT1A3**2 31T>C (rs3821242) and 140T>C (rs6431625) were genotyped by polymerase chain reaction amplification and the subsequent direct sequencing using Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster, CA) as described previously [28, 29].

Population PK analysis

Population pharmacokinetic analysis of plasma concentration-time profiles of ATV and its major metabolite, ATV-LAC were performed through nonlinear mixed effects modeling tool using NONMEM® (version 7.2.0, ICON Development Solutions, Ellicott City, MD, USA) as described previously with modifications [30, 31]. A graphical user interface, PLT Tools (PLTsoft, San Francisco, CA, USA) was used to facilitate NONMEM analysis.

To account differences in the molecular weight of ATV and metabolite, the concentrations of ATV-LAC were expressed as equivalent of the parent (the concentration of the metabolite was multiplied by the ratio of the molecular weights of ATV and ATV-LAC metabolite). Total of 639 concentrations (1-11 per patient) were included in the population PK analysis.

The basic PK model of parent drug without covariates was developed by evaluating various PK models including one-, two- and three- compartment as well as different estimation methods including the first-order (FO), the first-order conditional estimation (FOCE) and the FOCE with interaction (FOCE-INTER). The inter-subject variability was assessed using additive, exponential and proportional models. Likewise, the residual variability was evaluated using additive, exponential, proportional and combined additive-proportional error models.

The correct PK model was selected according to the following criteria: (i) a minimum value of the objective function; (ii) a low estimate of between- and within-subject variability; (iii) physiological plausibility of the estimates; (iv) goodness-of-fit and (v) normal distribution of weighted residuals.

After the development of the base model for ATV, a combined parent-metabolite model was developed by incorporating lactone concentrations based on the previously described mechanism [6]. The mixed effects were determined from a combined model. The influential covariates affecting the PK parameters of parent and metabolite were examined through PLT Tools. Potential covariates were added one at a time and reduction in the objective function value, and between-subject variability along with improvement in the fit were recorded. The effect of continuous covariates including age, body weight, glycosylated haemoglobin (HbA1c), glucose, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), γ -glutamyl transferase (GGT), cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, serum creatinine, total protein and serum albumin on the PK parameter was modeled using a power model scaled to median covariate value of the population. On the other hand, the effects of categorical covariates including the presence diabetes mellitus status or renal transplant as well as gender, ethnicity, concomitant use of prednisone, mycophenolic acid, sirolimus, tacrolimus and genetic polymorphism were modeled using a proportional model.

Forward stepwise addition of significant covariates was performed until no longer the objective function value was reduced (<3.84 , $p<0.05$, $df=1$). These covariates were then incorporated in the base model to form the full model. Removal of each covariate from the full model was carried out in backward elimination method, and increase in the objective function value was examined. The covariate that increased the objective

function value by 7.9 or more ($p < 0.005$) upon removal from the full model was retained in the model.

The validation of the final model was performed to assess its stability and predictive performance using nonparametric bootstrap analysis and visual predictive check (VPC). Visual predictive check is based on model-simulated data predictions including random effects, which visually examine the distribution of observed plasma concentration-time data and predictions at each sampling time. The final model and parameter estimates were used to simulate the data for 1000 virtual patients for VPC. A 95% prediction interval and median of the simulated concentrations were plotted along with observed plasma concentration–time data within 2.5th and 97.5th percentiles of the simulated model-simulated data. For bootstrap analysis, new datasets ($n=1000$) of the same size as the original dataset were generated by sampling random subjects with replacement from the original dataset and each of them was fitted using the final model. Median and 95% confidence interval of each PK parameter were calculated from bootstrap analysis.

Results

A total of 639 concentrations including both atorvastatin acid (n=322) and lactone metabolite (n=317) from 132 patients were available for the population PK modeling. The detailed demographic information of study participants is listed in **Table IV-1**. The mean oral dose of steady state treatment of ATV was 24.5 ± 19.0 mg. Moreover, a single 40 mg oral dose of Lipitor was administered to the ten kidney transplant recipients.

Initially, a base model for ATV was determined, thereafter ATV-LAC metabolite was added, and a combined parent-metabolite model was explored. Pharmacokinetic characteristics were best described using a two-compartment model with first-order oral absorption for ATV while a one-compartment with linear elimination was used for ATV-LAC as described previously [30, 31]. A schematic diagram representing the structural PK model for a combined parent drug and major metabolite used to model plasma concentration time profiles of ATV and ATV-LAC is illustrated in **Figure IV-1**. The model was developed based on the mechanism described by Jacobson et al. [6] with some interconversion of lactone to an acid form. However, for simplicity, no other complex pathways of elimination of ATV were incorporated in the model.

Subroutines ADVAN13 TRANS1, FOCE-INTER and double-precision model were selected as they helped to meet the selection criteria described in the method section. The interindividual and the residual variability of the PK parameters for both the parent drug and metabolite were modeled using an exponential and proportional error model, respectively.

Significant covariates for V_2/F , CL/F , Q/F , CL_M/F and V_M/F obtained after performing univariate analysis are listed in **Table IV-II**. Forward step wise addition of significant

covariates resulted in the full model incorporating status of the kidney transplant and LDH for CL_M/F , and gender for V_M/F . The final model obtained after performing backward elimination included the same covariates as obtained in the full model. The mean estimates of model parameters such as absorption rate constant (K_a), apparent volume of distribution of ATV in the central compartment (V_2/F), apparent oral clearance of ATV to ATV-LAC (CL/F), apparent volume of distribution of ATV in the peripheral compartment (V_3/F), apparent inter-compartmental clearance of ATV (Q/F), apparent total oral clearance of ATV-LAC (CL_M/F), apparent volume of distribution of ATV-LAC in the central compartment (V_M/F), apparent inter-compartmental clearance of ATV-LAC to ATV (Q_M/F) including bootstrap median and 95% confidence intervals of the final model are shown in **Table IV-III**. The final model for the typical values of various pharmacokinetic parameters is shown below:

$$K_a = \theta_1$$

$$V_2 = \theta_2$$

$$CL = \theta_3$$

$$V_3 = \theta_4$$

$$Q = \theta_5$$

$$CL_M = \theta_6 \times \theta_9^{(TRAT - 1)} \times \left(\frac{LDH}{167.5} \right)^{\theta_{11}}$$

$$V_M = \theta_7 \times \theta_{10}^{(GEND - 1)}$$

$$Q_M = \theta_8$$

The goodness-of-fit plots of the final model for ATV and ATV-LAC are presented in **Figure IV-2 and IV-3**, respectively. It can be depicted from the goodness-of-fit plots that all the points are close to the line of unity indicating good agreement between observed

and individual as well as population predicted plasma concentrations of parent and metabolite. The model is successful to describe the observed PK data. No specific trend was observed in the plot of conditional weighted residuals with interaction (CWRESI) versus time after a dose of ATV or population predicted concentrations for both parent and metabolite. The CWRESI are randomly distributed with a mean of close to zero and most of them lay within ± 2 units of the null ordinate of perfect agreement. The results of VPC evaluation contained simulated (n=1000) concentration-time profiles for both ATV and ATV-LAC at steady state and after a single dose are presented in **Figure IV-4**. The prediction interval (2.5th and 97.5th percentiles) incorporated most of the concentrations of both an acid and lactone forms of ATV and indicated that the final model provided an adequate fit to observed data.

Discussion

In this study, we found renal transplantation, LDH and gender as the significant covariates for clearance and volume of distribution of lactone metabolite, respectively.

The assessment of demographic and clinical characteristics of patients indicated the status of transplant as the most statistical significant covariate for CL_M , and it decreased between subject variability from 72% to 52%. Moreover, gender and LDH level influences V_M/F and CL_M/F , which reduced inter-patient variability from 78% to 53% and 72% to and 59%, respectively.

The effect of kidney transplant covariate was examined as a dichotomous variable (non-transplant=1 transplant=2). According to our study finding, the clearance of ATV-LAC is significantly decreased in patients with the kidney transplant. Nevertheless, an earlier published population PK study [30] reported a decrease in clearance of ATV-LAC metabolite in patients with diabetes mellitus. The study population only contained the stable kidney transplant recipients with or without diabetes mellitus. Lack of non-transplant subjects limited its ability to assess transplant as a covariate. The present study included of the stable kidney transplant (n=33) and non transplant (n=99) recipients.

Several different mechanisms could be proposed for lower metabolite clearance in transplant patients including oxidative stress, pro-inflammatory cytokines and elevated parathyroid hormone levels. Previous studies have reported coexistence of an inflammatory condition with elevated oxidative stress in the stable kidney transplant recipients [32-35]. Moreover, elevated parathyroid hormone levels remain in a significant number of renal transplant recipients after transplantation due to persistent secondary hyperparathyroidism [36-38].

A few *in vitro* studies have demonstrated down regulation of CYP3A4, the main metabolizing enzyme of ATV-LAC metabolite, by various cytokines such as interleukin IL-1 β , IL-6, [39] interferon- γ , and hepatocyte growth factor [40]. Furthermore, oxidative stress reduces CYP3A activity, and the more likely mechanism involves P450 metabolism of fatty acid hydroperoxides [41] and activation of nuclear factor- κ B leading to subsequent protein denaturation [42]. A previous *in vitro* and rat studies described the role of parathyroid hormone in the down regulation of CYP enzyme family [43].

Furthermore, transplant patients receive multiple interacting medications other than immunosuppressive therapy such azole antifungals, amiodarone, macrolide antibiotics, nefazodone, HIV protease inhibitors, mibefradil, digoxin, verapamil, nicotinic acid, warfarin, and diltiazem. Concomitant use of these medications may affect the metabolism and clearance of statins. Several cases of severe rhabdomyolysis have been reported in the kidney transplant recipients secondary to concurrent use of statin with immunosuppressant and other medications [20-22, 24-26].

The LDH level was also a significant covariate for the clearance of ATV-LAC. Likewise, prior published studies also found LDH and AST liver enzyme levels as significant covariates for the clearance of an acid and lactone forms of ATV, respectively [30, 31]. Atorvastatin acid undergoes significant first-pass effect and therefore altered liver functions may affect its oral bioavailability. As LDH level increases, the clearance of metabolite decreased exponentially, which suggests that patients with liver disorders may have greater systemic exposure of lactone metabolite. Infections and hepatic disorders are the common complications in the kidney transplant recipients attributed to impaired resistance because of immunosuppressive treatment [44, 45]. Furthermore, few studies

have indicated elevated levels of liver enzymes in the kidney transplant recipients with mycophenolate mofetil therapy [46, 47].

Significantly higher plasma levels of ATV-LAC metabolite in patients experiencing statin-related myopathy were observed previously [14]. Moreover, ATV-LAC had a 14-fold higher potency to induce myotoxicity as compared to an acid form [15]. These previous results indicate that patients with reduced clearance of ATV-LAC are more likely to experience ATV related muscle side effects such as myopathy. This finding warrants careful treatment of ATV in the kidney transplant population.

The population PK analysis showed significantly higher volume of distribution of metabolite in male as compared to female. The variability in the volume of distribution of several drugs between females and males is attributed to several factors including plasma volume, plasma proteins and tissue binding, fat proportion, body weight, organ blood flow, body composition and body mass index. Atorvastatin acid is a highly protein-bound drug; more than 98% binds to plasma proteins [5]. Moreover, main plasma proteins that bind to drugs in human plasma are altered by sex hormone and therefore protein binding is affected by gender differences resulting in variability in volume of distribution of drugs [48].

Genetic polymorphisms of various transporters and drug metabolizing enzymes (listed in the method section) involved in the disposition of ATV, which significantly affects its PK were evaluated. Single nucleotide polymorphism of PON1 and 3 enzymes were the only significant covariates found for the clearance of ATV-LAC in univariate search. Riedmaier et al. have demonstrated that polymorphisms of PON1 -108T>C and PON3, 63C>T esterases are associated with changes in ATV-LAC hydrolysis and increased

expression of PON1 mRNA in human liver tissues [19]. Furthermore, *SLCO1B1*, 521T>C and *ABCB11*, 1331C>T were significant covariates for the clearance of parent drug. However, statistical significance was not obtained for these covariates during multivariate analysis. Additional studies with diverse and large population are necessary to thoroughly assess the effects of SNP on the PK of both an acid and lactone forms of ATV.

The current study has several limitations: (a) Because of lack of a diverse population, probably it was not possible to thoroughly evaluate the effect of genetic polymorphism, age and ethnicity on the PK properties of ATV and metabolite; (b) We did not determine whether the transplant patients included in this study were affected by non-alcoholic steatohepatitis due to lack of noninvasive method; (c) Sparse sampling limited the ability of the model to accurately predict absorption phase of ATV; (d) absolute bioavailability of ATV was not determined due to unavailability of intravenous data.

Conclusion

In summary, a combined parent-metabolite population pharmacokinetic model of ATV was developed. The pharmacokinetic analysis reported 50% reduction in clearance of ATV-LAC in the kidney transplant recipients. This finding should be taken into account while prescribing ATV treatment in the kidney transplant population. However, a further study to investigate pharmacokinetic of ATV over a 24 h dosing interval in a large number of the kidney transplant and non-transplant patients is required to confirm this finding.

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Figure IV-1. Schematic diagram of the proposed structural combined parent-metabolite pharmacokinetic model for orally administered ATV and its major metabolite, ATV-LAC. A two-compartment model with first-order oral absorption and one-compartment with linear elimination was used to describe the PK of ATV and ATV-LAC, respectively with some interconversion between two forms. Elimination of ATV-LAC only from the central compartment was assumed and no other pathways of elimination of ATV were accounted. K_a = absorption rate constant; V_2/F = apparent volume of distribution of the parent drug in the central compartment; CL/F = apparent oral clearance of the parent drug to the metabolite; V_3/F = apparent volume of distribution of the parent drug in the peripheral compartment; Q/F = apparent inter-compartmental clearance of the parent drug; CL_M/F = apparent total oral clearance of the metabolite; V_M/F = apparent volume of distribution of the metabolite in the central compartment; Q_M/F = apparent inter-compartmental clearance of the metabolite to the parent drug.

Figure IV-2. The goodness-of-fit plots of the final model for atorvastatin acid: (a) OBS vs PRED (b) OBS vs IPRED (c) CWRESI vs PRED (d) CWRESI vs time post-dose at steady state. (e) CWRESI vs time after single dose. OBS = observed concentrations of atorvastatin acid; PRED = population predicted concentrations of atorvastatin acid; IPRED = individual predicted concentrations of atorvastatin acid; CWRESI = conditional weighted residuals with interaction. Logarithmic scale was used for clarity.

Figure IV-3 The goodness-of-fit plots of the final model for atorvastatin lactone: (a) OBS vs PRED (b) OBS vs IPRED (c) CWRESI vs PRED (d) CWRESI vs time post-dose at steady state. (e) CWRESI vs time after single dose. OBS = observed concentrations of atorvastatin lactone; PRED = population predicted concentrations of atorvastatin lactone; IPRED = individual predicted concentrations of atorvastatin lactone; CWRESI = conditional weighted residuals with interaction. Logarithmic scale was used for clarity.

Figure IV-4. The plots of the visual predictive checks for the final model from the simulated data for plasma concentrations of (a) atorvastatin acid at steady state (b) atorvastatin acid after single dose (c) atorvastatin lactone at steady state (d) atorvastatin lactone after single dose. Observed concentrations ($\circ$) compared to the 97.5th (upper dashed line), 50th (middle solid line) and 2.5th (lower dashed line) percentiles of the 1000 simulated datasets. Logarithmic scale was used for clarity.

Table IV-1. Characteristics of the patients included in the NONMEM analysis.

	Mean	Median	Range
<i>Demographics</i>			
Gender [male/female]	77/55		
Age [years]	56.44	58	19-77
Ethnicity [Caucasian/Hispanic/African American/others]	120/2/6/4		
Diabetes [non-diabetic/diabetic]	64/68		
Weight [kg]	90.1	88.6	45-145
Transplantation [non-transplant/transplant]	99/33		
Time post transplant [months]	56.15	47	3-140
Atorvastatin acid dose in mg	20	25.7	5-80
Prednisone dose [mg/day]	5.85	5	2.5-30
Mycophenolic acid dose [mg/day]	1000	1000	1000-2000
Tacrolimus dose [mg/day]	2.16	2	0.5-5
Sirolimus dose [mg/day]	1.79	2	1-3
<i>Clinical</i>			
Albumin [g/dl]	4.31	4.30	4-5
Protein, total [mg/dl]	6.87	6.90	6-8
Cholesterol, total [mg/dl]	169.5	168	73-285
High-density lipoproteins cholesterol [mg/dl]	53.2	50.5	17-147
Low-density lipoproteins cholesterol [mg/dl]	85.8	87.2	11-182
Triglycerides [mg/dl]	167.2	135.5	44-811
Creatinine [mg/dl]	1.13	1.04	1-3
Creatinine kinase [IU/l]	135.9	92.5	16-3118
Glucose [mg/dl]	132.6	113	65-601
Hemoglobin A1c [%]	6.34	5.8	5-12
Lactate dehydrogenase [IU/l]	173	167.5	81-324
Aspartate aminotransferase [IU/l]	24.9	23.5	12-93
Alkaline phosphatase [IU/l]	77.80	74	32-184
Alanine aminotransferase [IU/l]	24.31	22	6-85
Gamma-glutamyl transferase [IU/l]	37.29	24.5	6-492

Table IV-2. Significant covariates for the univariate and multivariate analyses.

	Δ Minimum objective function value	P values
<i>Univariate analysis</i>		
Effect of transplant status on CL _M /F	-41.4	<0.001
Effect of lactate dehydrogenase on CL _M /F	-36.2	<0.001
Effect of dose of tacrolimus on CL _M /F	-18.5	<0.001
Effect of PON3, 63C>T on CL _M /F	-9.0	<0.005
Effect of PON1 -108T>C on CL _M /F	-7.5	<0.01
Effect of dose of sirolimus on CL _M /F	-7.2	<0.01
Effect of alanine aminotransferase on CL _M /F	-7.2	<0.01
Effect of cholesterol on V ₂ /F	-19.2	<0.001
Effect of status of transplant on V ₂ /F	-14.5	<0.001
Effect of gender on V ₂ /F	-11.4	<0.001
Effect of triglycerides on V ₂ /F	-11.3	<0.001
Effect of ethnicity on V ₂ /F	-11.0	<0.001
Effect of dose of sirolimus on CL/F	-12.7	<0.001
Effect of <i>SLCO1B1</i> , 521T>C on CL/F	-11.0	<0.001
Effect of status of transplant on CL/F	-10.8	<0.005
Effect of g-glutamyl transferase on CL/F	-9.1	<0.005
Effect of dose of tacrolimus on CL/F	-8.9	<0.005
Effect of <i>ABCB11</i> , 1331C>T on CL/F	-6.8	<0.01
Effect of hemoglobin A _{1C} on CL/F	-5.3	<0.01
Effect of gender on V _M /F	-13.7	<0.001
Effect of total bilirubin on Q/F	-14.5	<0.001
Effect of total protein on Q/F	-7.4	<0.01
<i>Multivariate analysis</i>		
Effect of transplant status on CL _M /F	+30.0	<0.001
Effect of gender on V _M /F	+19.4	<0.001
Effect of lactate dehydrogenase on CL _M /F	+8.9	<0.005

CL_M/F, apparent total oral clearance of ATV-LAC; V₂/F, apparent volume of distribution of ATV in the central compartment; CL/F, apparent oral clearance of ATV to ATV-LAC; V_M/F, apparent volume of distribution of ATV-LAC in the central compartment; Q/F, apparent inter-compartmental clearance of ATV, PON, paraoxonase; *SLCO1B1*, gene encoding organic anion-transporting polypeptide; *ABCB11*, gene encoding breast cancer resistant protein

Table IV-3. The parameter estimates for the atorvastatin and metabolite population models and the results of bootstrap validation of the final model.

Model parameter (units)	Estimate	Bootstrap median (95% CI)
ka (hr ⁻¹)	0.771	0.762 (0.489, 1.02)
V ₂ /F (L)	481	456 (159, 1020)
CL/F (L/hr)	1126	1120 (723, 1550)
V ₃ /F (L)	5462	5220 (1990, 9410)
Q/F (L/hr)	343	341 (164,682)
CL _M /F (L/hr)	506	499 (401, 597)
V _M /F (L)	2349	2280 (1100, 4070)
Q _M /F (L/hr)	748	754(407, 1171)
Effect of lactate dehydrogenase on CL _M /F	0.479	0.496 (0.356, 0.689)
Effect of gender on V _M /F	0.327	0.337 (0.140, 0.691)
Effect of transplant on CL _M /F	-0.944	-0.979 (-1.66, -0.352)
<i>Random effects</i>		
Inter-individual variance (ω^2)		
ω^2 V ₂ /F	2.99	2.90 (0.544, 1.99)
ω^2 CL/F	0.340	0.332 (0.580, 0.934)
ω^2 Q/F	1.41	1.42 (0.105, 0.971)
ω^2 CL _M /F	0.531	0.520 (0.978, 2.17)
ω^2 V _M /F	0.712	0.677 (0.813, 1.38)
Intra-individual variability (σ^2)		
Atorvastatin acid (ng/mL)	0.373	0.373 (1.79, 2.12)
Atorvastatin lactone (ng/mL)	0.287	0.283 (1.73, 1.98)

CL_M/F, apparent total oral clearance of ATV-LAC; V₂/F, apparent volume of distribution of ATV in the central compartment; CL/F, apparent oral clearance of ATV to ATV-LAC; V_M/F, apparent volume of distribution of ATV-LAC in the central compartment; Q/F, apparent inter-compartmental clearance of ATV

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**Development of Physiologically-Based Pharmacokinetic Model for Atorvastatin
Acid and Rosuvastatin Acid with their Metabolites: *In vitro and in vivo studies***

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Abbreviations: Advanced Compartmental Absorption and Transit (ACAT), Area under the plasma-concentration time curve (AUC), Atorvastatin acid (ATV), Atorvastatin lactone (ATV-LAC), Breast cancer resistant protein (BCRP), Cytochrome P450 (CYP), Effective jejunal permeability (P_{eff}), Fraction unbound in plasma (f_{up}), Human liver microsomal fractions (HLMs), *In silico* effective jejunal permeability ($S+P_{\text{eff}}$), Liquid chromatography-tandem mass spectrometry (LC-MS/MS), Maximum plasma concentration (C_{max}), Organic anion-transporting polypeptide (OATP), P-glycoprotein (P-gp), Physiologically-based pharmacokinetic (PBPK), Rosuvastatin acid (RST), Rosuvastatin-5S-lactone (RST-LAC), Time to reach maximum plasma concentration (T_{max}), Tissue:plasma partition coefficients (K_{ps}), UDP-glucuronosyltransferase (UGT)

ABSTRACT

Aim: To develop a whole-body physiologically-based pharmacokinetic model to predict oral pharmacokinetics of atorvastatin acid as well as rosuvastatin acid with their respective metabolites in the stable kidney transplant patients with diabetes mellitus using *in silico* and experimentally measured input parameters.

Subjects and methods: A clinical study was conducted in the stable kidney transplant recipients with diabetes mellitus to obtain plasma concentration-time profiles of the parent drug and its metabolites. The kinetic parameters of metabolic clearance of atorvastatin acid previously determined using human liver microsomal fractions obtained from donors with diabetes mellitus were integrated in the model. *In vitro* dissolution studies of both statins were carried out in different pH media to assess pH dependent release profile. Simulations were implemented using the built-in Advanced Compartmental Absorption and Transit (ACAT) model and Population Estimates for Age-Related (PEAR™) physiology in GastroPlus™ software package (Simulations Plus, Inc., Lancaster, CA, USA). The essential input parameters to construct physiologically-based pharmacokinetic model of statins were measured experimentally, *in silico* predicted and/or obtained from the literature.

Results:

The simulated plasma concentration-time profiles of both statins and their metabolites were in a good correlation with mean plasma concentrations observed in study patients. Berezhkovskiy algorithm utilized to determine tissue distribution of perfusion-limited tissues as it successfully estimated observed high volume of distribution for both statins. Parameter sensitivity analysis revealed that systemic

exposure of both statins is most sensitive to change in intestinal transit time. The stochastic simulation performed using virtual trial feature of the software showed that the observed mean plasma concentration-time curves of both statins lie between 90% confidence interval, maximal and minimal simulated concentrations of ten virtual patients.

Conclusion: A whole-body physiologically-based pharmacokinetic model was constructed to simulate systemic exposure of orally administered atorvastatin acid and rosuvastatin acid with their metabolites in stable kidney transplant patients with diabetes mellitus. This study also demonstrated that disease specific *in vitro* metabolic clearance data are superior for an appropriate prediction of systemic exposure of the drug that undergoes extensive metabolism, which might have changed due to altered activity of drug metabolizing enzymes in specific disease state.

INTRODUCTION

The 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors (statins) are widely prescribed medication for treatment of hypercholesterolemia. Statins effectively decrease elevated levels of low-density lipoproteins and total cholesterol and hence the mortality rate has been drastically reduced in all over the world thereby, it remains the drug of choice to prevent coronary heart diseases.¹ Several lipid-lowering agents from statins class including, fluvastatin, atorvastatin, pravastatin, rosuvastatin, lovastatin, simvastatin pitavastatin are commercially available. Atorvastatin acid (ATV) is the largest-selling prescribed medication while rosuvastatin acid (RST) is the most efficacious among all statins.²

An active acid form of atorvastatin undergoes extensive first-pass metabolism after an oral administration (10-80 mg/day). The drug is highly bound to plasma protein (>98%) with an absolute oral bioavailability of 14%.¹ It is extensively metabolized mainly by intestinal and liver cytochrome P450 (CYP) 3A4 enzyme and forms pharmacologically active *ortho*- and *para*-hydroxylated (*o*-OH-ATV and *p*-OH-ATV) metabolites.³ The parent drug and its hydroxy acid metabolites remain in equilibrium with their respective pharmacologically inactive lactone forms (ATV-LAC, *o*-OH-ATV-LAC and *p*-OH-ATV-LAC),⁴ which have 83-fold higher affinity for CYP3A4 and exhibit higher metabolic clearance.^{3, 5} Thus, it has been postulated that the elimination of the parent drug occurs primarily via hydroxylation of ATV-LAC rather than hydroxylation of the parent drug.³ Moreover, previous study reported nearly equal systemic exposure of lactone metabolite as compared to an acid form.⁶ The formation

of lactone is attributed to glucuronidation via UDP-glucuronosyltransferase (UGT)1A1 and 1A3,⁷ low pH environment⁴ and intermediate product of coenzyme A-dependent pathway.⁸ On the other hand, lactones are hydrolyzed either chemically or enzymatically⁹ mainly by paraoxonase 1 and 3 and esterases.¹⁰⁻¹² The results of *in vitro* study from our group indicated a predominant role of CYP3A4 in the biotransformation of ATV-LAC,⁵ which is in consistent with previous findings.³ The parent drug and its metabolites mainly excreted into the bile and approximately 1% of the administered dose is excreted through renal route.¹

Rosuvastatin acid has a modest absolute bioavailability (approximately 20%). It is reversibly bound to 88% of plasma protein and also exhibits limited metabolism. Rosuvastatin acid mainly excreted unchanged (76.8% of the dose) in feces.¹³ It is primarily metabolized by isoenzyme CYP2C9 and to the minor extent through CYP2C19 and CYP3A4,¹⁴ thereby it generates pharmacologically active principal metabolite, N-desmethyl derivative.¹³ Furthermore, the formation of an inactive 5S-lactone metabolite of rosuvastatin acid (RST-LAC) occurs via the glucuronidation pathway.¹⁴ Fecal (approximately 90% of the dose) and renal (approximately 10% of the dose) are the major and minor route of excretion of RST, respectively.¹³

Hepatic uptake transporter, organic anion-transporting polypeptide (OATP) 1B1, plays a major role in an active uptake of ATV and its metabolites.^{15, 16} Atorvastatin acid and its metabolites are also substrates for efflux transporters including P-glycoprotein (P-gp),^{17, 18} and breast cancer resistant protein (BCRP),¹⁹ which govern their intestinal absorption and hepatic elimination. Similarly, RST is transported into the liver by

multiple uptake transporters including OATP1A2, OATP2B1, OATP1B3, OATP1B1.^{20, 21} It is also a substrate of various efflux transporters such as BCRP, P-gp, and MRP2 that mediate its intestinal and biliary excretion.^{19, 21, 22}

Statins are well tolerated by most patients although skeletal muscle-related toxicity is the most common reported side effect that develops after commencing statin therapy. Myotoxicity is ranging from a mild condition called myalgia to rare but potentially fatal rhabdomyolysis usually requiring hospitalization. Because of elevated incidence of statin-associated rhabdomyolysis, cerivastatin was voluntarily withdrawn from the United States market in 2001.²³ A meta analysis study conducted by the United States FDA including 130, 865 patients indicated three times higher incidence of fatal rhabdomyolysis in diabetic patients.²⁴ Significantly higher plasma level of ATV-LAC was found in atorvastatin-treated patients experiencing myopathy.²⁵ Moreover, an *in vitro* study conducted using human skeletal muscle cell culture exposed to an acid and lactone forms of statin showed that lactone form is more toxic to muscle cells than an acid form.²⁶ Furthermore, Skottheim et al. suggested that the lactone/acid concentration ratio can potentially be utilized as a specific diagnostic tool to determine patients at higher risk of developing statin-induced skeletal muscle toxicity.²⁷ Recently, we published both *in vivo* and *in vitro* studies that assessed the effect of diabetes mellitus on the biotransformation of ATV.⁵ We found 3.56 times reduced clearance of ATV-LAC metabolite in the stable kidney transplant recipients with diabetes mellitus⁵ because of down regulation of CYP3A4, the main metabolizing enzyme of ATV-LAC in this population.²⁸ It is essential to construct physiologically-based pharmacokinetic (PBPK) models for statins to simulate its distribution in the liver and skeletal muscle to

estimate their pharmacological and toxicological effects in patients with diabetes mellitus.

Recently, *in silico* prediction by advanced computation technology has been widely preferred for drug discovery and development to reduce the time and investment of research by decreasing the need of extensive experimental work. Mechanistic modeling tools integrate various information about the drug including estimated *in silico* physicochemical and biopharmaceutical properties, experimentally measured *in vitro* drug metabolism kinetic parameters and intestinal permeability to predict fraction absorbed, oral bioavailability, intestinal and hepatic extraction. Several software packages are available for mechanistic simulation including GastroPlus^{TM}, SimCYP, PKSim and IDEA.

GastroPlus^{TM} is a mechanistically based simulation software program design for the prediction of advanced absorption, physiologically-based pharmacokinetics/dynamics, *in vivo* and *in vitro* extrapolation and drug-drug interactions. GastroPlus^{TM} software uses the Advanced Compartmental Absorption and Transit (ACAT) model,²⁹ which is the modified version of previously elucidated the original Compartmental Absorption and Transit (CAT) model³⁰⁻³² to incorporate a whole-body related various physiological parameters to each interconnected hypothetical compartment. The ACAT model comprised of total eighteen compartments including nine enterocytes and nine gastrointestinal compartments. The gastrointestinal compartment starts from stomach; followed by six small intestinal compartments, caecum and colon. Each compartment defines several events including disintegration, dissolution, release,

precipitation, luminal degradation, absorption/desorption, gut wall metabolism, uptake of the drug, which are repeated in each compartment as it travels through different segments of the gastrointestinal tract. Furthermore, physiological values of gastrointestinal pH, mean transit times, permeability, fluid volume, dimension, bile salt and pore size are incorporated in each compartment. The inter-compartmental transit of the drug is illustrated by integrated series of linear and nonlinear differential equations. To perform each simulation, the drug specific input parameters including pH dependent solubility, logP, pKa, particle size, dose, *in vitro* kinetic data of enzymatic biotransformation and transport mechanisms were fed into the software.

The aim of the study was to build PBPK models for ATV and RST to predict plasma concentration-time profiles and tissue distribution of the parent drug and its metabolites in the stable kidney transplant subjects with diabetes mellitus using physiological and drug-related parameters.

MATERIALS AND METHODS

Study design

The open-label crossover pharmacokinetic study of ATV and RST was carried out in the diabetic stable kidney transplant recipients. The study protocol was approved by the Institutional Review Board of Rhode Island Hospital (Providence RI, USA). The procedures of the study were explained verbally to the study subjects, and thereafter their signed informed consent was obtained.

Patient population

The ten stable kidney transplant male (n=7) or female (n=3) subjects with documented diabetes mellitus (type 1 or 2) were recruited. The study participants comprised of mainly Caucasian population with 51 years of mean age and 90.39 Kg mean body weight. The patients were receiving a triple immunosuppressant regimen consisting of mycophenolic acid either from mycophenolate mofetil (Cellcept™, Roche, Nutley, NJ) or mycophenolate sodium (Myfortic, Novartis, East Hanover, NJ), tacrolimus and prednisone. Patients with severe clinical gastroparesis, liver diseases, pregnancy, abdominal surgery (within past 3 months), and history of inflammatory bowel disease were excluded from the study. Moreover, patients taking concomitant medications, which may alter gastric pH and gastrointestinal motility, were not recruited in the study.

Pharmacokinetic study

Study subjects were notified to stop their statin medication at least 3 days prior the study. Moreover, they were instructed to fast the night before the study day. The participants were subjected to routine physical examination including height, weight

and blood pressure measurement on each day of the study. Thereafter, patients administered a single 20 mg oral dose of rosuvastatin calcium (Crestor®, AstraZeneca Pharmaceuticals LP, DE, USA) along with their routine medications including immunosuppressant regimens. All patients were served two standardized diabetic meal at the end of 6 h and 10 h after administration of statin medications including morning low fat breakfast bar. Blood samples were collected in vacutainer tubes containing lithium heparin as an anticoagulant at various time points (0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 h) post-dose. The blood samples were centrifuged immediately at 1400xg and plasma samples were diluted 1:1 with 0.1 M, pH 4.0 sodium acetate.³³ The study samples were stored at -80°C until analysis. The similar study procedures were followed by the same participants after administering a single 40 mg oral dose of atorvastatin calcium (Lipitor®, Pfizer Inc, NY, USA) after at least two weeks. For 12 h post-dose pharmacokinetics study of ATV, blood samples were collected in vacutainer tubes containing fluoride/potassium oxalate as an anticoagulant.³³ The blood samples were immediately centrifuged at 1400xg and plasma samples were stored at -80°C until analysis.

Quantitative analysis of ATV, RST and their metabolites in human plasma

Plasma levels of ATV and its metabolites (ATV-LAC, *o*-OH-ATV and *o*-OH-ATV-LAC) were determined using previously validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay.³³ Briefly, the analytes and corresponding deuterium (*d*5) labeled internal standards were extracted from 50 µL of human plasma using 200 µL of 0.1% *v/v* glacial acetic acid in acetonitrile as protein precipitating solvent. The chromatographic separation of the analytes was achieved using a Zorbax-SB Phenyl

column (2.1 mm × 100 mm, 3.5 μm) within 7.0 min using a flow rate of 0.35 mL/min. Mobile phase consisting of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B). Mass spectrometry detection was carried out in positive electrospray ionization mode, with multiple reaction monitoring scan. Calibration curves for all the analytes over the concentration range of 0.05-100 ng/mL were used.³³ Likewise, quantitative determination of RST and its metabolite, RST-LAC in human plasma was carried out using a previously reported validated LC-MS/MS assay.³⁴ In brief, all the analytes and the corresponding deuterium-labeled (*d*6) internal standards were extracted from 50 μL of buffered human plasma by protein precipitation. The analytes were chromatographically separated using a Zorbax-SB Phenyl column (2.1 mm×100 mm, 3.5 μm). The mobile phase comprised of a gradient mixture of 0.1% v/v glacial acetic acid in 10% v/v methanol in water (solvent A) and 40% v/v methanol in acetonitrile (solvent B). The analytes were separated at baseline within 6.0 min using a flow rate of 0.35 mL/min. Mass spectrometry detection was carried out in positive electrospray ionization mode. Calibration curves for both the analytes were linear ($R \geq 0.9964$, $n=3$) over the concentration range of 0.1–100 ng/mL for RST and RST-LAC. Mean extraction recoveries ranged within 88.0–106%. Intra- and inter-run mean percent accuracy were within 91.8–111%, and percent imprecision was $\leq 15\%$. Stability studies revealed that all the analytes were stable in matrix during bench-top (6 h on ice–water slurry), at the end of three successive freeze and thaw cycles and during storage at -80°C for 1 month.

***In vitro* dissolution study**

In vitro dissolution studies of ATV and RST were carried out using USP apparatus II-paddle type, LID-8D dissolution tester (Vanguard Pharmaceutical Machinery, Inc., Spring, TX, USA). A single tablet dosage form of 40 mg of ATV and 20 mg of RST per dissolution bath was used for dissolution experiments. The dissolution study was performed using 0.1 M hydrochloric acid (1.2 pH) and 0.05 M ammonium acetate buffer with 3, 4.5, 6.8 and 8 pHs as dissolution media. The pH of each dissolution media was adjusted using glacial acetic acid or ammonium hydroxide. The dissolution of ATV was carried out at 75 rpm speed, and samples were collected at 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. Similarly, dissolution of RST was carried out at 50 rpm speed and samples were collected at 10, 20, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min. The volume of dissolution medium was 900 mL, and it was replaced with fresh buffer equilibrated at 37 °C after each sampling. All dissolution experiments were carried out in triplicates. The collected samples were immediately kept on dry ice to minimize interconversion between acid and lactone forms and were analyzed using HPLC-UV assay with slight modification of the methods described by Macwan et al.³³,

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Physiologically-based pharmacokinetic (PBPK) model structures

Computer hardware and the software

The simulations for disposition and absorption were performed using GastroPlus^{TM} version 8.0.0002 simulation software (Simulations Plus, Inc., CA, USA) on a Dell laptop computer with Intel core i3 CPU M350 (2.27 GHz).

A whole-body disposition

A whole-body PBPK modeling approach was utilized through the ACAT model in PBPK^{TM} module of the software. A separate whole-body PBPK models were built for the disposition of the parent drug and each metabolite, and these were coupled via gut/hepatic metabolism and/or transport, allowing simultaneous simulations of both the parent drug and its metabolites. Built-in age, body weight, height, and gender-dependent Population Estimates for Age-Related physiology^{TM} (PEAR) module was used to generate human physiology for 51 years (mean age of study subjects) old an American male patient with body weight of 90.4 kg (mean weight of study subjects) based on the study population. The PEAR is generated based on data from the National and Nutrition Examination Survey.³⁵ The volume, weight and rate of blood perfusion of each body tissue were computed from default balance model.

Prediction of disposition parameters of ATV

Intestinal permeability

Experimentally measured human jejunal permeability of many drugs using *in vivo* perfusion methods is very limited. Computer based *in silico* prediction based on chemical structure of the drug is widely preferred. The permeability of the drug changes as it transit through different regions of the gastrointestinal tract due to alteration of several parameters including regional pH, surface area, ionization, density of villi and microvilli. Built-in optimized logD model scales regional permeability based on these parameters. Furthermore, regional gastrointestinal absorption of the drug is determined by human *in silico* effective jejunal permeability ($S+P_{\text{eff}}$) and the concentration gradient at the apical membrane of the enterocytes. Integrated ADMET^{TM} predictor module of GastroPlus^{TM} computed human $S+P_{\text{eff}}$ based on built-

in quantitative structure-property relationship model. Apparent jejunal permeability of ATV was studied by rat infusion technique³⁶ and the calculated human effective jejunal permeability (P_{eff}) value was equivalent to estimate of ADMET^{TM} predictor.

CYP3A4, UGT1A1/3, OATP1B1 and P-gp kinetic constants

Atorvastatin acid is predominantly metabolized in the liver and gut mainly by CYP3A4 enzyme. Recently, we determined enzymatic metabolism kinetics of an acid and lactone forms of atorvastatin using human liver microsomal fractions (HLMs) obtained from donors with and without diabetes mellitus.⁵ Briefly, microsomal fractions were prepared as illustrated previously²⁸ from human livers obtained from donors with and without diabetes mellitus (Xenotech LLC, Lenexa, KA, USA) and were stored at -80°C until analysis. Several concentrations of an acid and lactone forms of atorvastatin were incubated with prepared HLMs. At the end of incubation, ice-cold acetonitrile containing an internal standard was added to cease the reaction. The samples were centrifuged, and the supernatant was injected onto an analytical column. The quantitative determination of ATV and its five metabolites were performed using previously described LC-MS/MS assay with slight modifications.³³ Enzyme kinetic parameters (K_m and V_{max}) of ATV and ATV-LAC were calculated using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

For the simulations, *in vitro* average K_m ($5.21\ \mu\text{M}$) and the sum of V_{max} values ($2593.7\ \text{pmol/min/mg}$ microsomal protein) were used as input parameters for the formation of CYP3A4 mediated *para*- and *ortho*-hydroxylated acid metabolites that obtained upon incubation of ATV with HLMs from livers of diabetes donors.⁵ Moreover, from available literature data, an average K_m of $14\ \mu\text{M}$ ^{7, 37} and fitted V_{max} values for the

formation of ATV-LAC metabolite mediated mainly through UGT1A3 and UGT1A1 enzymes were integrated in the model to account for glucuronidation mediated metabolism of ATV. The enzyme kinetic parameters of CYP3A4 and UGT1A1 were applied to both the gut and liver while UGT1A3 was only applied to liver tissue due to negligible expression in the intestine.³⁸ All *in vitro* $V_{\max}$ values were scaled to *in vivo* $V_{\max}$ based on either GastroPlus^{TM} built-in or literature based absolute quantification data of each enzyme. To incorporate OATP1B1 mediated hepatic uptake clearance of ATV, an average of 0.85 μM and 5.225 pmol/min/mg^{39, 40} of *in vitro* K_m and $V_{\max}$, respectively were fed into the model. The fitted $V_{\max}$ and average K_m of 112.5 μM ^{17, 18} were applied only to canalicular membrane of liver for P-gp efflux transporter. To run simulations, liver was considered a permeability-limited tissue, on the other hand, the rest of the body tissues were perfusion-limited.

Volume of distribution

PBPK^{TM} plus module of GastroPlus^{TM} was utilized to determine tissue:plasma partition coefficients (K_{ps}) of perfusion-limited tissues (non-hepatic tissues) using Berezhkovskiy's algorithm,⁴¹ which calculated K_{ps} based on free concentrations of the drug in plasma/tissue as well as the volume fractions of lipids including phospholipids and neutral lipids, and body water. However, the partition of the drug between blood and interstitial space for permeability-limited liver was determined using Poulin and Theil method (extracellular)^{42, 43} that calculated K_{ps} based on hematocrit and the fraction unbound in plasma as well as in tissues.

Systemic clearance

Atorvastatin acid is significantly metabolized in both the gut and liver mainly by CYP3A4 enzyme. Furthermore, it is a substrate of hepatic OATP1B1 uptake transporter that can modulate clearance of the drug, which is eliminated extensively by hepatic metabolism; therefore, hepatic elimination plays a major role in overall clearance of ATV. In the present study, as mentioned previously, CYP3A4 mediated *in vitro* metabolism of ATV in HLMs obtained from livers of diabetes donors' were interpolated to the model to illustrate metabolic clearance of ATV and the formation of the two hydroxylated metabolites. The kinetic parameters were converted to per mg protein using a conversion factor of 0.37 nmol total CYP/mg protein.⁴⁴

Scaling of *in vitro* metabolic clearance data to *in vivo* clearance was based on measured amount of microsomal protein per gm liver and average liver size. The *in vitro* $V_{\max}$ values were scaled to *in vivo* by considering 111 pmol of CYP3A4/mg microsomal protein,⁴⁵ 17.3 pmol of UGT1A3/mg microsomal protein,⁴⁶ 33.2 pmol of UGT1A1/mg microsomal protein,⁴⁶ 38 mg of microsomal protein/gm of liver tissue⁴⁷ and 1400 gm of average weight of liver.⁴⁸ All the values were corrected for non specific binding to microsomes by *in vitro* unbound fraction of 40.5%⁴⁹ for accurate prediction of metabolic clearance.

Prediction of in vivo dissolution rate

Actual *in vivo* solubility could be different from *in vitro* aqueous buffer solubility because of presence of bile salt and lipids in the intestinal fluids. Biorelevant solubility was determined to account for altered solubility with changes in bile salt concentrations in different regions of intestine. The most accurate prediction of absorption of the lipophilic drugs can be made by measuring biorelevant solubility in

biologically relevant gastrointestinal media such as fasted state simulated intestinal fluid (FaSSIF), fed state simulated intestinal fluid (FeSSIF) and fasted state simulated gastric fluid (FaSSGF). An equilibrium solubility of ATV measured in FaSSIF was 0.3 mg/mL,⁵⁰ and it was used to run simulations along with *in silico* solubility of 0.0011 mg/mL and 0.69 mg/mL in simulated gastric fluid (SGF) and FeSSIF dissolution medium, respectively due to unavailability of experimentally measured values. We utilized default Johnson dissolution model in GastroPlus^{TM} to predict dissolution rate.

Gastrointestinal absorption model

The default human fed physiology and optimized logD model SA/V 6.1 in the ACAT model of the software was selected to calculate the changes in the rate of passive absorption from gut lumen to enterocytes of dissolved drug as it transit through the gastrointestinal tract. The default properties of individual gut compartment such as pH, transit time, volume, length, radii, bile salt concentration, surface area enhancement factors, and radius of pores between two adjacent cells were implicated in the model. The ACAT model parameters are listed in **Table V-1**. Liver blood flow rate was increased to 120 L/h for fed condition.⁵¹

The absorption rate coefficient of the drug in each segment of the gastrointestinal tract was determined based on human $S+P_{eff}$. The relative distribution of CYP3A4 and P-gp in nine compartments of the gastrointestinal tract in the ACAT model was explained previously.⁴⁴ The quantitative expression of UGT1A1 in different segments of the gastrointestinal tract was assumed based on available limited information.³⁸

Prediction of disposition parameters ATV major metabolites

ATV-LAC

The pH dependent distribution coefficients for tissue partition were calculated based on experimentally measured $\log D_{7.0}$ of 4.2 previously reported by Ishigami et al.⁵² Other properties such as pK_a , f_{up} (fraction unbound in plasma), blood/plasma concentration ratio and human P_{eff} estimated by ADMET^{TM} were interpolated to the model. Our recent work demonstrated metabolic clearance of ATV-LAC and the formation of *o*- and *p*-OH-ATV-LAC metabolites by using HLMs obtained from livers of donors with diabetes mellitus.⁵ An average K_m of 6.0 μM ⁵ and fitted V_{max} were utilized to simulate concentration-time profile of *o*-OH-ATV-LAC. An *in vitro* K_m value was corrected for unbound fraction using default Austin factor.

***o*-OH-ATV**

In silico estimates of ADMET^{TM} predictor were implicated in the model due to unavailability of experimental data. Previous work identified glucuronide metabolite of *o*-OH-ATV in bile of rat and dog.⁵³ Furthermore, to reflect low permeability of glucuronide metabolite, human $S+P_{eff}$ was decreased from 1.12×10^4 to 0.8×10^4 cm/s. Moreover, like ATV, *o*-OH-ATV metabolite is also a substrate of hepatic OATP1B1 uptake and intestinal P-gp efflux transporter.⁵⁴ Fitted K_m and V_{max} constants for OATP1B1 and P-gp were applied to basolateral side of liver and apical side of gut, respectively due to lack of data.

***o*-OH-ATV-LAC**

Due to deficiency of experimentally measured input parameters, *in silico* estimates by ADMET^{TM} predictor were included for mechanistic modeling of *o*-OH-ATV-LAC. Unlike, *o*-OH-ATV further metabolic clearance of *o*-OH-ATV-LAC was not reported in the literature. To simulate limited absorption of *o*-OH-ATV-LAC, fitted K_m and

$V_{\max}$ parameters for P-gp efflux transporter at apical side of gut were included. An active hepatic uptake of *o*-OH-ATV-LAC was not considered due to relative more lipophilicity than an acid form.

Prediction of disposition parameters of RST

Intestinal permeability

Similar to ATV, human $S+P_{\text{eff}}$ determined by ADMET^{TM} predictor module was incorporated to run all simulations for prediction of RST oral absorption and pharmacokinetics.

UGT1A1, UGT1A3, OATP and BCRP pharmacokinetic constants

Rosuvastatin acid exhibit minimal hepatic metabolism, however, to simulate concentration-time curve of lactone metabolite that forms via glucuronidation, enzyme kinetic constants for UGT1A1 (applied to the liver and gut) and 1A3 (only applied to liver) available in literature were integrated in the model¹⁴. The significant contribution of hepatobiliary transport in disposition of RST mediated by several sinusoidal hepatic OATP uptake transporters and BCRP canalicular efflux transporter were incorporated in mechanistic modeling. The simulations utilized an average K_m (4.93 μM) and the sum of $V_{\max}$ (12.3 pmol/min/mg) of OATP2B1, OATP1A2 and OATP1B3 reported by Ho et al.²⁰ Liver was considered a permeability-limited tissue. Similarly, for BCRP efflux transporter, $V_{\max}$ of 304 pmol/min/mg and an average K_m value of 0.473 μM ^{22, 55} obtained from the literature²¹ were added to canalicular membrane of liver tissue. To simulate limited intestinal absorption, an average K_m of 0.473 μM ^{22, 55} for BCRP efflux transporter was fed to gut tissues along with fitted $V_{\max}$.

Volume of distribution

Mechanistic tissue composition equations defined by Berezhkovskiy⁴¹ were selected for calculation of K_{ps} from blood into cells for perfusion rate limited tissues because it provided better estimate of observed high volume of distribution as compared to Rodgers and Rowland method.^{56, 57}

Berezhkovskiy assumed homogenous distribution of the drug into tissue and plasma. It calculated tissue-plasma partition coefficient based on experimentally measured or *in silico* predicted compound specific biopharmaceutical properties mainly pK_a, logP, f_{up}, blood/plasma concentration ratio and species-specific tissue composition. The partition of the drug between blood and interstitial space for permeability-limited liver tissue was estimated using Poulin and Theil method (extracellular).^{42, 43}

Systemic clearance

Limited metabolic clearance of RST through lactonization mediated by glucuronidation pathway involving mainly UGT1A1 and 1A3¹⁴ enzymes was integrated in the ACAT model for gut and PBPK tissues to estimate its overall clearance. As mentioned in the previous section, *in vitro* enzyme kinetic constants were scaled to *in vivo* by assuming 17.3 pmol of UGT1A3/mg microsomal protein,⁴⁶ 33.2 pmol of UGT1A1/mg microsomal protein,⁴⁶ 38 mg of microsomal protein/gm of liver tissue⁴⁷ and 1400 gm of average weight of liver.⁴⁸ Similarly, *in vitro* data for multiple OATP transporters mediated active uptake clearance were applied to permeability-limited liver tissue.²⁰ The value was scaled to *in vivo* assuming 85 mg membrane protein/gm liver.⁵⁸ The relative regional distribution of BCRP efflux transporter at the apical side of gut was studied earlier by Englund et al.⁵⁹ Renal clearance was estimated from f_{up} and glomerular filtration rate.

Prediction of in vivo dissolution rate

Experimentally measured *in vitro* dissolution release vs time profile was incorporated and default Johnson dissolution model was employed. *In silico* estimates of solubility in SGF, FaSSIF and FeSSIF dissolution medium were used for mechanistic modeling due to lack of data.

The physiologically-based ACAT model of the human gastrointestinal tract

Because of cross over study design, the same subjects received 20 mg of Crestor®; therefore, human fed physiological model was selected as described in the prior section of atorvastatin acid. The ACAT physiological model parameters employed in the simulation of RST and RST-LAC are showed in **Table V-2**.

Gastrointestinal absorption model

In silico human P_{eff} was chosen for PBPK model building of RST. Lactonization of RST is mediated by UGT1A1 enzyme in both the gut and liver. The relative expression of UGT1A1 in each compartment of the gastrointestinal tract in the ACAT model was determined based on prior information.³⁸ Furthermore, intestinal absorption at apical side that is governed by BCRP efflux transporter was included by feeding its expression levels throughout the gastrointestinal tract⁵⁹ along with kinetic constants.

Prediction of disposition parameters RST metabolite

RST-LAC

Because of unavailability of human P_{eff} and other biopharmaceutical properties such as pK_a , f_{up} , blood/plasma concentration ratio and reference solubility, estimates of ADMET^{TM} predictor module were used to perform simulations. The $\log D_{7.4}$ of 1.2 was reported previously.⁶⁰

Virtual trial simulations

The population simulator mode in GastroPlus^{TM} permits the user to evaluate the combined effects of inter-individual variability in population physiology and the predicted disposition parameters along with formulation/compound specific properties using a whole-body PBPK model linked with Monte Carlo simulation. A stochastic simulation generates variability in each variable by random sampling of each variable parameter from predefined distribution for each simulation. The trial was performed for ten virtual patients, which is equal to the number of patients participated in our clinical study. The virtual trial studies were conducted using GastroPlus^{TM} standard physiological conditions and compound-specific characteristics, which were sampled randomly from log-normal distributions via Monte Carlo method. Moreover, predefined coefficient variations that randomly created based on their mean values in GastroPlus^{TM} were used in simulations.

Parameter sensitivity analysis

Parameter sensitivity analysis feature of GastroPlus^{TM} was utilized to assess the sensitivity of predicted disposition parameters to key input elements. The key input parameters, which are likely to affect plasma concentration-time curve based on available literature information were separately varied over a broad range of values to conduct parameter sensitivity analysis.

Sensitivity of pharmacokinetic parameters of ATV including maximum plasma concentration ($C_{\max}$), time to reach $C_{\max}$ ($T_{\max}$) and area under the plasma concentration-time curve (AUC) were assessed with changes in transit time of the stomach and small intestine, stomach pH, kinetic parameters of CYP3A4 and

OATP1B1. Similarly, using PBPK model of RST, the effect of variations in input parameters including transit time of the stomach and small intestine, stomach pH, K_m and V_{max} of BCRP and OATP1B1 on simulated pharmacokinetic parameters was evaluated.

Unit converter

Metabolism and transporter module in GastroPlus^{TM} comprises a convenient unit converter. Built-in unit converter is a conversion tool for simple unit conversion that converts units of all K_m (mg/L) and V_{max} (mg/s/mg-enzyme) values obtained from the literature to get suitable units of inputs for simulations in GastroPlus^{TM}.

RESULTS

PBPK modeling of ATV and its three metabolites

GastroPlus^{TM} default PBPK^{TM} module along with *in vitro* clearance data was used to describe oral absorption and pharmacokinetics of ATV and its three metabolites.

In silico or experimentally measured physiochemical/biopharmaceutical properties

Intestinal permeability

The ADMET^{TM} predictor estimated value of intestinal permeability based on chemical structure of the compound predicted the disposition parameters of ATV.

pKa, blood/plasma concentration ratio, fup and logD

Several biopharmaceutical properties of the drug including blood/plasma concentration ratio, fup and logD strongly influence hepatic bioavailability and thus hepatic clearance.

Blood/plasma concentration ratio of 0.25 for ATV reported in the literature was selected for the modeling.⁶¹ Atorvastatin acid is highly bound to plasma protein. The plasma unbound fraction of 2% was reported by Lennarnas et al.¹ The pKa value of 4.6 was obtained from Pfizer (in house data). The logD₇ of 1.53 previously measured by Ishigami et al.⁵² was utilized in the simulations. For mechanistic modeling of all three metabolites, *in silico* estimates of physiochemical properties were utilized due to lack of experimentally measured values. The key physicochemical and biopharmaceutical parameters are presented in **Table V-3**.

PBPK Modeling

Prediction of systemic clearance

Previously our group demonstrated *in vitro* metabolism of an acid and lactone forms of ATV using HLMS. The clinical observations were collected from patients with diabetes mellitus; therefore, model building was performed using metabolic intrinsic clearance values of diabetes livers reported by Dostalek et al.⁵ These *in vitro* values were scaled to calculate *in vivo* K_m and V_{max} except for *in vitro* V_{max} of ATV-LAC as explained in detail in the method section. An attempt was made to assess the effect of metabolic intrinsic clearance data of ATV for non diabetic livers⁵ on its disposition. As expected, the model under-predicted the observed plasma concentration-time profile.

Transporter mediated an active uptake clearance is the rate-limiting step for overall hepatic elimination of ATV;⁶² therefore, permeability-limited liver was selected in the proposed model to predict overall hepatic clearance. Metabolic and uptake clearance pathways for overall elimination of ATV incorporated in the model successfully described the observed clinical plasma concentration-time profile of the parent drug following oral dosing within 20% error (**Table V-5**) together with three metabolites depicted from **Figure V-1a** and **Figure V-1b**.

Prediction of volume of distribution

Initially Rodgers and Rowland equations^{56, 57} were assessed for K_{ps} calculation of perfusion-limited tissues. However, the estimated volume of distribution of ATV was very low as compared to observed; therefore, Berezhkovskiy algorithm was utilized to calculate K_{ps} to determine tissue distribution of perfusion-limited tissues as it adequately predicted observed large volume of distribution of ATV. The estimated volume of distribution determined based on *in silico* K_{ps} , tissue volumes and blood perfusion successfully explained extensive peripheral tissue binding of ATV.¹

In vitro dissolution study and prediction of *in vivo* solubility

Atorvastatin acid was completely dissolved in dissolution medium with high pH such as ≥ 6.8 as shown in **Figure V-2a**. The dissolution studies performed at low pHs including 1.2 and 3 indicated poor dissolution along with the formation of lactone form (**Figure V-2b**), which was in accordance with results shown by Kearny et al.⁴ The formation of ATV-LAC was higher at very low pH, 1.2 as compared to pH 3 and its absence at higher pH reflected instability in basic medium (**Figure V-2c**). The systemic exposure of ATV was under-predicted when measured *in vitro* dissolution *vs* time data was integrated in dissolution model could be because of significant differences between its aqueous and biorelevant solubility.

Gastrointestinal absorption model

A whole-body PBPK simulation approach in GastroPlus^{TM} allowed determining the relative contribution of different gastrointestinal regions to the overall absorption of the drug. *In silico* simulated compartmental absorption of ATV in the human gastrointestinal tract following an oral administration of 40 mg tablet resulted in total fraction of dose absorption of 99.6%. The predicted highest absorption (58.1%) of ATV occurred in jejunum followed by duodenum (17.7%) and ileum (13.6%) of total fraction of dose absorbed.

Virtual trial simulations

Simulations after an oral dosing of 40 mg of ATV in ten virtual patients were compared with mean observed plasma concentration-time curve. The result of virtual trial simulations is presented in **Figure V-3**. The figure shows mean plasma concentrations *vs* time profile of ten virtual patients along with observed

concentrations. Moreover, green shaded area represents 90% confidence interval of the simulated data around the mean of the predictive values. The solid blue, dashed, and dotted lines show individual simulated results that include 100, 95, 90, 75, 50, 25, and 10% of the range of simulated data. The observed mean plasma concentration-time curve lay between 90% of confidence interval, maximal and minimal virtual patients and was adequately well illustrated by the generated concentration-time curves of virtual population.

Parameter sensitivity analysis

The results of parameter sensitivity analysis indicated that AUC and $C_{\max}$ parameters relatively slight sensitive to *in vitro* transporter kinetic parameters contrary, insensitive to *in vitro* enzymatic clearance data. The parameter sensitivity analysis identified that AUC and $C_{\max}$ parameters were the most sensitive to changes in small intestinal transit time and stomach pH, respectively.

PBPK modeling of rosuvastatin acid and its lactone metabolite

GastroPlus^{TM} default PBPK^{TM} module in combination with the drug-related properties and *in vitro* clearance data was employed to predict oral pharmacokinetics of RST and its lactone metabolite.

In silico estimated or experimentally measured physiochemical and/or biopharmaceutical properties

Intestinal permeability

The human P_{eff} of RST and RST-LAC, estimated by ADMET Predictor^{TM} module of GastroPlus^{TM} based on quantitative structure–property relationship was 0.74×10^{-4} and 1.09×10^{-4} cm/s, respectively.

pKa, blood/plasma concentration ratio, fup and logD

Rosuvastatin acid is a hydrophilic statin and $\log D_{7.4}$ of -0.33⁶⁰ was used for simulation to reflect its poor membrane permeability. The fup (9.4%) and blood/plasma concentration ratio (0.56) reported by Jones et al. were incorporated in the model.⁶³ The key physicochemical and biopharmaceutical parameters for simulations are included in **Table V-4**.

PBPK Modeling

Prediction of systemic clearance

The recent *in vitro* study published by Jones et al. measured hepatic metabolic and uptake clearance of various OATP substrates including RST using HLMs and sandwich culture human hepatocytes, respectively.⁶³ According to study results, no measurable CYP enzyme mediated metabolism in HLMs was found for RST, was in agreement with observations of previous work⁶⁴ indicating its insignificant metabolism. Furthermore, study reported by Prueksaritanont et al. demonstrated glucuronidation metabolism of RST and the subsequent formation of lactone metabolite mainly by UGT1A1 and UGT1A3 enzymes.¹⁴ Moreover, carrier-mediated uptake clearance by several hepatic OATP transporters is the major clearance pathway for RST.⁶⁵ An average K_m and the sum of V_{max} for multiple OATP transporters including OATP1A2, 2B1 and 1B3 reported by Ho et al.²⁰ were chosen to determine overall hepatic uptake clearance. These *in vitro* values were scaled as explained in detail in the method section to calculate *in vivo* K_m and V_{max} to determine overall systemic clearance. There was a good agreement between model predicted and

clinically observed mean plasma concentration-time profile (within 1.5 fold) of RST following an oral dosing (**Figure V-4** and **Table V-5**).

Prediction of volume of distribution

Rodgers and Rowland equations were assessed for Kps calculation of perfusion-limited tissues in the beginning. However, the predicted volume of distribution of RST was very low than the observed value; therefore, Berezhkovskiy algorithm was utilized to calculate Kps to determine tissue distribution of perfusion-limited tissues as it well described the observed large volume of distribution of RST. The estimated volume of distribution calculated based on *in silico* Kps, tissue volumes and blood perfusion adequately explains extensive peripheral tissue binding of RST.¹

In vitro dissolution study and prediction of in vivo solubility

Rosuvastatin acid is a biopharmaceutics classification system (BCS) and biopharmaceutical drug disposition classification system (BDDCS) class III drug with high solubility and low permeability. It was completely dissolved in dissolution medium of all pH ranges as shown in **Figure V-5a and b**. The minor formation of lactone form occurred at pH 1.2 as shown in **Figure V-5c**.

Default Johnson dissolution model, which represents the Nernst-Brunner dissolution equation along with *in vitro* release input used to compute RST dissolution rate.

Gastrointestinal absorption model

The PBPK module of GastroPlus^{TM} permits to assess the relative contribution of various regions of the gastrointestinal tract to the absorption of the drug. *In silico* simulated human gastrointestinal compartmental absorption of RST following an oral administration of 20 mg tablet predicted maximum absorption of total fraction of

absorbed dose in jejunum followed by ileum and duodenum regions of the small intestine (data are not presented).

Virtual trial simulations

Like ATV, simulated plasma concentration-time profiles followed by administration of an oral dose of 20 mg of RST in virtual subjects were compared with observed plasma concentration-time profile as described above. The observed plasma concentration-time curve lay between 90% of confidence interval, maximal and minimal virtual patients (**Figure V-6**). The observed plasma concentration-time graph matches adequately by the created concentration-time curves of virtual population.

Parameter sensitivity analysis

The result of parameter sensitivity analysis indicated that AUC and $C_{\max}$ pharmacokinetics parameters were the most sensitive to changes in small intestinal transit time. Moreover, they are less likely to be affected by changes in stomach pH and are not sensitive to change in stomach transit time, kinetic parameters of BCRP and OATP1B1 transporters (data are not shown).

DISCUSSION

The objective of the study was to develop a whole-body PBPK model that mechanistically characterized observed plasma concentration-time courses of two widely prescribed statins, which disposition governs by various complex phenomenon including gut-liver first-pass effect and/or biliary secretions and active hepatic uptake. A whole-body PBPK modeling of both statins were performed using GastroPlus™ software through single simulation mode by required input parameters, which were experimentally measured, *in silico* estimated and/or obtained from the available literature. The present mechanistic model accurately described the observed concentrations in spite of the complex mechanism governing the absorption and elimination especially in case of ATV.

Atorvastatin acid, a BCS and BDDCS class II⁵⁰ drug, is highly soluble and permeable and therefore, complete absorption was predicted.¹ Atorvastatin acid, a hydroxy acid form with an acidic pKa of 4.6 showed significant pH dependent Caco-2 permeability with highest permeability at low apical pH. The pH in the proximal portion of the small intestine is low as compared to the distal end; therefore, the region specific rate of absorption of ATV estimated from *in silico* simulations was higher in the duodenum and jejunum as compared to ileum and caecum. Jejunum being the highest absorption site as compared to duodenum could be because of large surface area due to the presence of more villi and microvilli. Unlike ATV, both hydrophilicity and BCRP intestinal efflux transporters played a significant role in limiting intestinal absorption of RST at apical side of intestinal lumen.

Atorvastatin acid is metabolized mainly by CYP3A4 and UGT1A1/3 in both the gut and liver. The lactone form of atorvastatin is more lipid soluble as compared to an acid form based on their reported differences in $\log D_{7.0}$.⁵² The major mechanism governing hepatic distribution of an acid and lactone forms of atorvastatin were an active uptake and passive diffusion, respectively, which is in accordance with their distribution coefficient at physiological pH.⁶⁶ Relative importance of hepatic OATP influx transporters for an active uptake of ATV was demonstrated. The results indicated hepatic uptake process as rate-limiting step for its hepatic clearance.⁶² Moreover, *in vitro* studies that characterized metabolic clearance⁵ and hepatic transport^{39, 40, 66} indicated an active hepatic uptake as a rate-determining step in overall hepatic elimination; therefore, a permeability-limited liver model was selected to elucidate hepatic disposition of ATV. Mechanistic modeling of ATV integrating only *in vitro* metabolic clearance data resulted in significant under-prediction of oral clearance, which is accordance with previous study.⁶⁷ The proposed whole-body PBPK model adequately predicted observed data considering the significant active hepatic uptake clearance. A previously reported PBPK model indicated permeability-limited hepatic disposition of various statins such as simvastatin⁶⁸ pravastatin,⁶⁹ which is in accordance with our observation. Moreover, an appropriate selection of available literature data for metabolic and uptake clearance allowed the model to predict observed plasma concentration-time profile satisfactorily without the need of additional scaling factors.

The overall hepatic intrinsic clearance of permeability-limited liver can be expressed by the following equation based on a clearance concept⁷⁰ :

$$CL_{int, overall} = PS_{u, influx} \times \frac{CL_{int, met}}{PS_{u, efflux} + CL_{int, met}}$$

Where,

$CL_{int, overall}$ = overall hepatic clearance

$PS_{u, influx}$ = membrane permeability–surface area products of free drugs across the basolateral membrane for the influx process

$PS_{u, efflux}$ = membrane permeability–surface area products of free drugs across the basolateral membrane for the efflux process

$CL_{int, met}$ = Intrinsic metabolic clearance

When $PS_{u, efflux}$ is significantly greater than $CL_{int, met}$, and transfer of the drug from in and out of cells are similar then overall hepatic clearance is determined by $CL_{int, met}$. Moreover, overall clearance predominantly driven by an active uptake clearance, when $CL_{int, met}$ is considerably more than $PS_{u, efflux}$ and therefore, an alteration of metabolic clearance might not have any significant impact on overall systemic exposure.

A clinical study observed similar plasma levels of an acid and lactone forms of atorvastatin.⁶ *In vitro* studies indicated metabolic clearance of lactone metabolite mainly through CYP3A4 as one of the major mechanisms for elimination of ATV.^{3, 5} Furthermore, our group revealed a significant reduction (3.56 times) in clearance of ATV-LAC in the stable kidney transplant recipients with diabetes mellitus and thereafter, observed significantly increased plasma levels of ATV-LAC due to down regulation of primary metabolizing enzyme, CYP3A4. As a result, an *in vitro* enzyme kinetic study conducted by Dostalek et al. revealed statistically significant reduction in

clearance of hydroxylated metabolites for both an acid and lactone forms of ATV in HLMs of donors with diabetes.⁵

For mechanistic modeling of ATV, observed plasma concentrations were obtained from the kidney transplant subjects with diabetes mellitus. To reflect significantly reduced clearance of an acid and lactone forms of ATV in diabetes study participants, *in vitro* data obtained from HLMs of donors with diabetes mellitus was selected. Besides our group, to the best of our knowledge, only a single *in vitro* study determined enzyme kinetic parameters for metabolic clearance of lactone form together with an acid form of atorvastatin. However, the detail characteristics of liver tissues were not defined.³ The model under-predicted the observed concentration-time curve of ATV when enzyme kinetic data for metabolic clearance of an acid form obtained from HLMs of donors without diabetes mellitus were integrated (data are not shown). Moreover, the optimized $V_{\max}$ value was significantly lower than experimentally measured *in vitro* value. The possible reasons could be : 1) the simulated concentrations in the cytoplasm of hepatocytes could be different from concentrations in HLMs; 2) contribution of isoform of CYP enzymes was not considered⁷¹; 3) substantial differences in activities of enzymes in non physiological media⁷²; 4) incorrect presumption of a rapid equilibrium between blood and hepatocytes⁷³; 5) inter-individual differences between *in vivo* and *in vitro* livers attributed to inherent variability.⁷⁴

We demonstrated that metabolic clearance data generated from HLMs of donors from patients with diabetes mellitus was superior for the prediction of concentration-time

graph of ATV in this population. Atorvastatin acid is also significantly metabolized to *ortho*- and *para*-hydroxylated glucuronides.⁵³ *Ortho*-hydroxy atorvastatin acid undergoes significant glucuronidation and excreted into bile of rat and dog.⁵³ It is believed that *ortho*-hydroxy glucuronidated metabolite pumped back out into the intestinal lumen and may exhibit enterohepatic recirculation.⁵³ They are believed to be a substrate of hepatic OATP uptake transporter because of its relatively low lipophilicity. The P-gp efflux transporter at intestinal apical side was applied to reflect limited intestinal absorption of observed levels of *o*-OH-ATV metabolite.⁵⁴ Similarly, restricted intestinal absorption of *o*-OH-ATV-LAC was predicted by considering efflux by P-gp at intestinal apical side.

Metabolic clearance is insignificant for elimination of RST. However, predicted biotransformation in lactone metabolite via UGT pathways was consistent with our clinical observations. Like ATV, various studies indicated dominant contribution of multiple hepatic OATP uptake transporters in hepatic elimination of RST.^{16, 20, 21, 75, 76} Moreover, a canalicular efflux transporter, BCRP, efficiently facilitates its biliary excretion.^{19, 22, 55} A permeability-limited liver model was chosen to determine overall hepatobiliary disposition of RST. The model over-predicted observed plasma concentrations when BCRP mediated intestinal transport was not incorporated. This observation suggested significant contribution of BCRP transporters in intestinal absorption.

Parameter sensitivity analysis revealed that small intestinal transit time and gastric pH can significantly affect the AUC and $C_{\max}$ of the ATV, respectively. Delayed small

intestinal transit time and significant intragastric pH differences in patients with diabetes have been reported previously.^{77, 78} These physiological changes may result in higher systemic exposure of ATV that can increase the risk of muscle toxicity in diabetes population.⁷⁹

Similar to ATV, parameter sensitivity analysis was performed to understand the effect of various input parameters on AUC and $C_{\max}$ of RST. The analysis showed that systemic exposure is the most sensitive to changes in small intestinal transit time. Delayed small intestinal transit time may have considerable implication on therapeutic and toxicological properties of RST in population with diabetes mellitus. On the other hand, previous study reported that changes in hepatic uptake activities markedly affect plasma concentration-time course of pravastatin in rat⁸⁰ and human.⁶⁹ The PBPK model of pravastatin, a hydrophilic statin resembles RST was constructed using ten virtual healthy volunteers.⁶⁹ However, the present PBPK modeling was performed in transplant patients with diabetes mellitus and was capable to capture influential gastrointestinal parameters that might have changed in disease conditions and can affect systemic exposure of statins.

CONCLUSION

In summary, a mechanistic modeling approach was used to predict oral pharmacokinetics of ATV and RST with their respective metabolites using a blend of input parameters obtained from *in silico*, *in vivo* and *in vitro* measurements without the need for scaling factors. The present PBPK model of ATV that integrated *in vitro* enzyme kinetic data generated using HLMs from donors with diabetes was superior for the prediction of plasma concentration-time curve of ATV in patients with diabetes mellitus. *In vitro* kinetic parameters obtained from tissues of donors with specific disease that may alter the activity of drug enzyme metabolizing enzyme and transporters as a result influence the pharmacokinetics may be superior for accurate prediction of absorption and metabolism in specific population.

The present work demonstrated the ability of the ACAT model within GastroPlus^{TM} to simulate observed plasma concentration-time profiles of statins for, which intestinal-hepatic metabolism and/or various influx-efflux transporters play a prominent role in their disposition. This work demonstrated the mechanistic and model-driven application of *in vitro* uptake and efflux kinetic data for predicting transporter mediated disposition of statins. A generic whole-body PBPK based modeling approach can advance the reasonable predictions of the parent drug with their metabolites in particular population.

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Figure V-1a. Simulated and observed human oral mean plasma concentration-time profiles for atorvastatin acid and atorvastatin lactone metabolite

Figure V-1b. Simulated and observed human oral mean plasma concentration-time profiles for an acid and lactone from of *ortho*-hydroxy atorvastatin metabolites

Figure V-2 (a) Mean (n=3) dissolution profiles of atorvastatin acid in different pH media. (b) Mean (n=3) concentrations of an acid form of atorvastatin in dissolution media with pH 1.2, 3, 4.5, 6.8 and 8 (c) Mean (n=3) concentrations of lactone form of atorvastatin in dissolution media with pH 1.2 and 3

Figure V-3. Virtual trial simulation for ten subjects following a 40 mg oral dose of atorvastatin acid. Solid line shows the mean of simulated concentrations of ten subjects. Square with error bar represents the clinical observations. The green highlighted area represents 90% confidence interval of simulated concentrations data. The solid blue, dashed, and dotted lines represent individual simulated results that incorporate 100, 95, 90, 75, 50, 25, and 10% of the range of simulated data.

Figure V-4. Simulated and observed human oral mean plasma concentration-time profiles for rosvastatin acid and its lactone metabolite.

Figure V-5. (a) Mean (n=3) dissolution profiles of rosuvastatin acid in different pH media. (b) Mean (n=3) concentrations of an acid form of rosuvastatin in dissolution media with pH 1.2, 3, 4.5, 6.8 and 8 (c) Mean (n=3) concentrations of lactone form of rosuvastatin in dissolution media with pH 1.2

Figure V-6. Virtual trial simulation for ten subjects following a 20 mg oral dose of rosuvastatin. Solid line shows the mean of simulated concentrations of ten subjects. Square with error bar represents the clinical observations. The green highlighted area represents 90% confidence interval of simulated concentrations data around mean. The solid blue, dashed, and dotted lines represent individual simulated results that incorporate 100, 95, 90, 75, 50, 25, and 10% of the range of simulated data.

Table V-1. The ACAT physiological model compartment parameters of default fed human physiology used in simulations of atorvastatin acid and three metabolites in GastroPlus™.

Compartment	ASF (cm ⁻¹)	pH	Transit time (h)	Volume (mL)	Length (cm)	Radius (cm)	P-gp expression	CYP3A4 expression	UGT1A1 expression
Stomach	0.0	4.9	1.00	1000	30	10	0.0	0.0	0.0
Duodenum	2.774	5.4	0.26	48.25	15	1.60	0.538	2.09E-3	6.3E-4
Jejunam1	2.760	5.4	0.95	175.3	62	1.50	0.645	3.26E-3	1.0E-3
Jejunam2	2.675	6.0	0.76	139.9	62	1.34	0.723	3.26E-3	1.0E-3
Ileaum 1	2.583	6.6	0.59	108.5	62	1.18	0.770	1.03E-3	3.2E-4
Ileaum 2	2.534	6.9	0.43	79.48	62	1.01	0.838	1.03E-3	3.2E-4
Ileaum 3	2.435	7.4	0.31	56.29	62	0.85	0.908	1.03E-3	3.2E-4
Cecum	1.160	6.4	4.50	52.92	13.75	3.50	1.000	3.1E-4	2.0E-4
Ascending colon	1.508	6.8	13.5	56.98	29.02	2.50	1.000	3.1E-4	2.0E-4

Table V-2. ACAT physiological model compartment parameters of default fed human physiology used in simulations of rosuvastatin acid and lactone metabolite in GastroPlus^{TM}.

Compartment	ASF (cm ⁻¹)	pH	Transit time (h)	Volume (mL)	Length (cm)	Radius (cm)	BCRP expression	UGT1A1 expression
Stomach	0.0	4.9	1.00	1000	30	10	0.0	0.0
Duodenum	2.778	5.4	0.26	48.25	15	1.60	0.60	6.3E-4
Jejunam1	2.763	5.4	0.95	175.3	62	1.50	0.60	1.0E-3
Jejunam2	2.674	6.0	0.76	139.9	62	1.34	0.60	1.0E-3
Ileaum 1	2.584	6.6	0.59	108.5	62	1.18	1	3.2E-4
Ileaum 2	2.540	6.9	0.43	79.48	62	1.01	1	3.2E-4
Ileaum 3	2.456	7.4	0.31	56.29	62	0.85	1	3.2E-4
Cecum	0.164	6.4	4.50	52.92	13.75	3.50	0.40	2.0E-4
Ascending colon	0.232	6.8	13.5	56.98	29.02	2.50	0.40	2.0E-4

Table V-3. Summary of key biopharmaceutical properties and *in vitro* data of atorvastatin acid (ATV), atorvastatin lactone (ATV-LAC), *ortho*-hydroxy atorvastatin acid (O-OH-ATV) and *ortho*-hydroxy atorvastatin lactone (O-OH-ATV-LAC).

Parameter	ATV		ATV-LAC		O-OH-ATV		O-OH-ATV-LAC	
	Value	Source	Value	Source	Value	Source	Value	Source
Molecular weight	558.65	a	540.64	a	574.65	a	556.64	a
logP/logD _{7.4}	1.53@pH 7	⁵²	4.2@pH 7	⁵²	4.42	a	5.22	a
pKa acid	11.36/4.6	a/*	11.16	a	10.97/9.14/4.7	a	10.83/9.02	a
Fraction unbound in plasma (%)	2	¹	2.71	a	0.93	a	4.51	a
Blood/plasma concentration ratio	0.25	⁶¹	0.66	a	0.54	a	0.65	a
Human P _{eff} (X10 ⁻⁴ cm/s)	1.11	a	1.48	a	0.8	b	1.48	a
Reference solubility (mg/mL) @ pH	0.021@2.1	⁴	4.4X10 ⁻³ @7	a	0.17@4.17	a	9.84X10 ⁻³ @6.8	a
Dose (mg)	40	NA	NA	a	NA	a	NA	a
Mean particle radius (μM)	25	a	25	a	25	a	25	a
Mean precipitation time (s)	900	a	900	a	900	a	900	a
Diffusion coefficient (X10 ⁻⁴ cm/s)	0.51	a	0.53	a	0.51	a	0.53	a
CYP3A4 K _m (μM)	1.179	⁵	0.604	⁵	NA	NA	NA	NA
CYP3A4 V _{max} : gut/PBPK [(mg/s)/(mg/s-mg enzyme)]	1.699/3.8x10 ⁻³	⁵	0.069/10 ⁻⁵	⁵	NA	NA	NA	NA
UGT1A1 K _m (μM)	3.168	^{7, 37}	NA	NA	3.057	b	NA	NA
UGT1A1 V _{max} : gut/PBPK [(mg/s)/(mg/s-mg enzyme)]	2/0.07	b	NA	NA	0.1/0.007	b	NA	NA
UGT1A3 K _m (μM)	3.168	^{7, 37}	NA	NA	3.057	b	NA	NA
UGT1A3 V _{max} : PBPK (mg/s-mg enzyme)	0.007	b	NA	NA	0.07	b	NA	NA
OATP1B1 K _m (μM)	0.0095	^{39, 40}	NA	NA	1.535	b	NA	NA
OATP1B1 V _{max} : PBPK [(mg/s)/(mg/s-mg transporter)]	0.584x10 ⁻⁵	^{39, 40}	NA	NA	0.012	b	NA	NA
P-gp K _m (μM)	1.257	¹⁷	NA	NA	25.03	b	20.03	b
P-gp V _{max} : gut or PBPK [(mg/s)/(mg/s-mg transporter)]	0.0005 (PBPK)	b	NA	NA	0.3 (Gut)	b	0.02 (Gut)	b

a=Estimated by ADMET Predictor^{TM}

b=Fitted value

NA=not applicable

*= Data obtained from Pfizer Inc

Table V-4. Summary of key biopharmaceutical properties and *in vitro* data of rosuvasatin acid and rosuvasatin lactone used in the PBPK simulations.

Parameter	Rosuvastatin acid		Rosuvastatin lactone	
	Value	Source	Value	Source
Molecular weight	481.55	a	463.53	a
logP/logD _{7.4}	-0.33@ pH 7.4	⁶³	1.2@7.4	⁶⁰
pKa acid or base	4.2/1.7 [*] /-3.17 [*]	⁶³ /a/a	1.31 [#] /-3.33 [#]	a
Fraction unbound in plasma (%)	9.4	⁶³	9.13	a
Blood/plasma concentration ratio	0.56	⁶³	0.76	a
Human P _{eff} (X10 ⁻⁴ cm/s)	0.74	a	1.09	a
Reference solubility (mg/mL) @ pH	0.79@3.72	a	0.12@7	a
Dose (mg)	20	NA	20	NA
Mean particle radius (μM)	25	a	25	a
Mean precipitation time (s)	900	a	900	a
Diffusion coefficient (X10 ⁻⁴ cm/s)	0.57	a	1.2	a
UGT1A1 K _m (μM)	11.72	¹⁴	NA	NA
UGT1A1 V _{max} : gut/PBPK [(mg/s)/(mg/s-mg enzyme)]	0.07077/0.00092	¹⁴	NA	NA
UGT1A3 K _m (μM)	11.72	¹⁴	NA	NA
UGT1A3 V _{max} : PBPK (mg/s-mg enzyme)	0.00023	¹⁴	NA	NA
BCRP K _m (μM)	0.471	^{22, 55}	NA	NA
BCRP V _{max} : gut/PBPK [(mg/s)/(mg/s-mg transporter)]	0.007/0.000293	²¹	NA	NA
OATP K _m (μM)	2.942	²⁰	NA	NA
OATP V _{max} : PBPK (mg/s-mg transporter)	7.13X10 ⁻⁵	²⁰	NA	NA

* and [#] = Base pKa estimated by ADMET Predictor^{TM}

a = Estimated by ADMET Predictor^{TM}

NA= not applicable

Table V-5. Summary of observed and predicted pharmacokinetic parameters of atorvastatin acid and rosuvastatin acid following an oral administration.

Parameter	Atorvastatin acid			Rosuvastatin acid		
	Observed	Predicted	PPE (%)	Observed	Predicted	PPE (%)
Mean $C_{\max}$ (ng/mL)	17.88	14.74	17.6	6.26	6.28	0.30
Mean $T_{\max}$ (h)	2	1.72	14.0	6	3.96	34.0
Mean AUC_{0-t} (ng/mL.h)	103.58	103.32	0.30	55.07	57.90	5.10

PPE=Percentage prediction error: $[|(\text{Predicted}-\text{Observed})/\text{Observed}|]*100$

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