CYP3A and drug transporters activity changes in Thai elderly with or without chronic kidney disease using a microdose cocktail

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การเปลี่ยนแปลงการทำงานของเอนไซม์ซิพสามเอและตัวขนส่งยาในผู้สูงอายุชาวไทยที่มีและไม่มีภาวะโรคไตเรื้อรังโดยใช้ไมโครโดสคอกเทล

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ปุณยาภร รัตนชีวร : การเปลี่ยนแปลงการทำงานของเอนไซม์ซิพสามเอและตัวขนส่งยาในผู้สูงอายุชาวไทยที่มีและไม่มีภาวะโรคไตเรื้อรังโดยใช้ไมโครโดสคอกเทล. (CYP3A and drug transporters activity changes in Thai elderly with or without chronic kidney disease using a microdose cocktail) อ. ที่ปรึกษาหลัก : ผศ.ดร. พญ.ปาจรีย์ จริยวิลาศกุล, อ.ที่ปรึกษาร่วม : ศ. นพ.ยิ่งยศ อวิหิงสานนท์

ในปัจจุบัน ประชากรชาวไทยมีแนวโน้มเข้าสู่สังคมผู้สูงอายุ คือผู้ที่มีอายุมากกว่า 60 ปีขึ้นไป และโรคไตเรื้อรังที่จัดเป็นหนึ่งในปัญหาหลักทางด้านสุขภาวะของคนไทยซึ่งมีความสัมพันธ์กับอายุที่เพิ่มมากขึ้น เป็นที่ทราบกันดีว่าภาวะสูงวัย การทำงานของไตบกพร่องตลอดจนสภาวะโรคไตวายเรื้อรังเป็นปัจจัยที่ส่งผลกระทบต่อกระบวนการทางเภสัชจลนศาสตร์ โดยกระบวนการทางเภสัชจลนศาสตร์เป็นกระบวนการที่เกี่ยวข้องกับระดับยาในกระแสเลือดและตำแหน่งของการออกฤทธิ์ ประสิทธิภาพ และความปลอดภัยของยา เนื่องจากกลไกจากผลกระทบของภาวะสูงวัยและ/หรือภาวะโรคไตเรื้อรังต่อกระบวนการทางเภสัชจลนศาสตร์ยังคงมีอยู่อย่างจำกัดและยังไม่เป็นที่แน่ชัด การศึกษาทางคลินิกในครั้งนี้จึงมุ่งเน้นค้นหาความเปลี่ยนแปลงด้านการทำงานของเอนไซม์ซิพสามเอและตัวขนส่งยาในคนไทยที่มีภาวะสูงวัยสุขภาพดี และภาวะสูงวัยที่มีโรคไตเรื้อรัง เปรียบเทียบกับคนหนุ่มสาว โดยใช้เครื่องมือที่ชื่อว่าไมโครโดสคอกเทล การวิจัยเชิงทดลองนี้ได้ทำการวิเคราะห์ในสามกลุ่มผู้เข้าร่วมวิจัยคือ กลุ่มที่ 1 หนุ่มสาวสุขภาพดี กลุ่มที่ 2 ผู้สูงวัยสุขภาพดี และกลุ่มที่ 3 ผู้สูงวัยที่มีโรคไตเรื้อรัง ผู้เข้าร่วมวิจัยทุกท่านได้รับประทานไมโครโดสคอกเทลหนึ่งครั้ง ประกอบด้วยยาที่เป็นตัวแทนซับสเตรดคือ มิดาโซแลม ขนาด 30 ไมโครกรัม ดาบิกาแทรน เอกทีซิเลต ขนาด 750 ไมโครกรัม อะทอวาสแตติน ขนาด 100 ไมโครกรัม พิทาวาสแตติน ขนาด 10 ไมโครกรัม และโรซูวาสแตติน ขนาด 50 ไมโครกรัม ภายหลังจากระยะพัก 14 วัน ผู้เข้าร่วมวิจัยกลุ่มที่ 1 เข้าสู่ช่วงที่ 2 ของงานวิจัยโดยได้รับประทานไมโครโดสคอกเทล 1 ครั้งร่วมกับยาไรแฟมพิซิน การประเมินค่าทางเภสัชจลนศาสตร์ของตัวแทนซับสเตรดในไมโครโดสคอกเทลถูกคำนวณสำหรับค่าพื้นที่ใต้กราฟระดับยาจากเวลาเริ่มต้นถึงความเข้มข้นสุดท้ายที่วัดได้ ค่าพื้นที่ใต้กราฟจากเวลาเริ่มต้นถึงอินฟินิตี้ ความเข้มข้นสูงสุดของยาในกระแสเลือด นอกจากนี้ได้ทำการวิเคราะจีโนไทป์ของตัวขนส่งยาโอเอทีพีวันบีวัน และบีซีอาร์พี ในผู้เข้าร่วมวิจัยเพื่อลดปัจจัยกวนที่อาจส่งผลต่อระดับยาในไมโครโดสคอกเทล เมื่อเปรียบเทียบกับผู้เข้าร่วมวิจัยกลุ่มที่ 1 ภาวะสูงวัยส่งผลเปลี่ยนแปลงเภสัชจลนศาสตร์ของยามิดาโซแลม โดยมีการเพิ่มขึ้นในกลุ่มผู้เข้าร่วมวิจัยกลุ่มที่ 2 และ 3 ของค่าพื้นที่ใต้กราฟระดับยาจากเวลาเริ่มต้นถึงความเข้มข้นสุดท้ายที่วัดได้ ซึ่งเป็นตัวแทนของระดับยาในเลือดภายในร่างกายประมาณ 2 เท่า และค่าความเข้มข้นสูงสุดของยาในกระแสเลือดประมาณ 3 เท่า อีกทั้ง 2 กลุ่มผู้เข้าร่วมวิจัยนี้ยังมีอัตราการกำจัดยาที่ลดลง และมีค่าครึ่งชีวิตที่ยาวนานมากขึ้นอีกด้วย เช่นเดียวกับยาอะทอวาสแตติน ซึ่งเป็นตัวแทนซับสเตรดของเอนไซม์ซิพสามเอสี่ ที่มีค่าพื้นที่ใต้กราฟระดับยาจากเวลาเริ่มต้นถึงความเข้มข้นสุดท้ายที่วัดได้ และค่าความเข้มข้นสูงสุดของยาในกระแสเลือด เพิ่มขึ้น 2 เท่าในผู้เข้าร่วมวิจัยกลุ่มที่ 2 และเพิ่มขึ้น 4 เท่าในผู้เข้าร่วมวิจัยกลุ่มที่ 3 อย่างไรก็ตามสำหรับการประเมินการทำงานของตัวขนส่งยาที่เกี่ยวข้องในงานวิจัยครั้งนี้ยังไม่สามารถเป็นที่สรุปได้แน่ชัด ผลงานวิจัยครั้งนี้ที่ใช้ไมโครโดสคอกเทลในการวิเคราะห์ระบุว่าอายุที่เพิ่มมากขึ้นมีความเกี่ยวข้องกับการทำงานของเอนไซม์ซิพสามเอสี่ที่ลดลง ถึงแม้ว่าการทำงานของตัวขนส่งยาโอเอทีพีวันบี พีจีพี และ บีซีอาร์พี ในประชากรเป้าหมายของงานวิจัยในครั้งนี้ยังไม่สามารถสรุปอย่างแน่ชัดได้ แต่ผลจากงานวิจัยสามารถชี้ให้เห็นถึงแนวโน้มของการเปลี่ยนแปลงทางเภสัชจลนศาสตร์ของยาในไมโครโดสคอกเทลซึ่งอันเป็นประโยชน์ต่อการพัฒนาการค้นหากลไกที่อาจเกี่ยวข้องเชิงลึกต่อไป

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Punyabhorn Rattanacheeworn : CYP3A and drug transporters activity changes in Thai elderly with or without chronic kidney disease using a microdose cocktail. Advisor: Asst. Prof. Pajaree Chariyavilaskul, M.D., PhD Co-advisor: Prof. YINGYOS AVIHINGSANON, M.D.

Thai general population has become more “aged society” (>60 years old) and chronic kidney disease (CKD) is one of major public health problems in Thailand. Ageing and CKD are known to affect pharmacokinetics which generally determines drug exposure in plasma and site of action. Due to the related mechanisms are still unclear, cytochrome P450 (CYP)3A and drug transporters activity changes were investigated in Thai elderly with or without CKD compared to healthy adults using a microdose cocktail.

This study was conducted in 3 groups of subjects: Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3; elderly with CKD. All subjects received single dose of microdose cocktail probe containing 30 µg midazolam, 750 µg dabigatran etexilate, 100 µg atorvastatin, 10 µg pitavastatin, and 50 µg rosuvastatin. After 14 days of washout period, Group 1 continued period 2 of study received microdose cocktail plus rifampicin. $AUC_{0\text{-}last}$ (area under the concentration-time curve from time zero to last quantifiable concentration), $AUC_{0\text{-}inf}$ (AUC time zero to infinity), and C_{max} (maximum concentration) were mainly estimated. Genotype analysis for Organic anion transporting polypeptide (OATP) 1B1 and breast cancer resistance protein (BCRP) transporters was analyzed for excluding confounding factor.

$AUC_{0\text{-}last}$ and C_{max} of midazolam, a CYP3A probe substrate, were increased ~2 to 3-fold in Group 2 and Group 3. Reduction in elimination rate constant and half-life prolongation were observed in both groups. $AUC_{0\text{-}last}$ and C_{max} of another CYP3A4 probe substrate, atorvastatin, was increased 2-fold in Group 2 and 4-fold in Group 3. CYP3A4 activity was reduced by advanced age resulting in decreased in midazolam clearance and prolonged half-life. The association of pharmacokinetics changes in other probe drugs with drug transporters activity including OATP1B, intestinal P-glycoprotein (P-gp), and BCRP was still inconclusive.

Microdose cocktail indicates the reduction in CYP3A4 activity associated with advanced age, especially in Thai population. Although OATP1B, P-gp, and BCRP activity of the special population cannot be concluded in this study yet, there was a trend of pharmacokinetic alterations in microdose cocktail probe drugs.

Field of Study: Medical Sciences Student's Signature .....
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Chapter 1

Introduction

Background and Rationale

In the past decades, Thai general population has become a more "aged society" as more than 10 percent of the population is >60 years old. There are several consequences associated with aging, such as the quality of life, socio-economics status of the country, and especially health problems.[1]

Elderly population not only have comorbidities and receive polypharmacy in long term therapy, but also have changes in pharmacokinetics and pharmacodynamics of drugs associated with aging. These lead to an increased in the risk of adverse drug events (ADR) and drug-drug interaction (DDI).[2, 3]

Pharmacokinetics determines drug exposure in plasma and its site of action. There are many factors influencing the changes in pharmacokinetics including age, ethnicity, and organ impairment. Recently, genetic variability in drug metabolizing enzymes and drug transporters were reported to alter pharmacokinetics.[4, 5] Hence, the exposure of drugs in plasma and its site of action may differ between younger adults (aged 20-60 years old) and the elderly. The understanding of the mechanisms underpinning PK changes in the elderly with and without comorbidities are still limited, especially information in regard to cytochrome P450 (CYP450) and drug transporter activities.

Chronic kidney disease (CKD) is a major public health problem in Thailand. Its prevalence is remarkably high (17.5% of the population) and mostly found with advancing age. CKD is itself a risk factor for cardiovascular disease mortality, and end stage renal disease (ESRD) which required hemo- or peritoneal dialysis.[6] Since the kidneys are responsible for drug excretion, CKD causes major changes leading unexpected drug responses.

Clinical studies performed to determine PK changes in special population is essential in dosage adjustment, especially in drug development. However, the clinical studies in the elderly (aged >60 years old) are underrepresented compared to the younger adults (aged 20-60 years old). The dosage recommendation and the extrapolation of the drug response listed in the small package insert may not be suitable for the elderly.[4, 7] Studies looking at mechanistic changes in PK and PD specifically for Thai elderly population is, therefore, very important. This would help adjust the optimal dosage regimen for Thai patients.

Hence, this study aimed to investigate PK changes of important drug metabolizing enzyme and drug transporter activity in the elderly with or without CKD compared to healthy controls using a microdose cocktail containing several probe substrates. The genetic polymorphisms of drug transporters were also investigated.

Chapter 2

Review of the literatures

1. Ageing society of Thailand

Due to a decreased in birth rate and mortality rate, Thai general population has currently been an "aged society", defined as more than 10 %of the Thai population is aged >60 years old, with aging index of 50-119.9, since 1990-1991. Life expectancy is expected to be increased from 72 and 79 years old for males and females, respectively, in 2014 to 76 and 83 years old in the next ten years.[1] The increased in life expectancy of Thai population affects their quality of life, society, national economy, and importantly, increased health problems. The major health problems in the elderly is associated with the deterioration of many organs that cause chronic and degenerative diseases, such as hypertension, diabetes mellitus, CKD, and cardiovascular disease, etc.[2] Moreover, the elderly usually has comorbidities and receive polypharmacy leading to the greater risk of DDI and ADR.[3]

2. Pharmacokinetic changes in the elderly

The differences in physiological changes are observed in the elderly, as advanced age influences on all processes of pharmacokinetic related to drug efficacy and safety. (Table 1)

Table 1 Pharmacokinetic alterations in the elderly[3, 4),(8-11)]

Pharmacokinetic process	**Pharmacokinetic alterations**	**Physiological changes in elderly**
Absorption	Depends on physiochemical drugs properties • Slightly decreased for drugs requiring low pH • Delayed time to maximum concentration (T_{max}) but unchanged absorption for passive intestinal permeability drugs	• Increased gastric pH • Delayed gastric emptying time
	• Decreased first pass metabolism for high extraction drug • Bioavailability may be either unchanged or increased, no consistent effect	• Decreased gastrointestinal blood flow
	• No clinically change	• Decreased in small bowel surface area
Distribution	• Increased in volume distribution and prolonged elimination half-life of lipophilic drugs • Decreased in volume distribution of hydrophilic drugs and increased in plasma concentration	• Increased body fat • Decreased muscle mass • Decreased total body water
	• Increased free forms of highly protein-bound acidic drug	• Decreased serum albumin
	• Decreased free forms of highly protein-bound basic drug	• Increased α-1 acid glycoprotein
Metabolism	• Higher bioavailability (especially, drugs with high first pass extraction rate) and decreased phase I metabolism	• Decreased liver mass • Decreased hepatic blood flow
	• Decreased hepatic metabolism	• Decreased cytochrome P450 activity (in some isozymes of CYP450 and genetic polymorphisms may be involved)
Excretion	• Decreased renal elimination	• Decreased glomerular filtration rate (serum creatinine may remain in normal range) • Decreased renal mass • Decreased renal blood flow • Decreased tubular function

3. Chronic kidney disease situation in Thailand

CKD is a risk factor for cardiovascular disease mortality and ESRD. Its prevalence is remarkably high (17.5% of the Thai population)[12] and mostly found with advancing age. Additionally, the increased in life expectancy tend to also increase the prevalence of CKD.[12]

CKD is defined by the Kidney Disease: Improving Global Outcomes (KDIGO) 2012[13] as abnormalities of kidney structure or function, present for >3 months, with implications for health and is classified into 5 stages according to estimated glomerular filtration rate (eGFR) (Table 2).[13]

Table 2 CKD stages, KDIGO 2012[13]

CKD stage	**eGFR (mL/min/1.73 m^2)**
Stage 1	≥90
Stage 2	60-89
Stage 3	30-59
Stage 4	15-29
Stage 5 (ESRD)	<15

4. Pharmacokinetic changes in chronic kidney disease

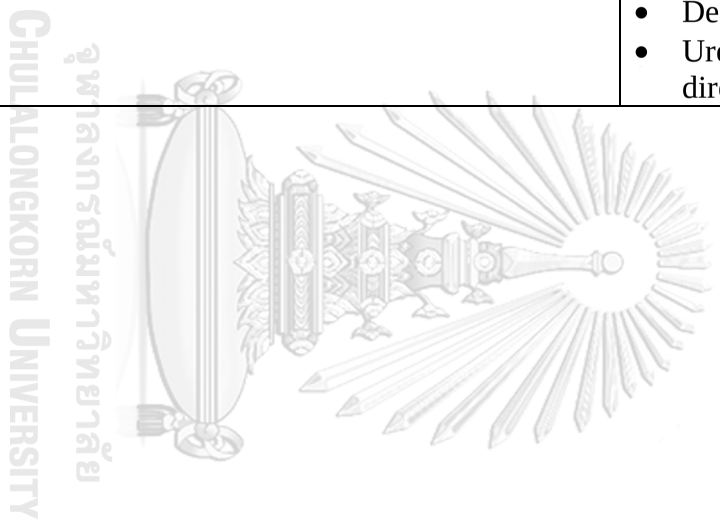
CKD alters pharmacokinetic process by reducing renal clearance, affecting protein binding and non-renal clearance of drugs.[14] Unfortunately**,** the effects of non-renal drug-metabolizing enzymes (especially in the liver) or transporters activity in CKD could not be definitely defined because of the complexity interplays among them and the other factors such as genetics.[15]

The pharmacokinetic changes in CKD were summarized in Table 3**.**

Table 3 Pharmacokinetic alterations in renal impairment[15-20]

Pharmacokinetic process	**Pharmacokinetic alterations**	**Physiological changes in renal impairment**
Absorption	• Altered drug absorption by either decreased bioavailability or decreased first-pass metabolism	• Gastrointestinal edema
	• Increased absorption and bioavailability especially in drug requiring presystemic elimination.	• Decreased intestinal barrier function (mediated by uremic toxin and inflammation) • Downregulation of intestinal P-gp, MRP2 transporter and CYP3A4
	• Slightly delayed T_{max} but unchanged bioavailability	• Decreased gastric emptying time • Decreased gastrointestinal motility
Distribution	• Increased free fraction of highly protein-bound acidic drug	• Decreased serum albumin
	• Increased free fraction drug	• Changed in the conformation of protein binding site • Competition for binding sites by endogenous substance accumulation
	• Decreased free fraction of highly protein-bound basic drug	• Increased α-1 acid glycoprotein
	• Increased volume distribution in hydrophilic drug • Decreased drug serum concentration	• Fluid overload
Metabolism	• Decreased hepatic metabolism	• Downregulation of hepatic OATP and CYP450 activity • Decreased phase II metabolizing enzyme (N-acetyltransferase, uridine diphosphate-glucuronosyltransferase)

Pharmacokinetic process	**Pharmacokinetic alterations**	**Physiological changes in renal impairment**
Excretion	• Decreased renal elimination • Prolonged half-life in some drugs	• Decreased renal mass • Decreased number of functioning nephrons • Decreased renal blood flow • Decreased glomerular filtration rate • Decreased tubular secretion rate • Uremic toxins inhibit drug transporter directly



5. Drug metabolizing enzymes and drug transporters

5.1. Drug metabolizing enzymes

Drug metabolizing enzymes are mainly responsible for metabolizing of xenobiotic chemicals such as drugs, carcinogens, pesticides, pollutants and endogenous compounds such as steroids, prostaglandins, and bile acids.[21] There are 2 main types of drug metabolizing reactions which primarily occur at liver.are listed in Figure 1

5.1.1. Phase I drug metabolisms

Phase I drug metabolisms include oxidation, reduction and hydrolysis. This type of metabolism generally converts drug molecules to enhance their polarity to be suitable for renal or biliary excretion. Drug oxidation is catalyzed by two large families of enzymes, cytochrome P 450 (CYP450) and flavin-containing mono-oxygenases (FMOs).[18] The others are monoamine oxidase (MAOs), and xanthine oxidase/aldehyde oxidase (XO/AO).[21]

5.1.2. Phase II biotransformation reactions

Phase II biotransformation reactions include glucuronidation, sulfonation, methylation, acetylation, amino acids, and glutathione (GSH) conjugation. The conjugative phase II metabolizing enzymes consist of uridine 5'-diphodpho (UDP)-glucuronosyltransferases (UGTs), sulfotransferase (SULTs), and glutathione *S*-transferases (GSTs). Many drugs or their phase I metabolites are also conjugated by phase II reactions. The conjugated metabolites are more polar and readily to be excreted via urine or bile.[21]

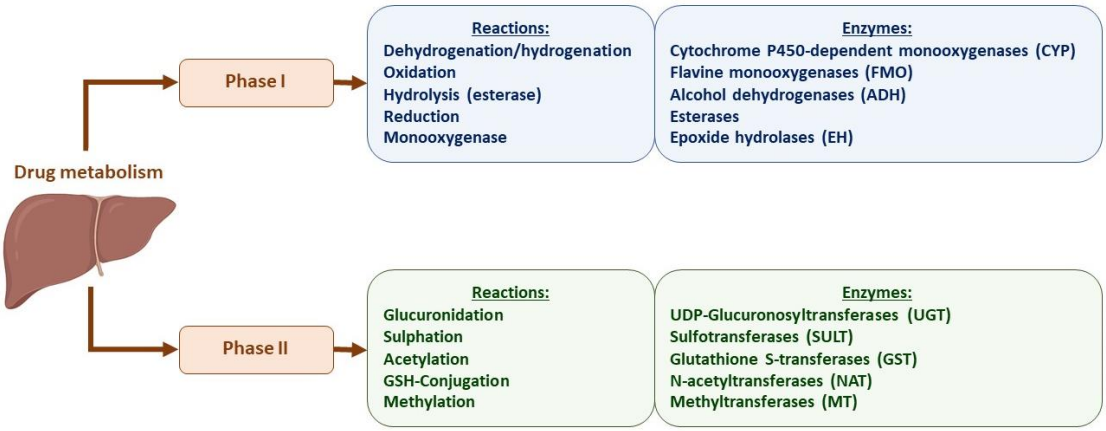


Figure 1 The metabolism processes and involving drug metabolizing enzymes
The primarily site of biotransformation is liver. Phase I and II metabolism reactions and involving metabolizing enzymes are listed.[22, 23]

The amount of drug metabolizing enzymes in phase I and II metabolism reactions are estimated into size of each pie in pie chart as shown in Figure 2.[24, 25]

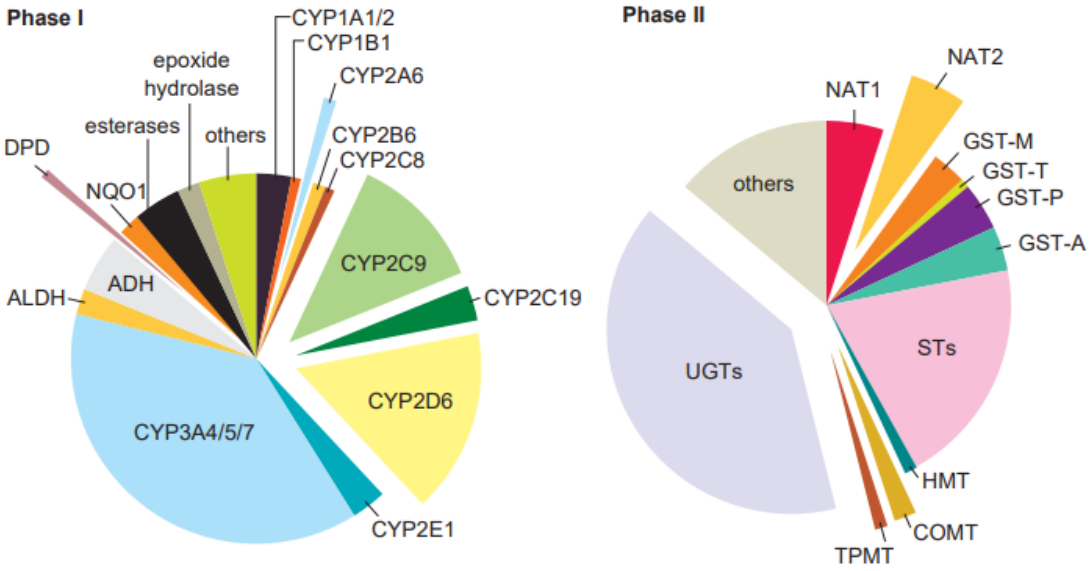


Figure 2 Pie chart of estimated amount of metabolizing enzymes involved in phase I and II metabolism reactions in liver.
It is described by relative size of each pie section. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine-dehidrogenase; NQOI, NADPH, quinone oxidoreductase; COMT, catechol O-methvltransferase; GST, glutathione S-transferase; HMT, histamine methvl-transferase; NAT, N-acetyl transferase; STs, sulfotransferases/SULTs; TPMT, thiopurine methyltransferse; UGTs, uridine 50 -triphosphate glucuronosyltransferases

CYP450 enzyme system is a superfamily of mixed-function mono-oxygenase that is a major catalyzing enzyme of phase I oxidative reaction. CYP450 isozymes are mostly located in liver but also found at other tissues including intestinal mucosa and kidneys. The CYP450 isozymes in liver and intestinal mucosa govern oral bioavailability and systemic metabolic clearance of drug molecules.

CYP450 isozymes consist of at least 14 human families, 22 human subfamilies, and 36 human CYP enzymes.[26] In human liver, approximately 70% of human hepatic CYP450 are CYP1, 2, 3 families including CYP3A4 and 3A5 (40% of total liver content), CYP2Cs (25%), CYP1A2 (18%), CYP2E1 (9%), CYP2A6 (2%), CYP2D6 (2%), and CYP2B6 (<1%). CYP450 enzymes are also found in human intestine including CYP3A4/5 (82% of total intestine content), CYP2C9 (14%), CYP2C19 (2%), CYP2D6 (0.7%) and CYP2J2 (1.4%). There are some CYPs isozymes in kidneys but much lower than those presented in liver and intestinal mucosa (Figure 3).[18, 27]

This study focuses on CYP3A, the most abundant isozymes in liver and intestine, activities changes in elderly with or without chronic kidney disease.

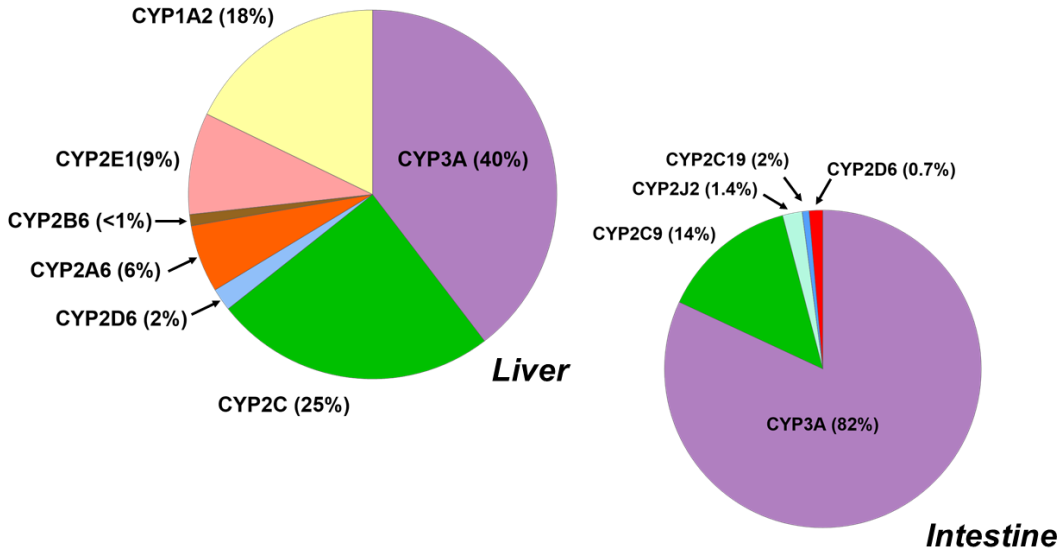


Figure 3 Pie chart of the estimated percentage of cytochrome P450 (CYP) isozymes found in human liver and intestine.
CYP3A is mostly found in both liver and intestine with 40% and 82% of their content, respectively.(adapted from Paine et al. 2006)[27]

5.2. Drug transporters

Drug transporters are membrane bound proteins that facilitate drugs and xenobiotics across biological barriers. They mostly express in liver, brain, intestine, and kidney to act as either efflux transporter or influx transporter. Therefore, they play a critical role in drug absorption, distribution, and excretion thereby affecting PK and drug exposure. Drug transporters can be classified into 2 superfamilies.[5, 28]

5.2.1. Adenosine triphosphate (ATP) binding cassette (ABC) transporters

ABC transporters are responsible for host detoxification or protection against xenobiotic substances by either influx or efflux substances from the cells. They require energy to transport various molecules across cell membrane against the concentration gradient using hydrolysis of ATP.[28, 29] The ABC transporters are characterized by homology of their ATP binding region. All families contain two ATP binding regions and two transmembrane domains, except ATP Binding Cassette Subfamily G 2 (*ABCG2*) that consists of several half transporters forming homo- or heterodimers to create active transporters. There are at least 49 ABC transporter genes that are divided into 7 different families (A-G) based on sequence similarity. Three of these are important for drug transport and multidrug resistance.[29]

- **P-glycoprotein or (P-gp, encoded by *ABCB1* gene)**: P-gp, also known as PGY1 or multidrug resistance protein (MDR1), generally expressed in cells that be able to confer resistance to multiple class of drugs, especially chemotherapy. P-gp protein structure is a single polypeptide chain containing two homologue transmembrane domains with two nucleotide binding domains, acts as ATP binding site. Each transmembrane domain consists of six alpha helices. The amine (N) and carboxy (C) terminal of single polypeptide chain are located inside cytoplasmic region (Figure 4).[30, 31] P-gp transporter is located at multiple organs and excretory cell types such as, blood-brain barrier that mainly transport toxic compounds out of the brain, the canaliculi of hepatocyte to excrete xenobiotics out of liver into bile, the apical surface of enterocyte at intestine lumen, the apical surface of proximal tubule at kidney, and epithelial cell of placenta.[28, 30] Drug absorption and oral bioavailability of drug substrates are often influenced by *ABCB1* gene on the apical surface of epithelial cell of lower gastrointestinal tract (jejunum, ileum, colon).[28] P-gp substrates are hydrophobic drugs with a polyaromatic skeleton and a neutral or positive charge such as cytotoxic chemotherapeutic agents, protease inhibitors, immunosuppressants, calcium channel blockers, beta-blockers, statins, steroids, antihistamine, anticonvulsants and antidepressants. The substrate specificity of P-gp is also shared with CYP3A4.[29]
- **Multidrug resistance proteins (MRPs, encoded by *ABCC* family):** There are 10 members of MRPs family and at least 7 members (MRP1 to MRP7). Their structure has two transmembrane domains as P-gp with one extra transmembrane domain containing five alpha-helices and two nucleotide binding domains (Figure 4).[30, 31] MRPs is associated with chemotherapeutic resistance, especially the most significant is MRP1. MRPs are located at the brain, liver, kidneys, and intestines with excretory function. MRPs substrates are often hydrophobic substances.[29]

- **Breast cancer resistance protein or (BCRP, encoded by *ABCG2* gene):** BCRP transporter is an ATP binding cassette half transporter which consists of one nucleotide binding domain and one transmembrane domain (Figure 4).[30] It is an unidirectional transporter that transports substrates from cytoplasm out of the cell.[28] Similar to P-gp, BCRP transporter expresses in gastrointestinal tract, liver kidney, brain, and placenta. BCRP expression can also induce mammary gland during lactation and secret nutrients (such as riboflavin) into milk.[28, 29] BCRP is also associated with hydrophobic anticancer resistance, similar to P-gp, and may cause either a reduction in drug absorption or increased biliary excretion. BRCP substrates include mitoxantrone, anthracyclines and camptothecin derivatives. Some drugs act as BCRP inhibitors such as antiviral nucleoside analogs (e.g. zidovudine, lopinavir, etc.)[29]

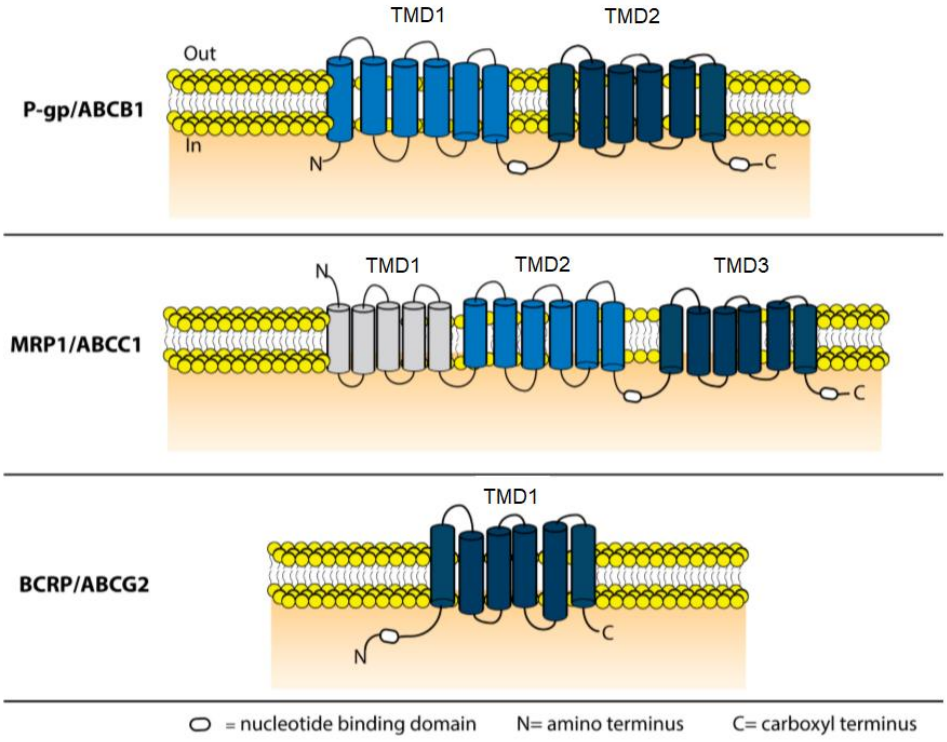


Figure 4 The structure of P-gp, MRPs including MRP1 to MRP7, and BCRP transporters.
P-gp has 2 TMDs containing six alpha-helices each with 2 NBDs, acts as ATP-binding sites. MRPs has 3 TMDs, containing 2 of six alpha-helices and 1 of five alpha helices, with 2 NBDs. BCRP has 1 TMD of six alpha-helices and 1 NBD. Both amino (N) and carboxy (C) terminal of P-gp and BCRP are located inside the cell whereas N-terminal of MRPs is located at out of cell. P-gp, P-glycoprotein; MRPs, multidrug resistance proteins; BCRP, breast cancer resistance protein; TMD, transmembrane domain; NBD, nucleotide binding domain. (Adapted from Jaramillo et al. 2018)[30]

5.2.2. Solute-carriers (SLCs) transporters

SLCs transporters are important in homeostasis maintenance. They transport ions and organic substances across the biological membrane as passive transporters, ion coupled transporters, and exchangers. Most of them is uptake transporters.[28] There are more than 40 SLC superfamilies which are important in transportation several drugs as well as endogenous substances such as steroid hormones, thyroid hormones, leukotrienes, and prostaglandins.[29] SLCs transporters can be classified into several groups known as organic anion transporters (OATs), organic cation transporters (OCTs), organic anion transporting polypeptides (OATPs,).[29]

- **Organic anion transporters (OATs, encoded by SLC22A gene family):** OATs mediate a transport of various range of low molecular weight compounds including steroid hormone conjugates, vitamins, flavonoids, and various drugs such as antivirals, nonsteroidal anti-inflammatory drugs, diuretics.[32, 33] OATs is located mostly at proximal tubule of kidney and also found at liver, skeletal muscle, and placenta.[32] OATs structure has twelve transmembrane domains (TMD) with intracellular N and C terminal. There is large extracellular loop (between TMD1 and TMD2) and intracellular loop (between TMD6 and TMD7) containing glycosylate site and phosphorylation site, respectively (Figure 5).

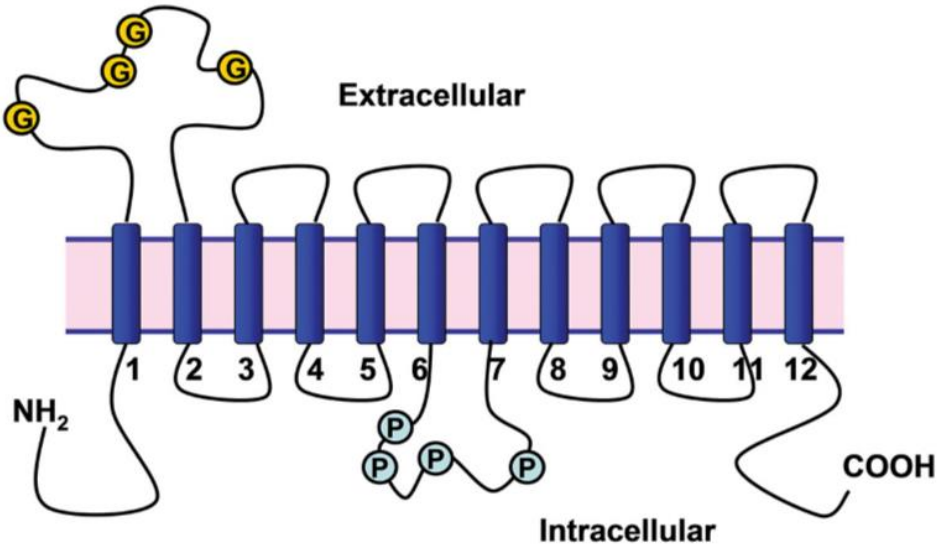


Figure 5 The structure of OATs transporter.
It consists of twelve TMDs with large extracellular and intracellular loop containing glycosylate site and phosphorylation site, respectively. The N and C terminal are located inside the cell. OATs, Organic anion transporters; TMD, transmembrane domain; G, glycosylate site; P, phosphorylation site; N or NH_2, amine terminal; C or COOH, carboxy terminal. (Adapted from Nigam et al. 2015)[33]

- **Organic cation transporters (OCTs, encoded by *SLC22A* gene family):** OCTs is also one of *SLC22A* gene family which mediates the transport organic cations by passive diffusion according to their electrochemical gradients.[32] These transporters include OCT1, OCT2, and OCT3.[34] OCTs is expressed in the liver (especially OCT1), intestine, kidney, placenta, and lung. It generally transports a wide variety of structurally unrelated small organic cation compounds including catecholamines, monoamine neurotransmitters and antiviral drugs.[32] All OCTs are similar in structure consisted of twelve transmembrane domains with intracellular both of N and C terminal and two large extracellular (between TMD1 and TMD2) and intracellular (between TMD6 and TMD7) loop (Figure 6).[35]

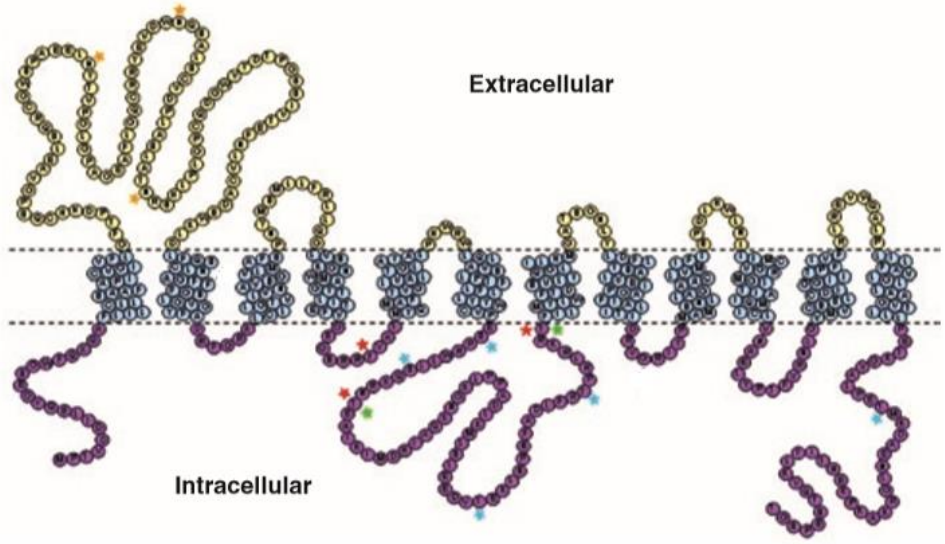


Figure 6 The structure of OCT1 consists of twelve TMDs.
There is large extracellular loop (between TMD1 and TMD2) containing glycosylation sites and intracellular loop (between TMD6 and TMD7) containing protein kinase C phosphorylation sites and protein kinase G phosphorylation sites. OCTs, Organic cation transporters; TMD, transmembrane domain; orange stars, proposed glycosylation sites; blue stars, proposed protein kinase C phosphorylation sites; green stars, protein kinase G phosphorylation sites. (Adapted from Engler et al. 2011)[35]

- **Organic anion transporting polypeptides (OATPs, encoded by *SLCO* gene family):** OATPs mediates a sodium-dependent transport of amphipathic organic compounds including steroids conjugates, thyroid hormones, bile salts, xenobiotics, and pharmaceuticals.[28] The structure of OATPs consists of one transmembrane domain (TMD) with twelve alpha-helices and both N and C terminal is located inside the cell (Figure 7).[36] There are 11 members in OATP family including OATP1B1, OATP1B3, OATP1A2, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, OATP5A1, and OATP6A1. OATP1B1 (encoded by *SLCO1B1* gene) is high expressed selectively at basolateral membrane of human hepatocyte. Although, OATP1B3 is also selectively at liver, it is primarily located at central vein. Therefore, the expression level of OATP1B1 is higher than OATP1B3.[37] There are a number of OATP1B1 drug substrates, including statins (pravastatin, pitavastatin, rosuvastatin, atorvastatin, etc.), enalapril, SN-38 (an active metabolite of irinotecan), methotrexate, pioglitazone, etc.[28]

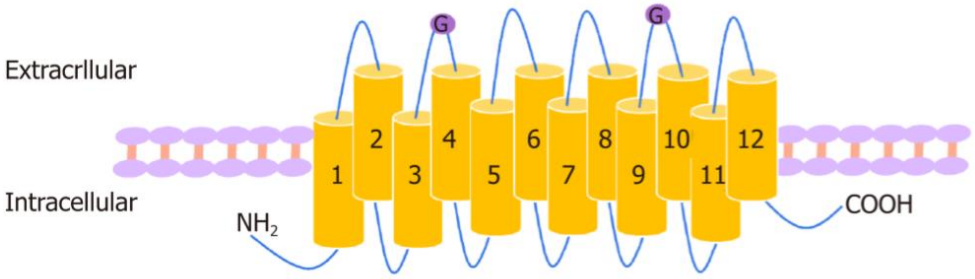


Figure 7 The structure of OATPs transporter.
It consists of twelve TDMs with intracellular N and C terminal. OATP1B1 has glycosylated site at the second and fifth extracellular loop. OATPs, Organic anion transporting polypeptides; TMD, transmembrane domain; G, glycosylate site; N or NH_2, amine terminal; C or COOH, carboxy terminal. (Adapted from Li et al. 2019)[36]

Drug transporters and their main organ expression are summarized in Table 4.

Table 4 ATP-binding cassette and Solute-carrier transporters and their main organ expression[18, 28, 33, 38-40]

Main organ expression	ABC transporters	SLC transporters
Intestine	P-gp (apical) BCRP (apical) MRP2 (apical) MRP3 (basolateral)	OATP (apical) OCT1 (basolateral)
Liver	BCRP (apical) P-gp (apical) MRP2 (apical) MRP3, MRP4, MRP6 (basolateral)	OATP1B1 (basolateral) OATP1B3 (basolateral) OATP2B1 (basolateral) OCT1 (basolateral) OAT2 (basolateral) NTCP (basolateral)
Kidney	P-gp (apical) MRP2, MRP4 (apical) MRP1, MRP3, MRP6 (basolateral)	OAT1, OAT2, OAT3 (basolateral) OCT2 (basolateral)
Blood-brain barrier	P-gp (apical) BCRP (apical) MRP1 (basolateral) MRP4, MRP5 (apical)	OATP1A2 (apical) OATP2B1 (apical) OAT3 (apical)
Placenta	P-gp (apical) BCRP (apical) MRP2 (apical)	OATP2B1 (basolateral) OATP4A1 (apical) OCT3 (basolateral) OAT4 (basolateral)

BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; OCT, organic cation transporter; OAT, organic anion-transporting polypeptides; OATP, Organic anion transporting polypeptide; NTCP, sodium-taurocholate cotransporting polypeptide

This study focuses on intestinal P-gp, BCRP, and OATP1B1 transporters activity in the elderly with or without CKD. According to the previous report that these transporters accounted for pharmacokinetic changes of drug substrate either drug interaction or genetic polymorphisms.[28] Gut-P-gp which is highly located at the apical side of enterocyte generally transports drug substrates into the intestinal lumen and is associated with drug absorption and bioavailability reduction.[28, 41, 42] BCRP is also located at intestinal lumen and canaliculi of the hepatocyte which has been reported to associated with drug absorption and hepatobiliary excretion.[43, 44] OATP1B1 is highly expressed at the basolateral membrane of the hepatocyte and is associated with drug uptake and drug exposure.[32, 36, 37]

Drug metabolizing enzymes and transporters expressed in human liver, kidneys and intestine is shown in Figure 8.

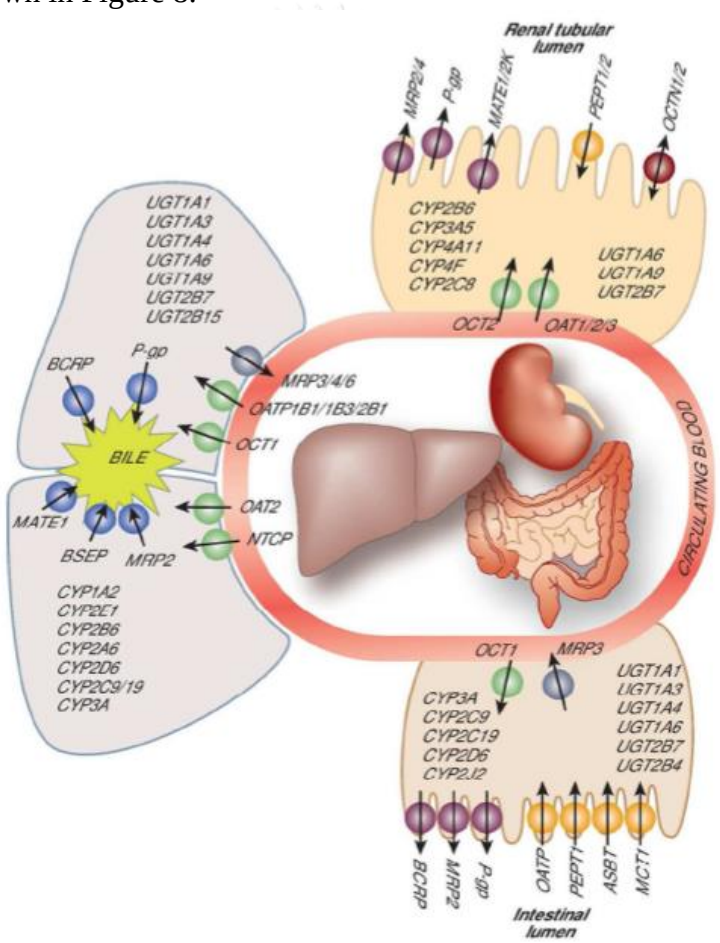


Figure 8 The summarized drug metabolizing enzymes and dug transporters that operate in human liver, kidneys, and intestine.[18]
CYP, cytochrome P 450; UGT, uridine diphosphate-glucuronosyltransferase; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; MATE, multidrug and toxin extrusion transporters; PEPT, peptide transporter, OCT, organic cation transporter; OAT, organic anion-transporting polypeptides; OATP, Organic anion transporting polypeptide; NTCP, sodium-taurocholate cotransporting polypeptide; ASBT, apical sodium dependent bile acid transporter; MCT, monocarboxylate transporters; BSEP, bile salt export pump

5.3. Chronic kidney disease is associated with enzymes and drug transporters changes in intestine and liver

The activity of non-renal drug-metabolizing enzymes (e.g. cytochrome P450 in some isoforms, N-acetyltransferase (NAT)) and transporters (e.g. P-gp, OATPs, multidrug resistance-associated protein) are reduced in CKD and may affect non-renal clearance of substrates and increased bioavailability of drug.[18],[15, 45-47] Many studies reported associations of CYP450 enzymes with PK alterations rather than drug transporters.

In CKD, 30 – 67% reduction of clearance was reported for the substrates of CYP3A4, CYP2D6, CYP2B6, and CYP2C9 enzymes.[15] For instance, clearance of intravenous and oral midazolam, a probe substrate of CYP3A4, are controversial which found a markedly higher AUC of intravenous midazolam in CKD on hemodialysis compared to healthy subjects, but not found in an oral midazolam.[48, 49] These might be associated with CYP3A4 mediated mechanism in enterocytes of oral midazolam that could be also affected by CKD resulting in possible masking the effect of hepatic clearance reduction.[49] For other CYP450 isozymes, the clearance of bupropion is reduced in CKD by a reduction of CYP2B6 activity.[50, 51] The hepatic CYP2C9 activity is reduced in CKD patients resulting in a 50% increase in the S/R-warfarin ratio.[52]

Phase II metabolizing enzymes are also altered in CKD. The clearance of isoniazid and procainamide is decreased due to a reduction of hepatic metabolism by NAT in patients with CKD.[53, 54] Area under the curve (AUC) of zidovudine, morphine and its metabolites (morphine-3-glucuronide and morphine-6-glucoronide) increased significantly in CKD patients suggesting a reduction of uridine diphosphate-glucuronosyltransferase (UGT) activity.[55, 56] In case of drug transporter activity, fexofenadine clearance is decreased reflecting a reduction of hepatic OATP activity.[48, 49]

5.4. Renal drug transporters activity in CKD

Renal drug transporters play a crucial role in drug elimination. A study in rat model reported a decrease in organic anion-transporter 1 (OAT1), OAT2, OAT3, organic anion-transporting polypeptides 4C1 (OATP4C1), and P-gp uptake transporters expression. Additionally, an increase in multidrug resistance-associated protein 2 (MRP2), MRP3, MRP4 efflux transporters was also observed.[57, 58] These alterations may be caused by an inhibition of uremic toxins which often affect the tubular secretion.[16]

5.5. Genetic polymorphism of drug metabolizing enzymes

Single Nucleotide Polymorphisms (SNPs) are the substitution of single base in deoxyribonucleic acid (DNA) sequence which occur approximately 1/1000 base pairs. SNPs are the most common type of genetic variation and commonly found more than 1% in population. Interestingly, SNPs can occur within gene and non-coding regions of DNA and more than 100 million SNPs in population have been discovered. Moreover, some SNPs are major in some region or race, others are generally found in population. Although most of them do not cause a disorder, some of them effect on certain health problem in term of drug response, PK alteration, etc. [59, 60]

SNPs of CYP450 have a large impact on CYP450 activity and affect drug/ substrate metabolism and drug efficacy.[61] For instance, statins is mainly metabolized by CYP3A4. Kajinami, et al.[62] showed that 290A>G variant of CYP3A4 caused a reduced response of atorvastatin leading to an ineffective reduction in low density lipoprotein (LDL)-cholesterol levels. In contrast, 445M>T, also known as 1334T>C or CYP3A4*3, polymorphism enhanced atorvastatin efficacy resulting in lower LDL-cholesterol levels in 445M>T polymorphism compared to wild type.

Wang, et al.[63] conducted a study to observe the effect of CYP3A4*4 (Ile118Val) on the efficacy of lipid-lowering of simvastatin and CYP3A4 activity. CYP3A4*4 was related to a functional decreased of CYP3A4 activity and seemed to increase lipid-lowering effect of simvastatin.

Goa, et al[64] investigated the effect of CYP3A4*1G polymorphism on statins efficacy. A group with CYP3A41*G showed an increase in atorvastatin efficacy in lowering lipid levels but this was not seen in simvastatin. CYP2D6 is also involved in statin metabolism.

Li, et al[65] showed that allele T of CYP2D6*10 is not related to hyperlipidemia, whereas CC genotype seemed to increase lipid lowering effect of simvastatin. Therefore, patients with CC genotype may require a lower dose of simvastatin than patients with CT/TT genotype.

In patients who are on statins, CYP450 polymorphisms are also associated with ADR from this drug. Frudakis, et al[66] suggested that the CYP2D6*4 allele is associated with muscle pain, myalgia, rhabdomyolysis, and creatine kinase evaluation without pain.

5.6. Genetic polymorphism of drug transporters

The genetic variations of drug transporter proteins may also cause interindividual variation in drug response.[18, 28, 67] In this study, we investigated the genetic variations of transporters in young healthy subjects and the elderly with and without CKD to exclude confounding factor which could affect plasma drug concentration. OATP1B1 and BCRP were focused.

5.6.1. Genetic variations of OATP1B1 transporter

OATP1B1 transporter is a hepatic influx transporter that uptakes substrates from the blood into hepatocytes. OATP1B1 transporter has more than 40 nonsynonymous SNPs identified on *SLCO1B1* gene and several of them are associated with functional changes. The major haplotypes in *SLCO1B1* gene which impact plasma drug concentration are *SLCO1B1*1A* (wild type), **1B* (wild type), **5*, **15*, **17* (Table 5) and its frequencies also differ between ethnic groups.[5, 28, 68]

Table 5 The major haplotypes in *SLCO1B1* gene

Nomenclature	**Allele function status**	**SNPs**			
		-11187	-10499	388	521
SLCO1B1*1A	Normal function	G	A	A	T
SLCO1B1*1B	Normal function	G	A	G	T
SLCO1B1*5	Decreased function	G	A	A	C
SLCO1B1*15	Decreased function	G	A	G	C
SLCO1B1*17	Decreased function	A	A	G	C

SLCO1B1, Solute carrier organic anion transporter family member 1B1; A, adenine; C, cytosine; G, guanine; T, thymine.

SNPs associated with OATP1B1 functional change is c.521 T>C variant that results in a decrease in OATP1B1 activity and changes in pharmacokinetics of OATP1B1 substrates. Statins is identified as OATP1B1 substrate (e.g. rosuvastatin, atorvastatin, pravastatin, pitavastatin). The c.521T>C and c.388A>G SNPs cause an increase in area under the concentration-time curve (AUC) and maximum plasma concentration (C_{max}) of statins due to a reduction of OATP1B1 activity. This may increase the risk of systemic toxicity, especially statin-induced myopathy.[67-70]

5.6.2. Genetic variations of BCRP transporter

BCRP transporter, encoded by *ABCG2* gene, transports substrates from the cytoplasm out of the cells. Several allelic variants may affect activity of gene products and modulate *ABCG2* gene. The two most frequent nonsynonymous mutations are c.421C>A and c.34G>A. c.421C>A is associated with a reduction of BRCP transporter activity, which has an important role in limiting the absorption of statins.[5, 18, 68, 71]

6. Clinical studies in elderly population

Clinical studies in elderly are underrepresented compared to younger adults. Hence, the dosage recommendation in the small drug package insert and the expected drug response may not be the same as young subjects.[4, 7]

At present, there was clinical review studies of pharmacotherapy in elderly population. For instance, Rhee et al. 2015[72] conducted the review study in HIV elderly patients using antiretroviral agents. Interestingly, they showed the association of pharmacokinetic alteration with advanced age and suggested for more clinical prospective further studies. In 2017-2018, cohort study and review study were conducted to discover antiretroviral management in elderly but the standard dosage adjustment has not been achieved yet.[73, 74] Likewise, Frank et al. 2014[75] performed a clinical review discussing the pharmacologic treatments in depression of the elderly found that there were several factors influencing on individual drug management such as comorbidities, drug-drug interaction, and clinical outcome.

Although there are several reasons related to underrepresent of elderly in clinical studies (such as the challenges of obtained inform consent, the effect of multiple comorbidities and polypharmacy to outcome assessment, the compliance to protocol, more supportive care requirement, and etc.), the results of clinical studies in elderly could especially be beneficial regard to the prediction of efficacy and risk in larger

elderly population, the appropriate dosage regimen for optimal outcome, and the standard guideline for special healthcare.(76)

To date, there were no clinical studies that demonstrate the mechanistic of pharmacokinetic changes in the elderly to help determine dosage adjustment specifically for this group of Thai patients.

7. Cocktail study

Generally, the cocktail approach has been widely used as a screening tool for DDI assessment in human early phases of clinical trial and drug development. Interestingly, the cocktail approach containing multiple probe substrates has been also used as a tool for simultaneously determining drug metabolizing enzymes and drug transporters activities. These could reduce the confounding influence of the inter- and intra-individual variability and also reduce the number of clinical trials and budget compared to the individual administration of specific probes in multiple studies.[77, 78]

For decades, U.S. FDA guidance and The EMA draft DDI guideline have recommend using cocktail approach that simultaneously administers a mixture of probe substrates of multiple CYP enzymes and transporters to evaluate a drug's inhibition or induction.[79, 80] In short, the probe drugs should be specific for individual CYP enzymes or transporters and do not interact with each other. The dosage should preferably be the dose in the validation study and the study should be conducted in a sufficient number of subjects. Negative results from a well-conducted cocktail study can rule out the need for evaluation of CYP enzymes or transporter. In contrast, if the results are positive, the additional studies for *in vivo* evaluation may be needed to provide definite quantitative exposure change of PK (such as AUC, C_{max}).[79, 80] Probe substrates of CYP enzymes and transporters for concomitant use clinical DDI studies and/or drug labeling are recommended by U.S. FDA are listed in Table 6 and Table 7.[81]

Table 6 Probe substrates of CYP enzymes for concomitant use clinical DDI studies and/or drug labeling

CYP Enzymes	Sensitive substrates	Moderate sensitive substrates
CYP1A2	alosetron, caffeine, duloxetine, melatonin, ramelteon, tasimelteon, tizanidine	clozapine, pirfenidone, ramosetron, theophylline
CYP2B6	bupropion	efavirenz
CYP2C8	repaglinide	montelukast, pioglitazone, rosiglitazone
CYP2C9	celecoxib	glimepiride, phenytoin, tolbutamide, warfarin
CYP2C19	S-mephenytoin, omeprazole	diazepam, lansoprazole, rabeprazole, voriconazole
CYP2D6	atomoxetine, desipramine, dextromethorphan, eliglustat, nebivolol, nortriptyline, perphenazine, tolterodine, R-venlafaxine	encainide, imipramine, metoprolol, propafenone, propranolol, tramadol, trimipramine, S-venlafaxine
CYP3A	alfentanil, avanafil, buspirone, conivaptan, darifenacin, darunavir, ebastine, everolimus, ibrutinib, lomitapide, lovastatin, midazolam, naloxegol, nisoldipine, saquinavir, simvastatin, sirolimus, tacrolimus, tipranavir, triazolam, vardenafil, budesonide, dasatinib, dronedarone, eletriptan, eplerenone, felodipine, indinavir, lurasidone, maraviroc, quetiapine, sildenafil, ticagrelor, tolvaptan budesonide, dasatinib, dronedarone, eletriptan, eplerenone, felodipine, indinavir, lurasidone, maraviroc, quetiapine, sildenafil, ticagrelor, tolvaptan	alprazolam, aprepitant, atorvastatin, colchicine, eliglustat, pimozide, rilpivirine, rivaroxaban, tadalafil

CYP, Cytochrome P450. (Adapted from US Food and Drug Administration. Drug development and drug interactions, Table of substrates, inhibitors and inducers.[81])

Table 7 Probe substrates of transporter for concomitant use clinical DDI studies and/or drug labeling

Transporter	Gene	Substrate
P-gp	*ABCB1*	dabigatran etexilate, digoxin, fexofenadine
BCRP	*ABCG2*	rosuvastatin, sulfasalazine
OATP1B1 **OATP1B3**	*SLCO1B1*, *SLCO1B3*	asunaprevir, atorvastatin, bosentan, cerivastatin, danoprevir, docetaxel, fexofenadine, glyburide, nateglinide, paclitaxel, pitavastatin, pravastatin, repaglinide, rosuvastatin, simvastatin acid
OAT1 **OAT3**	*SLC22A6*, *SLC22A8*	adefovir, cefaclor, ceftizoxime, famotidine, furosemide, ganciclovir, methotrexate, oseltamivir carboxylate, penicillin G
MATE1, **MATE-2K, OCT2**	*SLC47A1*, *SLC47A2*, *SLC22A2*	metformin

BCRP, breast cancer resistance protein, P-gp, P-glycoprotein, MATE, multidrug and toxin extrusion transporters, OCT, organic cation transporter, OAT, organic anion-transporting polypepties. (Adapted from US Food and Drug Administration. Drug development and drug interactions: Table of substrates, inhibitors and inducers.[81])

At first, the development of cocktail drugs mostly focused on the drug metabolizing enzyme to explore the route of drug metabolism. The developed cocktails were also applied to determine phenotypic drug metabolizing enzyme activities and drug metabolizing enzyme-mediated drug-drug interactions in drug development and special population such as patient with liver disease. The development of drug metabolizing enzyme probes cocktail studies is summarized in Table 8.

Several clinical studies have reported the association of DDI and CYP450, but mostly limited to drug transporters.[82] In 2015, Digoxin, a P-gp probe substrate, was added in validated cocktail as 'Modified Cooperstown 5+1 cocktail' and 'Modified Inje cocktail' to determine cytochrome P450 and P-gp transporter mediated pharmacokinetic drug-drug interaction (Table 9).[83, 84] So far, drug transporter cocktail studies has increased considerably (Table 9).

In 2017, digoxin was substituted by dabigatran etexilate as intestinal P-gp probe substrate in microdose cocktail study.[85] Because digoxin has low sensitivity to P-gp inhibitor and has no selectivity for P-gp *in vitro* and *in vivo* related to use for studying intestinal P-gp. Digoxin is not only mediated by P-gp lacated at intestine, but also involved in P-gp located at urinary and bile efflux.[86] Dabigatran etexelate has become a clinical probe for studying intestinal P-gp due to higher selectivity for intestinal P-gp and solely mediated by P-gp at intestine.[87] The development of drug metabolizing enzyme and transporter probes cocktail studies are summarized in Table.9.

Table 8 The development of drug metabolizing enzyme probes cocktail studies

Cocktail drugs	Substrate	Objectives	Key points	References
Antipyrine Theophylline	Hepatic drug metabolizing enzymes	To determine the correlation of cocktail drugs metabolic pathways whether mediated by the same or closely cytochrome P450 isozymes.	• The first cocktail study in human • Cocktail approach could explore drug metabolic pathways correlation.	Teunissen et al. 1985[88]
Antipyrine Tolbutamide	Hepatic drug metabolizing enzymes	To investigate the selective inhibitory effect of sulphaphenazole, cimetidine and primaquine on the disposition of cocktail drugs.	• Cocktail approach could investigate the selective drug metabolism pathway and inhibitory effect of drugs.	Back et al.1988[89]
Antipyrine Hexobarbitone Theophylline	Hepatic drug metabolizing enzymes	To determine the involved cytochrome P450 isozymes metabolism relationship between cocktail drugs.	• Cocktail approach could characterize and correlate activities of different cytochrome P450 isozymes and investigate substrate selectivity. • The influence of intra-individual variation in drug oxidation could be eliminated.	Schellens et al. 1988[90]
Antipyrine Hexobarbitone Aminophylline	Hepatic drug metabolizing enzymes	To investigate the effect of liver disease to cocktail drugs oxidation and antipyrine metabolite formation.	• The first application of cocktail approach in patients • Cocktail approach could determine cytochrome P450 substrate selectivity and activity related to liver disease.	Schellens et al. 1989[91]

CYP, Cytochrome P450; NAT, N-acetyltransferase; XO, xanthine oxidase

Cocktail drugs	Substrate	Objectives	Key points	References
Antipyrine Nifedipine Sparteine Mephenytoin	Hepatic drug metabolizing enzymes	To assess the inductive effect and inhibitory effect of phenobarbital and cimetidine, respectively, on drug metabolizing enzyme activities in subjects with or without poor and extensive metabolizer polymorphism.	• Cocktail approach could investigate the effects of environmental factors on the drug oxidation of multiple probe substrates.	Schellens et al. 1989[92]
Dapsone Debrisoquin Mephenytoin	CYP3A, NAT CYP2D6 CYP2C19	To characterize a population of healthy individuals for their ability to *N*-hydroxylate dapsone.	• Cocktail approach could determine enzyme activities by measurement of cocktail drug metabolites lead to understand route of metabolism and reduce adverse events.	May et al. 1994[93]
Caffeine Chlorzoxazone	CYP1A2 CYP2E1	To evaluate the specificity of these two probe substrates	• Intra- cocktail drug interaction was occurred.	Berthou et al. 1995[94]
'Pittsburgh cocktail'				
Caffeine Chlorzoxazone Dapsone Debrisoquin Mephenytoin	CYP1A2 CYP2E1 CYP3A, NAT CYP2D6 CYP2C19	To validate cocktail probe for using as a metabolic cocktail to estimate CYP1A2, CYP2E1 CYP3A, CYP2D6, CYP2C19, NAT activities	• The first cocktail study used five combinations of probe drugs. • No intra-cocktail drug interaction in low dose. • Limitation was specificity to CYP3A of dapsone seemed not enough because CYP2E1 might be involved.	Frye et al.1997[95]

CYP, Cytochrome P450; NAT, N-acetyltransferase; XO, xanthine oxidase

Cocktail drugs	Substrate	Objectives	Key points	References
‘Greenford-Ware (GW) cocktail’				
Caffeine Chlorzoxazone Midazolam Debrisoquin Mephenytoin Diclofenac	CYP1A2 CYP2E1 CYP3A CYP2D6 CYP2C19 CYP2C9	To validate and develop method for extraction and analysis probes of proposed ‘GW cocktail’ and to minimize limitation of ‘Pittsburgh cocktail’	• Dapsone in ‘Pittsburgh cocktail’ was substituted by midazolam for probe of CYP3A • Diclofenac was added as a probe for CYP2C9	Scott et al. 1999[96]
Modified from ‘Pittsburgh cocktail’				
Caffeine Chlorzoxazone Midazolam Metoprolol Mephenytoin	CYP1A2 CYP2E1 CYP3A CYP2D6 CYP2C19	To validate the modified cocktail probe for simultaneous phenotyping CYP1A2, CYP2E1, CYP3A, CYP2D6, CYP2C19 activities.	• Using debrisoquin as CYP2D6 probe was not approved by US FDA. • Debrisoquine and dapsone in ‘Pittsburgh cocktail’ were substituted by metoprolol and oral midazolam, respectively • Oral midazolam provided more CYP3A specificity.	Zhu et al. 2001[97]
‘Cooperstown cocktail’				
Caffeine Omeprazole Dextromethorphan Intravenous (IV) midazolam	CYP1A2, NAT2, XO CYP2C19 CYP2D6 CYP3A4	To validate Cooperstown cocktail probe for simultaneous phenotyping CYP1A2, CYP2C19, CYP2D6, CYP3A, NAT2, and XO activities	• The limitation of IV midazolam was the need for multiple blood sampling and not represented CYP3A4 activity	Streetman et al. 2000[98]

CYP, Cytochrome P450; NAT, N-acetyltransferase; XO, xanthine oxidase

Cocktail drugs	Substrate	Objectives	Key points	References
'Cooperstown 5+1 cocktail'				
Caffeine Omeprazole Dextromethorphan IV midazolam Warfarin plus vitamin K	CYP1A2, NAT2, XO CYP2C19 CYP2D6 CYP3A4 CYP2C9	To validate Cooperstown 5+1 cocktail probe for simultaneous phenotyping CYP1A2, CYP2C19, CYP2D6, CYP3A, CYP2C9, NAT2, and XO activities	• Provided additional phenotypic measurement for CYP2C9 activity. • The use of intravenous midazolam was still a limitation.	Chainuvati et al. 2003[99]
'Cooperstown 5+1 cocktail'				
Caffeine Omeprazole Dextromethorphan IV midazolam Warfarin plus vitamin K	CYP1A2, NAT2, XO CYP2C19 CYP2D6 CYP3A4 CYP2C9	To assess drug interaction of apivaroc by using 'Cooperstown 5+1 cocktail'	• Cocktail approach could determine drug interaction potential in drug developement.	Johnson et al. 2006[100]
'Karolinska cocktail'				
Caffeine Losartan Omeprazole Debrisoquin Quinine	CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	To validate 'Karolinska cocktail' for simultaneous phenotyping CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 activities and to design a few sampling collection schedule	• The inhibition of debrisoquin metabolism by concurrent drugs was occurred, given debrisoquin separately was suggested	Christensen et al. 2003[101]
'Inje cocktail'				
Losartan Oral midazolam Caffeine Omeprazole Dextromethorphan	CYP2C9 CYP3A CYP1A2 CYP2C19 CYP2D6	To develop five cocktail probe drugs to overcome the limitation of debrisoquine in 'Karolinska cocktail'and IV midazolam in 'Cooperstown cocktail'.	• This modified cocktail was developed to determine CYP2C9, CYP3A, CYP1A2, CYP2C19, CYP2D6 activities	Ryu et al. 2007[102]

CYP, Cytochrome P450; NAT, N-acetyltransferase; XO, xanthine oxidase

Table 9 The development of drug metabolizing enzyme and transporter probes cocktail studies

Cocktail drugs	Substrate	Objectives	Key points	References
'Modified Cooperstown 5+1 cocktail'				
Caffeine Omeprazole Dextromethorphan IV midazolam Warfarin plus vitamin K Digoxin	CYP1A2 CYP2C19 CYP2D6 CYP3A4 CYP2C9 P-gp	To determine the effect of etravirine on pharmacokinetics of 'Modified Cooperstown 5+1 cocktail'.	• Cocktail approach could determine PK drug-drug interaction in drug development.	Kakuda et al. 2014[83]
'Modified Inje cocktail'				
Losartan Oral midazolam Caffeine Omeprazole Dextromethorphan Digoxin	CYP2C9 CYP3A CYP1A2 CYP2C19 CYP2D6 P-gp	To evaluate potential inhibitory perpetrator of felodipine causing PK drug-drug interactions involving cytochrome P450 and P-gp.	• Cocktail approach could determine PK drug-drug interaction in drug development.	Synder et al. 2014[84]
Digoxin Metformin Rosuvastatin Furosemide	P-gp OCT, MATE BCRP, OATP OAT1, OAT3	To propose cocktail probes for determination of transporter-mediated drug-drug interaction	• The proposed transporter cocktail drugs for further clinical study.	Ebner et al. 2015[103]

CYP, Cytochrome P450; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein; MATE, multidrug and toxin extrusion transporters; OATP, Organic anion transporting polypeptide; OCT, organic cation transporter; OAT, organic anion-transporting polypepties; NAT, N-acetyltransferase; XO, xanthine oxidase

Cocktail	Substrate for	Objective	Key points	References
Digoxin Metformin Rosuvastatin Furosemide	P-gp OCT, MATE1, MATE2-K BCRP, OATP1B1/1B3 OAT1, OAT3	To explore the evaluation of proposed cocktail in Ebner et al. 2015[103] to determine transporter-mediated drug-drug interaction	• The first transporter cocktail clinical study. • Intra-interaction between digoxin and furosemide. • Increased in rosuvastatin with unknown mechanism.	Stopfer et al. 2016[104]
Digoxin Metformin Rosuvastatin Furosemide	P-gp OCT, MATE1, MATE2-K BCRP, OATP1B1, OATP1B3 OAT1, OAT3	To eliminate intra-cocktail interaction in Ebner et al. 2015[103] and Stopfer et al. 2016[104].	• Dose of metformin and furosemide was reduced to avoid drug-drug interaction with rosuvastatin	Stopfer et al. 2018[105]
Digoxin Metformin Rosuvastatin Furosemide	P-gp OCT, MATE1, MATE2-K BCRP, OATP1B1, OATP1B3 OAT1, OAT3	To validate a developed cocktail drugs from Stopfer et al. 2018[105].	• This cocktail has sensitivity and specificity and has potential to use as a tool in the evaluation of clinically relevant transporter-mediated drug-drug interaction.	Wiebe et al. 2020[106]

CYP, Cytochrome P450; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein; MATE, multidrug and toxin extrusion transporters; OATP, Organic anion transporting polypeptide; OCT, organic cation transporter; OAT, organic anion-transporting polypepties; NAT, N-acetyltransferase; XO, xanthine oxidase

Cocktail	Substrate for	Objective	Key points	References
Oral midazolam Dabigatran etexilate Pitavastatin Rosuvastatin Atorvastatin	CYP3A Intestinal P-gp OATP1B BCRP, hepatic OATP, P-gp CYP3A, Hepatic OATP, BCRP, intestinal P-gp,	To validate a microdose cocktail as a tool to assess clinical DDI related to drug transporters and CYP3A	• This cocktail has sensitivity and specificity and has potential to use as a tool in the evaluation of clinically relevant transporter and CYP3A-mediated drug-drug interaction. • Used 1/100th of pharmacological dose, so called 'microdose cocktail' lead to have more safety	Prueksa-ritanont et al. 2017[85]
Oral midazolam Dabigatran etexilate Pitavastatin Rosuvastatin Atorvastatin	CYP3A4 Intestinal P-gp OATP1B BCRP, hepatic OATP CYP3A4, hepatic OATP, BCRP, P-gp	To evaluate the impact of renal impairment on drug transporter and CYP3A4 activities by using validated microdose cocktail in Prueksa-ritanont et al. 2017[85]	• This microdose cocktail has efficacy and safety to use as a tool to phenotyping transported and CYP3A4 activities in renal impairment patients.	Tatosian et al. 2020[107]

CYP, Cytochrome P450; BCRP, breast cancer resistance protein; P-gp, P-glycoprotein; MATE, multidrug and toxin extrusion transporters; OATP, Organic anion transporting polypeptide; OCT, organic cation transporter; OAT, organic anion-transporting polypepties; NAT, N-acetyltransferase; XO, xanthine oxidase

8. Pharmacokinetics of cocktail containing probe drugs

In this study, the validated microdose cocktail containing oral midazolam, dabigatran etexilate, pitavastatin, rosuvastatin, and atorvastatin in Prueksaritanont et al. 2017[85] was used. This microdose cocktail was further co-administered with inhibitors (rifampicin, itraconazole, and clarithromycin). The group found that PK of drug substrates was comparable to previous reports in pharmacological dose, except non-linear PK was observed in dabigatran.[85] To understand more about drug profile, the pharmacokinetics of probe drugs are described below.

Midazolam (probe of CYP3A)

- **Absorption**

After oral administration, midazolam is absorbed rapidly and completely with reached maximum concentration ~ 1 hour. Linear relationship is appeared between oral dosage and plasma concentration. The bioavailability (BA) is low with ranged between 40-50% due to first-pass metabolism at intestine and liver.[108, 109]

- **Distribution**

Midazolam rapidly distributes to tissue within 1-2 hours after oral administration. Volume of distribution (Vd) is 0.8-1.5 L/kg. Midazolam is highly bound to plasma proteins with 96-98% protein binding primarily to albumin.[108, 109]

- **Metabolism**

Midazolam has intermediate hepatic extraction ratio (E_H 0.3-0.6) depending on liver blood flow, enzyme activity, and unbound fraction for hepatic excretion.[110, 111] It is exclusively eliminated by biotransformation and primarily metabolized by CYP3A isozymes. Both CYP3A4 and CYP3A5 are involved in midazolam oxidative metabolism. The hydroxylation of CYP3A is occurred rapidly after oral administration to form 1'-hydroxymidazolam (major active metabolite), 4-hydroxymidazolam, and 1',4-hydroxymidazolam.[108, 112]

Although there was an evidence confirmed that midazolam is predominantly mediated by cytochrome P450 3A4 isozymes in human liver, the regioselectivity of CYP3A4 and CYP3A5 was related to the formation of 1'-hydroxymidazolam. It seemed that α-hydroxymidazolam, an active metabolite, and 4-hydroxymidazolam is mainly formed by CYP3A5 and CPY3A4, respectively.[113] The pharmacologically active of 1'-hydroxymidazolam has only 10% of active midazolam and has no clinically significant relevance.[114] These metabolites are further rapidly metabolized by glucuronidation and excreted as conjugated metabolites. UDP-glucuronosyltransferase (UGT) is major mediated enzyme in 1'-hydroxymidazolam, 4-hydroxymidazolam, and 1',4-hydroxymidazolam conjugation.[115]

In intestine, approximately 70% of cytochrome P450 at luminal is CYP3A4 isozyme.[116] CYP3A4-mediated intestinal first-pass metabolism of midazolam is a major isozyme and has a variable role with intestinal extraction ranged 0 to 0.77.[117, 118] The potential significant CYP3A-mediated first-pass metabolism between intestine and liver is comparable (mean extraction ratio ~0.44) whereas glucuronidation is only occurred in liver.[117] Thus, small intestine is one of the major sources of interindividual

variability in oral midazolam bioavailability.[117] Using midazolam as a probe substrate of CYP3A could represent both CYP3A mediated-intestine and hepatic metabolism. The metabolism pathway of midazolam is shown in Figure 9.

- **Excretion**

After oral administration, midazolam is rapidly eliminated as conjugated metabolites into urine within 24 hour.[110] 1'-hydroxymidazolam glucuronide, major metabolite, is found in urine ~60-70% of dose excreted in urine. 4-hydroxymidazolam glucuronide and 1',4-hydroxymidazolam glucuronide, two minor metabolites, are only found ~3% and 1% of dose excreted in urine, respectively.[119] The elimination in urine as unchanged form is very low, approximately <1% of dose excreted in urine.[110] The elimination half-life ranged from 1.5 to 2.5 hours.[109]

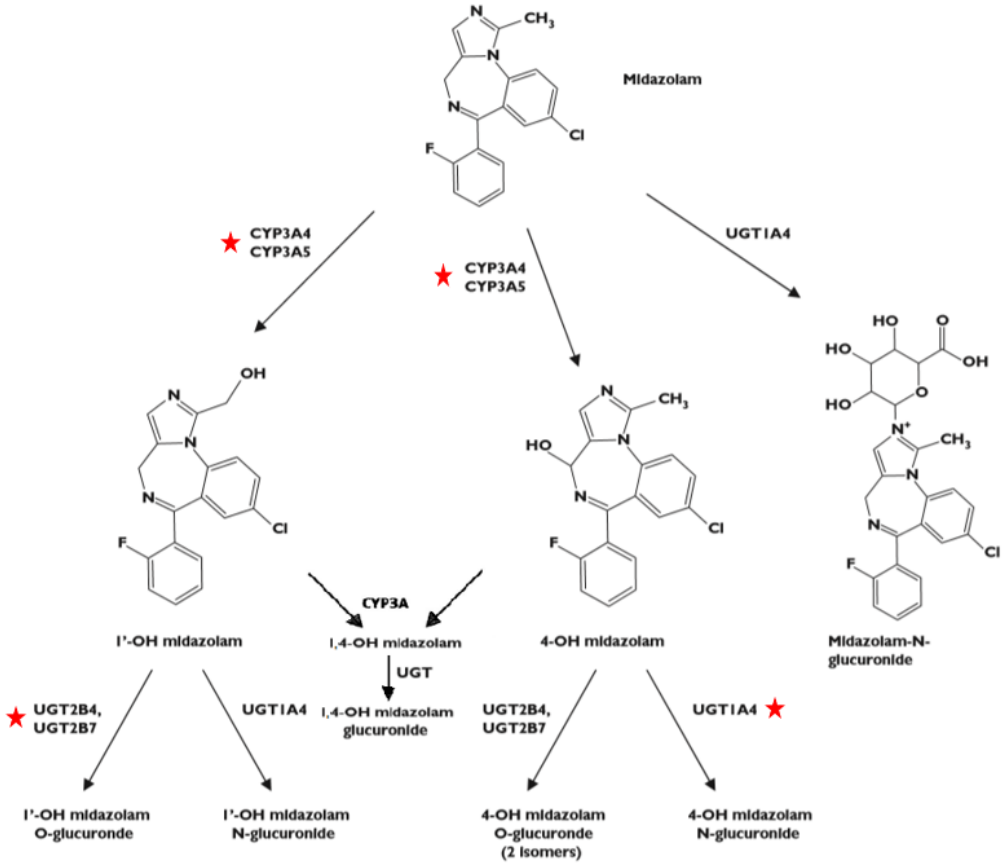


Figure 9 The metabolism pathway of midazolam.
Midazolam is primarily metabolized by both CYP3A4 and CYP3A5 and then converted to major metabolite (1'-OH midazolam) and two minor metabolites (4-OH midazolam, 1', 4-OH midazolam). These metabolites are rapidly conjugated with glucuronic acid. 1'-OH midazolam and 4-OH midazolam are mainly mediated by UGT2B4, UGT1B7 and UGT1A4, respectively.[120] The glucuronide conjugated metabolites are excreted into urine with 24 hours after oral midazolam administration. The alternative metabolism pathway is midazolam conjugation mediated by UGT1A4 directly when poor metabolism or inhibition of CYP3A4/5 are occurred.[121] CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; 1'-OH midazolam, 1'-hydroxymidazolam; 4-OH midazolam, 4-hydroxymidazolam; 1', 4-OH midazolam, 1', 4-hydroxymidazolam; red star indicates the main metabolism route. (Adapted from Hyland et al. 2009 and Nguyen et al. 2016)[115, 122]

Dabigatran etexilate (probe substrate of intestinal P-gp)

- **Absorption**

Dabigatran has high lipophilicity resulting in unabsorbed after oral administration.[123] Capsule of dabigatran etexilate, a prodrug of dabigatran, consists of pellets containing tartaric acid at the core and encapsulated with dabigatran etexilate.[124] Dabigatran etexilate, can increases bioavailability because tartaric acid can control acidic pH itself independent of gastric pH environment lead to increase drug dissolution and absorption.[124, 125]

Dabigatran exetilate, but not dabigatran, is a substrate of efflux P-gp transporter at intestine with high selectivity.[124] After oral administration, dabigatran level reaches maximum plasma concentration within 1 hour, it could be delayed when administered with high-fat meal.[126] The absolute bioavailability of dabigatran is 3-7%.[126] Despite the low bioavailability, dabigatran has linear pharmacokinetics between dosage and plasma concentration.[123]

- **Distribution**

Volume of distribution (Vd) of dabigatran is 50-70 L. Plasma protein binding is approximately 35%.[126]

- **Metabolism**

Dabigatran etexilate is rapidly converted to dabigatran, active metabolite, by esterase enzymes found in enterocyte, plasma and liver.[127] Dabigatran etexilate is a double prodrug involving two steps of hydrolysis and has different pathways depending on carboxyesterase (CES) enzymes.[128] The transformation of dabigatran etexilate to active metabolite is mediated by CES1 and CES2 with relatively substrate nonspecific.[129]

The bioconversion could appear in plasma, enterocyte, and liver (minor).[129, 130] In plasma, dabigatran etexilate is converted by nonspecific esterases into active metabolite.[130]

In intestine, the conversion of dabigatran etexilate initially occured at enterocyte by carbamate hydrolysis using CES2 to BIBR0951 (M2), then transferred to liver and hydrolyzed by CES1 to active metabolite.[129]

Another pathway is prodrug directly enters into liver and converted by ester hydrolysis into BIBR1087 (M1), then hydrolyzed to active metabolite by CES1. [129] Therefore, prodrug, M2, and active metabolite can be found in the portal vein, but the bioconversion to active metabolite is completed once in the liver.[127] The bioconversion of dabigatran etexilate to dabigatran was shown in Figure 10.

Cytochrome P450 is not associated with dabigatran metabolism. Approximately 20% of dabigatran is conjugated with UGT2B15 (major), UGT1A9 and UGT2B7 (minor) to dabigatran acylglucuronides existing less than 10% of total dabigatran in plasma and then excreted in bile.[126, 131]

- **Excretion**

The conjugated dabigatran is excreted in feces. The remaining 80% of dabigatran in systemic circulation is excreted unchanged in urine primarily via glomerular filtration. The elimination half-life ranged from 12 to 17 hours.[124, 126, 131]

The disposition of dabigatran etexilate and dabigatran is shown in Figure 11.

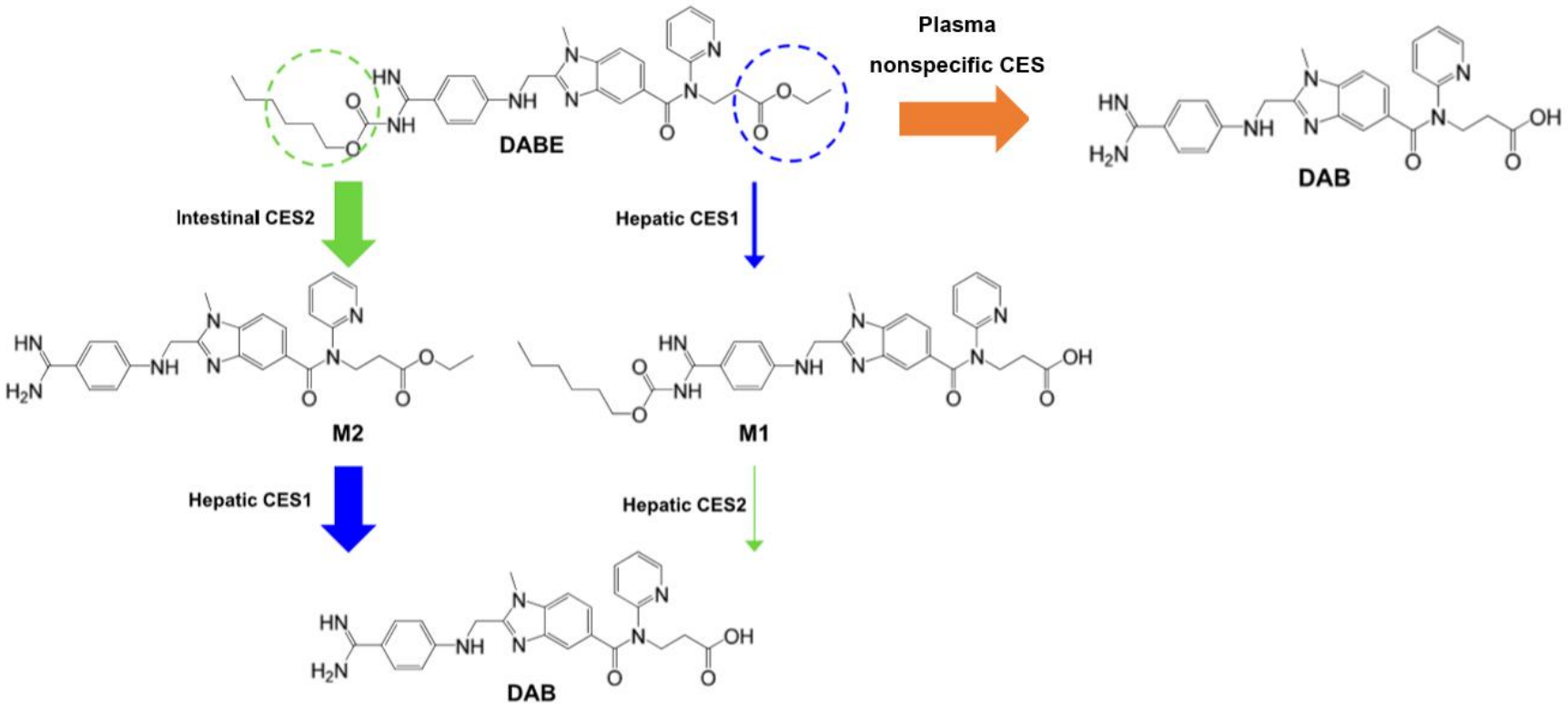


Figure 10 The bioconversion of dabigatran etexilate to dabigatran.
The circles indicate the hydrolysis sites (blue: hydrolyzed by CES1, green: hydrolyzed by CES2). The bioconversion in plasma is mediated by two steps of nonspecific CES with inconclusive pathways indicated in orange color. The thickness of arrow indicates relative contribution of transformation DABE into DAB. *DABE*, dabigatran etexilate; DAB, dabigatran; CES, carboxyesterase. (Adapted by Laizure et al. 2014 and Spinler et al. 2013).[129, 130]

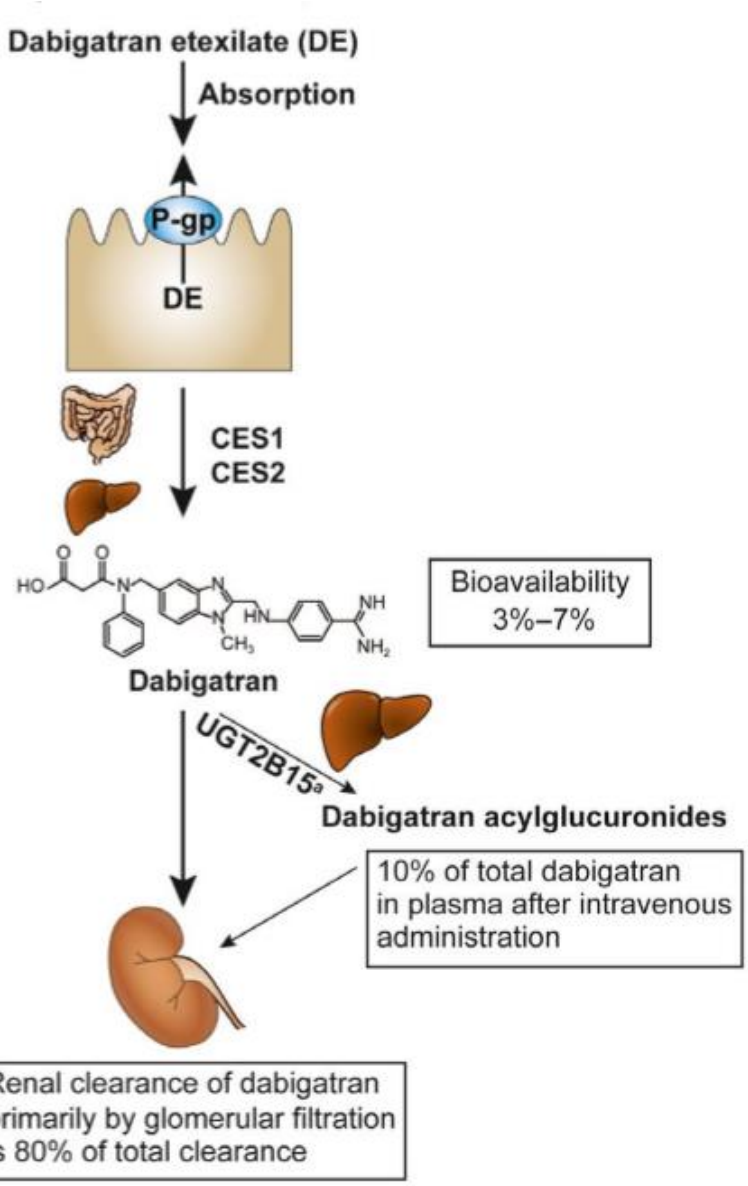


Figure 11 The disposition of dabigatran etexilate and dabigatran
Dabigatran etexilate is highly selective to P-gp at the intestine, so it was widely used as a probe substrate of intestinal P-gp. It is converted into dabigatran by CES1 and CES2 at intestine and liver. Dabigatran is absorbed with 3%-7% of absolute bioavailability and then 20% of dabigatran conjugated with UGT2B15 to dabigatran acyl glucuronide and 80% of dabigatran is excreted unchanged in urine primarily via glomerular filtration. [a]UGT2B15 (major) is not only involved in dabigatran conjugation, but UGT1A9 and UGT2B7 also have minor contribution. CES, carboxylesterase; P- gp, P-glycoprotein; UGT, uridine 5′- diphosphoglucuronosyl transferase. (Adapted by Chu et al. 2018)[(131)]

Pitavastatin (Probe substrate of OATP1B)

- **Absorption**

After oral administration, pitavastatin is rapidly absorbed and reached maximum plasma concentration ~1 hour.[132, 133] Maximum concentration decreased by 43% when taken with high fat meal. The absolute bioavailability is 51%. It has linear pharmacokinetic relationship between dosage and plasma concentration.[132]

Pitavastatin enters enterocyte by passive diffusion due to high lipophilicity and could be minimally transported via OATP1A2 and OATP1B1. In portal vein, the interconversion of pitavastatin and its inactive metabolite (pitavastatin lactone) could be occurred. Despite, pitavastatin in plasma is entirely stable, pitavastatin lactone is not.[134, 135] Pitavastatin, then, transports into liver mediated by OATP1B1 (mainly with 87% of total uptake), OATP1B3, and NTCP transporter whereas pitavastatin lactone is not mediated by drug transporter.[134]

- **Distribution**

Pitavastatin is highly bound to plasma proteins (more than 99%), mainly both albumin and alpha 1-acid glycoprotein. Volume of distribution (Vd) is 148 L.[132]

- **Metabolism**

High lipophilic drugs are usually metabolized by cytochrome P450 at the liver. Although pitavastatin has high lipophilicity, it is classified to non-CYP metabolized type and mainly undergoes rapid glucuronidation to pitavastatin lactone.[136] Pitavastatin is mainly metabolized by UGT1A1, UGT1A2, and UGT2B7 for pitavastatin glucuronide and subsequently non-enzymatically converted into pitavastatin lactone. CYP2C9 has minimally involved in pitavastatin metabolism but has no significant.[137]

Pitavastatin lactone is scarcely metabolized by CYP3A4 to form pitavastatin metabolite-M3 and negligible for CYP2D6 mediated pitavastatin metabolite-M13 formation. The conversion of pitavastatin lactone to pitavastatin could appear by hydrolysis.[136, 138] The metabolism pathway of pitavastatin is shown in Figure 12.

- **Excretion**

Approximately 80% of pitavastatin and pitavastatin lactone is primarily and rapidly excreted into bile within 2 hours after dosing through BCRP and P-pg efflux transporter, respectively, at hepatic basolateral membrane.[136, 139] The elimination half-life of pitavastatin is 12 hours.[132] The enterohepatic circulation of pitavastatin and pitavastatin lactone could prolong half-life.[139] Only 15% of total dose is excreted in urine.[132, 134, 139]

Drug transporter-mediated pitavastatin disposition is shown in Figure 13.

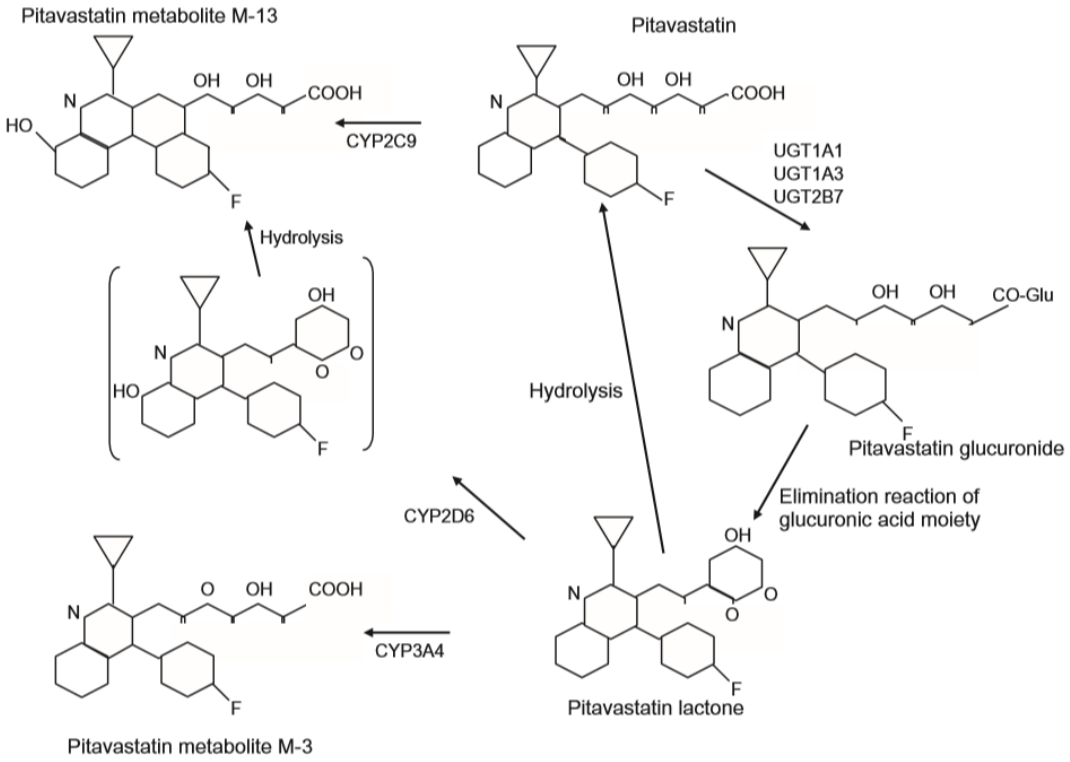


Figure 12 The metabolism pathway of pitavastatin
UGT1A1, 1A3, and 2B7 is mainly associated with pitavastatin conjugation. The conjugated metabolite, pitavastatin glucuronide, is continuously eliminated glucuronic acid moiety to form pitavastatin lactone. The conversion of pitavastatin lactone to pitavastatin is driven by hydrolysis. Pitavastatin is scarcely metabolized by CYP2C9 to pitavastatin metabolite M-13. Pitavastatin lactone is slightly metabolized by CYP3A4 and CYP2D6 to pitavastatin metabolite M-3 and M-13, respectively. CYP, cytochrome P450; UGT, uridine 5′- diphosphoglucuronosyl transferase (Adapted from Alagona et al. 2015)[136]

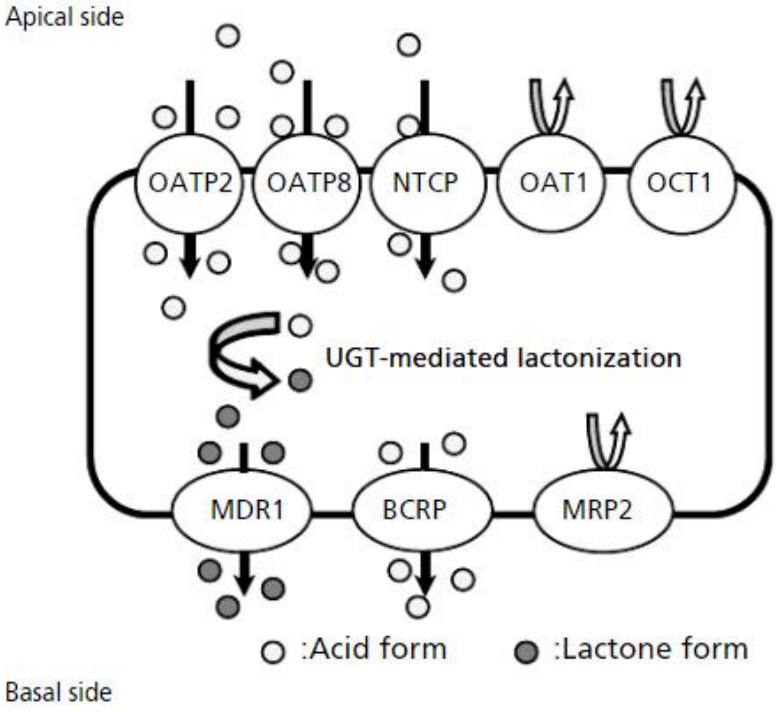


Figure 13 Drug transporter-mediated pitavastatin and pitavastatin lactone disposition.
Pitavastatin is transported into hepatocyte by OATP2 (OATP1B1), OATP8 (OATP1B3), and NTCP (minor) uptake transporters located at apical side. Pitavastatin lactone is not substrate of hepatic uptake transporter. Pitavastatin is conjugated to its metabolite, pitavastatin lactone, by UGT-mediated lactonization. Transporter-mediated biliary excretion for pitavastatin and pitavastatin lactone is BCRP and MDR1 (or P-gp), respectively. UGT, uridine diphosphate-glucuronosyltransferase; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; P-gp, P-glycoprotein; OCT, organic cation transporter; OAT, organic anion-transporting polypeptides; OATP, Organic anion transporting polypeptide; NTCP, sodium-taurocholate cotransporting polypeptide. (Adapted from Fujino et al. 2005).[134]

Rosuvastatin (probe substrate of BCRP and hepatic OATP1B1)

- **Absorption**

After oral administration, rosuvastatin level in plasma achieves maximum concentration approximately 3 to 5 hours. The absolute bioavailability is 20%. There is rosuvastatin dose proportion to plasma level as linear pharmacokinetics.[140] Taken with food could affect rate of absorption but not extent bioavailability.[141] Rifampicin has low ability diffusion through membrane. At intestinal lumen, rifampicin is transported into and out of enterocyte by OATP2B1 uptake transporter (minor), BCRP (major) and MRP2 (minor) efflux transporter.[142-145] From portal vein to liver, OATP1B1, OATP1B3, and NTCP transporter located at apical membrane of hepatocyte uptake rosuvastatin with contribution to 70%, 20%, and 10%, respectively.[145] OATP2B1 and MRP4 could minimally mediate rosuvastatin transportation in liver.[144, 145]

- **Distribution**

Rosuvastatin is approximately 90% protein bound mainly to albumin. Volume of distribution (Vd) is 134 L.[141]

- **Metabolism**

CYP-mediated metabolism is a minor route of clearance. Only 10% of dose is recovered as metabolites. Two metabolites of rosuvastatin are identified: (1) N-desmethyl rosuvastatin (major metabolite) mediated by CYP2C9 and, to a lesser extent, by CYP2C19 and CYP3A4; (2) rosuvastatin-5S-lactone mediated by UGT1B1 and UGT2B7.[140, 146, 147] The interconversion between rosuvastatin and rosuvastatin-5S-lactone also appears in both liver and plasma.[148] The metabolism pathway is shown in Figure 14.

- **Excretion**

Approximately 90% of rosuvastatin is excreted in feces, primarily with unchanged form (~76.8%).[146] Drug transporter-mediated biliary excretion of rosuvastatin includes BCRP (major) and MRP2. Only 10% of rosuvastatin is renal excretion by primarily tubular secretion. Rosuvastatin is a substrate of renal transporters including OAT3 located at basolateral side to pump rosuvastatin into proximal tubule and BCRP, MRP2, P-gp efflux transporter at apical side transporting rosuvastatin into collecting duct.[145] The elimination half-life is 19 hours.[140]

Rosuvastatin is mediated by several drug transporters located at intestine, liver, and kidney. Rosuvastatin especially has high selectivity and specificity to intestine BCRP and hepatic OATP1B1 which has been extensively used as clinical probe to assess BCRP and OATP1B1 role in drug disposition.[142, 149] Drug transporter-mediated rosuvastatin disposition is shown in Figure 15.

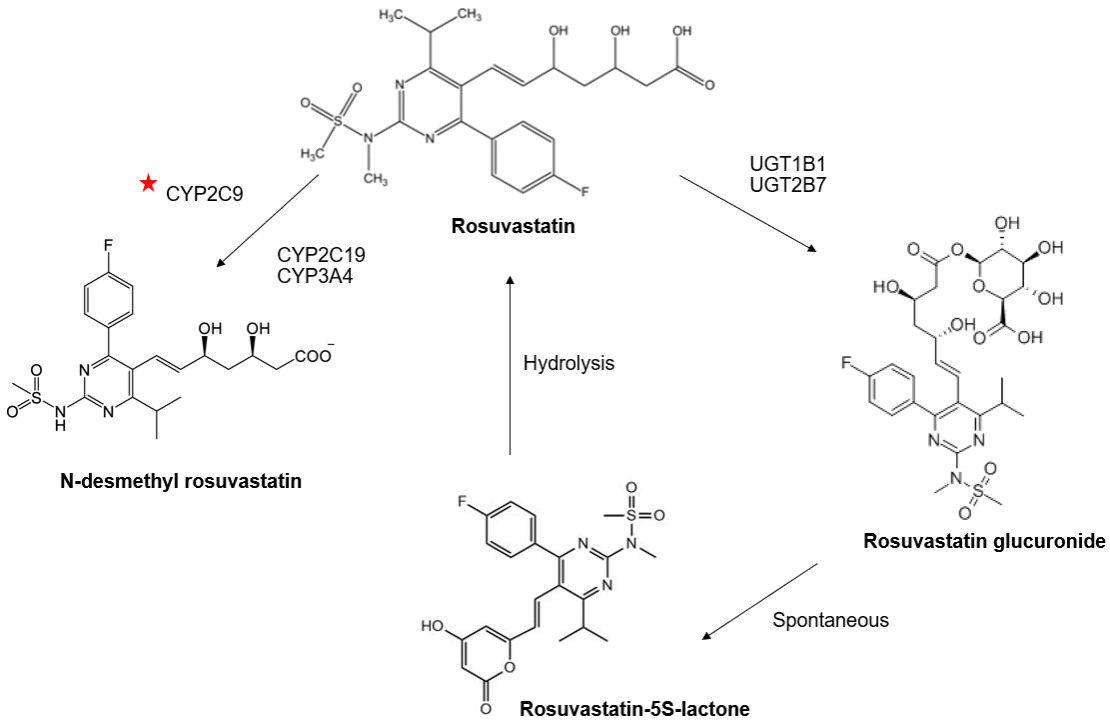


Figure 14 The metabolism pathway of rosuvastatin
The hepatic metabolism is a minor clearance pathway of rosuvastatin. CYP2C9 is a main metabolic enzyme metabolized to N-desmethyl rosuvastatin with a lesser contribution of CYP2C19 and CYP3A4. Lactonization is mediated by UGT1B1and UGT2B7 to produce rosuvastatin glucuronide and subsequently rosuvastatin-5S-lactone as a minor metabolite. The interconversion of rosuvastatin-5S-lactone to rosuvastatin could occur via hydrolysis. CYP, cytochrome P450; UGT, uridine 5′-diphosphoglucuronosyl transferase.

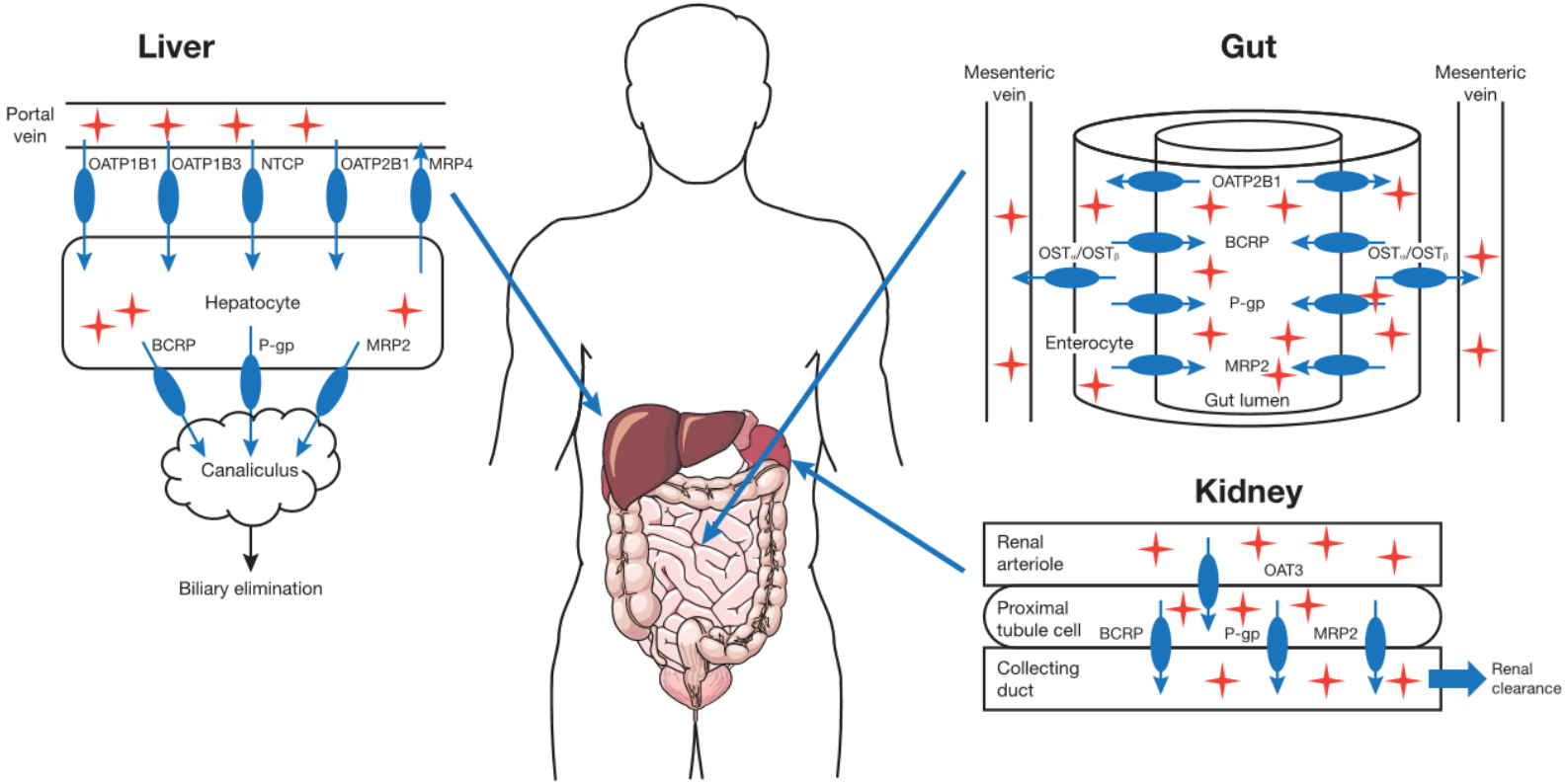


Figure 15 The possible drug transporters involving in rosuvastatin drug disposition in intestine, liver, and kidney
At enterocyte, rosuvastatin is a specifically substrate to BCRP and lesser contribution to other drug transporters. At liver, rosuvastatin is mainly transported into enterocyte by OATP1B1 (major), OATP1B3 and minimally NTCP. To excrete into canaliculus for biliary excretion, BCRP (major) and MRP2 is responsible for rosuvastatin efflux. Renal clearance of rosuvastatin is also mediated by OAT3 transporter, a rate-limiting step of renal clearance, and BCRP (key efflux of rosuvastatin), P-gp, and MRP2 transporter. Red star, rosuvastatin; Blue circle with arrow; drug transporters with their direction; ,BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; NTCP, sodium-taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; OST, organic solute transporter; P-gp, P-glycoprotein. (Adapted from Wang et al. 2017)[145]

Atorvastatin (Probe substrate of CYP3A4, hepatic OATP, BCRP, P-gp)

- **Absorption**

After oral administration, atorvastatin is rapidly absorbed and achieves maximum plasma concentration within 1 to 2 hours. The absolute bioavailability of atorvastatin is 14%.[150] Low systemic bioavailability is caused by presystemic intestinal and hepatic first-pass metabolism.[150]

Atorvastatin, a high lipophilic statin, enters to enterocyte either via passive diffusion or mediated minimally by OATP1A2 transporter (~7%) and ~70% of atorvastatin is pumped back to lumen by BCRP (major) and P-gp transporter. Atorvastatin is metabolized to two major active metabolites, 2-hydroxy atorvastatin (2-OH atorvastatin) and 4-hydroxy atorvastatin (4-OH atorvastatin), by CYP3A4 at enterocyte.[143, 151-153]

In portal vein, interconversion to its lactone metabolites is occurred lead to reduce bioavailability.[154] Atorvastatin is transported into the liver mainly via uptake transporter including OATP1B1 (94%) and possibly OATP1B3 transporter due to low contribution of OATP1B3 in liver. Both active metabolites are also substrate to hepatic OATP1B1 and OATP1B3 transporter.[143, 151-153]

- **Distribution**

Volume of distribution (Vd) is 381 L. Atorvastatin is highly bound to plasma proteins ≥98%.[150]

- **Metabolism**

Atorvastatin is primarily metabolized to 2-OH atorvastatin and 4- OH atorvastatin by CYP3A4 and undergoes further lactonization for 2-OH atorvastatin lactone and 4-OH atorvastatin lactone. Lactonization of atorvastatin is also directly metabolized by UGT1A3 to atorvastatin glucuronide and subsequently atorvastatin lactone. Atorvastatin lactone undergoes CYP3A4-mediated transformation to inactive metabolites either 2-OH atorvastatin lactone or 4-OH atorvastatin lactone. The lactone metabolites, including atorvastatin lactone, 2-OH atorvastatin lactone, and 4-OH atorvastatin lactone, could be hydrolyzed to their parent metabolites as interconversion process.[155-157] The metabolism pathway of atorvastatin is shown in Figure 16.

- **Excretion**

Atorvastatin and its metabolites are extensively excreted via biliary excretion mediated by efflux transporters including BCRP, P-gp, and MRP2 (minor). The elimination half-life is 14 hours but the activity of HMG-CoA reductase inhibitory effect of active metabolites extends half-life to 20-30 hours due to ~70% of circulating inhibitory activity contribute to active metabolites.[150, 153] Approximately <2% of atorvastatin dosage excreted into urine.[150]

Atorvastatin is a substrate to intestinal and hepatic CYP3A4, and several drug transporters including intestine and hepatic BCRP and P-gp, hepatic OATP1B1 and OATP1B3, and hepatic MRP2 transporter.[158] To use atorvastatin as probe substrate, previous studies have reported the affinity to drug transporters and summarized that

atorvastatin is a suitable probe substrate for intestinal BCRP, hepatic OATP1B1, intestinal and hepatic CYP3A4, and intestine P-gp (possibly at liver).[143, 158-160] Enzyme and drug transporter-mediated atorvastatin disposition is shown in Figure 17.

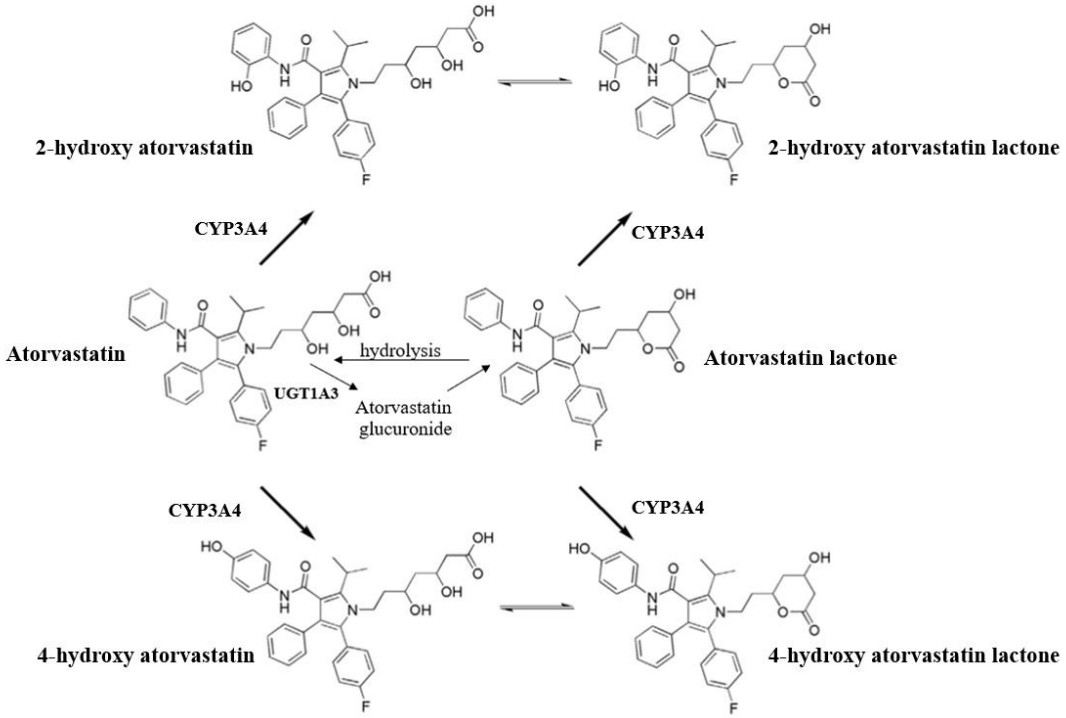


Figure 16 ***The metabolism pathway of atorvastatin***
CYP3A4 is a major atorvastatin metabolizing enzyme to two major active metabolites, 2-hydroxy atorvastatin and 4-hydroxy atorvastatin. These two metabolites undergo lactonization to their inactive lactone metabolite including 2-hydroxy atorvastatin lactone and 4-hydroxy atorvastatin lactone. Atorvastatin is also directly transformed to atorvastatin lactone, an inactive metabolite, by lactonization via atorvastatin glucuronide, an intermediate metabolite, mediated by UGT1A3 conjugation. The lactone metabolites are able to converse to their parent metabolites by hydrolysis. (Adapted from Hoffart et al. 2012)[155]

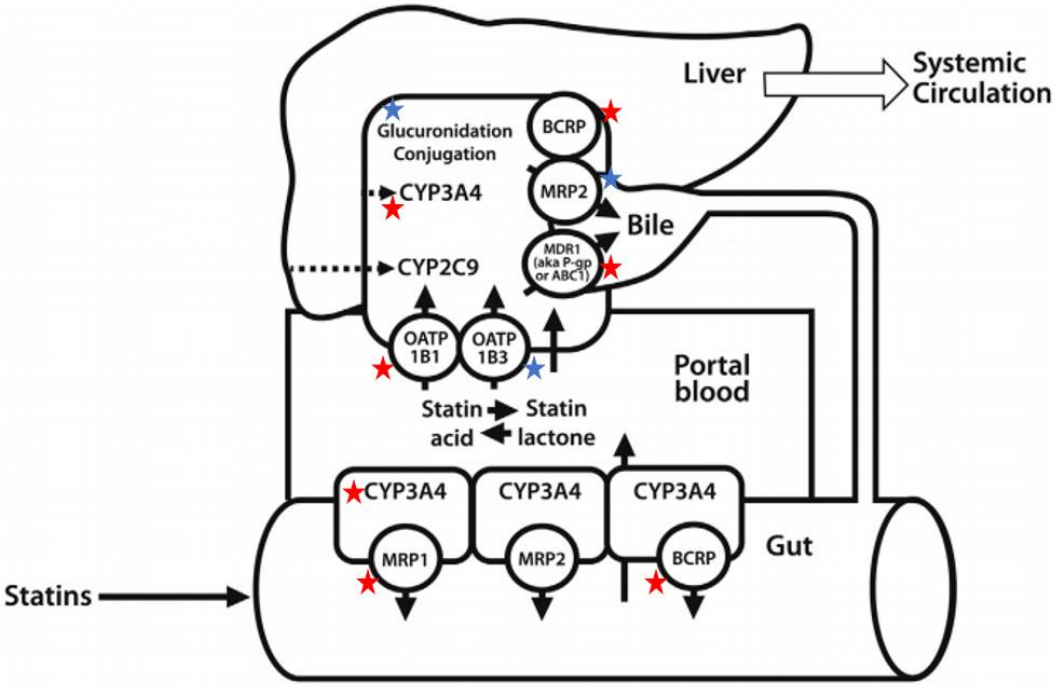


Figure 17 Enzyme and drug transporter-mediated atorvastatin drug disposition
The involved drug transporters in atorvastatin disposition mostly located at intestine/intestinal and liver indicated in star. At intestinal, CYP3A4 at apical membrane, MRP1 (or P-gp) and BCRP efflux transporter at basolateral membrane of enterocyte are associated with atorvastatin transportation. At liver, OATP1B1 and OATP1B3 uptake transporter at basolateral membrane of hepatocyte and BCRP, MRP1 (or P-gp), MRP2 efflux transporter at apical membrane are mediated in atorvastatin disposition. Red and blue color indicates major and minor role in enzyme and drug transporter-mediated atorvastatin disposition, respectively. (Adapted from Kellick et al. 2014)[143]

Research question

- What are the changes in PK determinants (e.g. drug transporters and enzymes) in Thai elderly with and without CKD?
- Are those PK determinants changes difference from Thai healthy young people?

Research Objectives

Primary objective

To investigate the changes in PK determinants in elderly subjects with and without CKD compared to healthy young subjects as a control using a microdose cocktail

Secondary objective

To determine the genotype variants of OATPs and BCRP transporter on PK in Thai elderly subjects with and without CKD and healthy young subjects

Hypothesis

There are the PK determinant changes among Thai elderly with and without CKD compared to healthy young subjects.

Conceptual framework

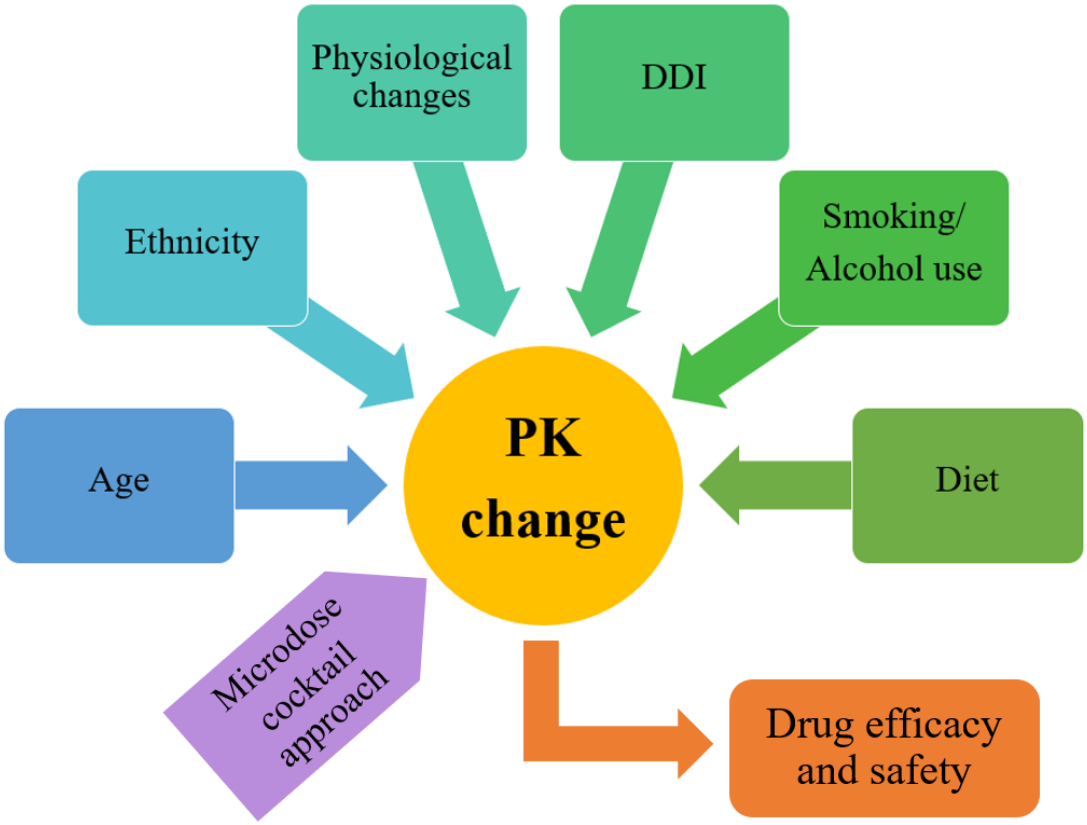


Figure 18 Conceptual framework of present study

The several factors including age, ethnicity, physiological changes, DDI, smoking, alcohol use, and diet could affect pharmacokinetic change lead to influence on drug response both of efficacy and safety.[161] In this study, Age and physiological change (CKD) in Thai population are investigated the association with CYP3A/transporter activity-mediated pharmacokinetic change by using microdose cocktail approach as assessment tool. Other factors would be controlled by study design. DDI, drug-drug interaction; PK, pharmacokinetic

Chapter 3

Methodology

1. Clinical pharmacokinetic study and Bioanalysis

1.1. Study design

This is an interventional study in three groups of subjects (Figure 19). Group 1 and 2 are the main for the study and Group 3 is for preliminary results.

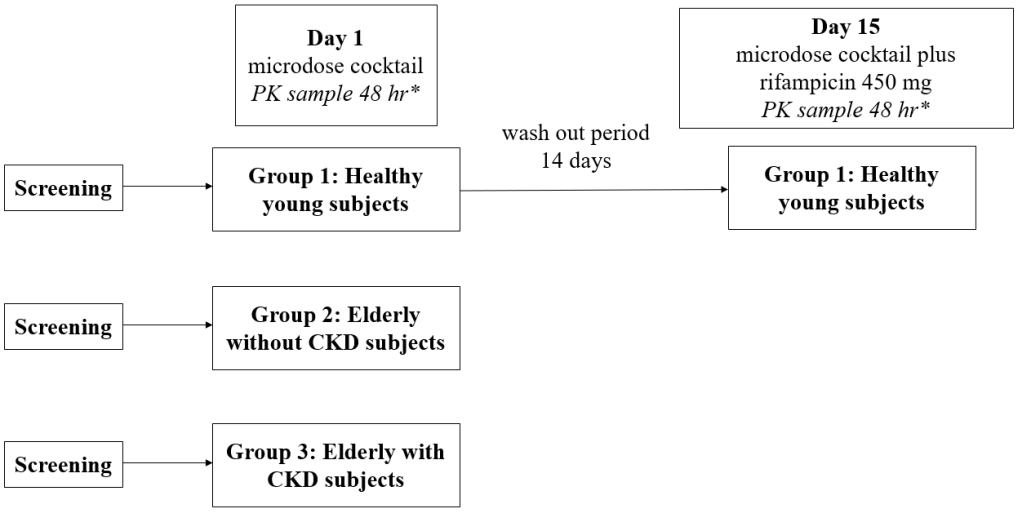


Figure 19 Diagram of study design

***PK sample:**

- *blood collection at predose and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 hours after dosing*
- *urine collection at predose and 0-4 hours, 4-8 hours, 8-12 hours, 12-24 hours, 24-36 hours and 36-48 hours*

Note: To confirm the effect of microdose cocktail on drug transporter, rifampicin which is well-known as OATP1B1, BCRP, intestinal P-gp inhibitor[162] was only administered in the second phase of Group 1 due to safety concern in elderly subjects.

1.2. Sample size

For testing two independent means (two-tailed test)[163, 164]:

$$n_1 = \frac{\left(z_{1-\frac{\alpha}{2}} + z_{1-\beta}\right)^2 \left[\sigma_1^2 + \frac{\sigma_2^2}{r}\right]}{\Delta^2}$$

$$r = \frac{n_2}{n_1}, \Delta = \mu_1 - \mu_2$$

Mean in Group 1 (C_{max} of midazolam) and standard deviation (SD) are obtained from Prueksaritanont, et al.[85] Mean in Group 2 is estimated from 70% lower than mean in Group 1.

Mean in Group 1 (μ_1) = 55.70, SD in Group 1 (σ_1) = 11.84
Mean in Group 2 (μ_2) = 38.99, SD in Group 2 (σ_2) = 11.84
Ratio (r) = 1.00

Alpha (α) = 0.05, Z (0.975) = 1.959964
Beta (β) = 0.100, Z (0.900) = 1.281552

From the calculation, the sample size is at least 11 per group.
Group 1 (n_1, healthy subjects) = 11
Group2 (n_2, elderly without CKD) = 11
In this study, the sample size of 20 in each group will be assigned.

1.3. Subjects

Subject recruitment was complied with inclusion and exclusion criteria (Table 10).

- Healthy young volunteers were recruited from Maha Chakri Sirindhorn Clinical Research Center under the Royal Patronage (ChulaCRC) volunteers's database.
- Healthy elderly volunteers were recruited from an out-patient of Comprehensive Geriatric Clinic of King Chulalongkorn Memorial Hospital.
- CKD patients were recruited from an out-patient clinic of King Chulalongkorn Memorial Hospital by nephrologists in study team

Table 10 Inclusion and exclusion criteria of the study

	Group 1: Healthy young subject	**Group 2: Relatively healthy elderly**	**Group 3: Elderly subject with CKD**
Inclusion criteria			
	Thai male or female, aged 20 to 40 years (inclusive).	Thai male or female, aged ≥60 years. (inclusive).	
	BMI is 18-25 kg/m^2 (inclusive).	BMI is 18-30 kg/m^2 (inclusive).	
	Healthy person by medical history, physical examination, and vital signs.		Diagnosed with CKD according to KDIGO 2012 criteria.
	Laboratory values at screening visit[a] of are within the normal range or showing no clinically significant abnormalities as confirmed by the clinical investigator.		
	eGFR[b] ≥90 mL/min/1.73 m^2	eGFR[b] >60 mL/min/1.73 m^2	eGFR[b] 15-60 mL/min/1.73 m^2
Exclusion criteria			
	History of drug allergy to dabigatran etexilate, midazolam, pitavastatin, rosuvastatin, atorvastatin and rifampicin		
	History of any illness that, in the opinion of the clinical investigator	History of hypertension, diabetes mellitus, other major systemic disease	History of HD, PD, renal/liver transplantation
	Receive any medical prescription[c] within 14 days before the first administration of the microdose cocktail, especially drug related to the study drug metabolizing enzyme		
	History of heavy smoking (> 10 cigarettes per day) or moderate smoking (< 10 cigarettes per day) and cannot omit smoking a least one day before the study and until the completion of the study phase.		
	History of alcoholic (more than 2 years) or moderate drinker (more than 3 drinks per day – one is equal to one unit of alcohol: - one glass of mine, half pine of beer or one measure of spirit) or have a history of any drug abuse.		
	Pregnancy or breast feeding (female).		

[a]Laboratory tests including complete blood count (CBC), fasting blood sugar (FBS), blood urea nitrogen (BUN), serum creatinine (Scr), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, total protein, albumin, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), electrolytes, parathyroid hormone (PTH), phosphate, urinalysis (UA), Hepatitis Bs-antigen, anti-HIV.

[b]Calculated by eGFR for Thai[(165)]

[c]Group 3 subjects can continue their chronic disease medicine.

BMI, body mass index; eGFR, estimated glomerular filtration rate; HD, hemodialysis; PD, peritoneal dialysis.

1.4. Screening

Within 4 weeks before the first treatment, subject underwent a pre-study examination which includes the following assessment:

- Evaluation of eligibility regarding inclusion and exclusion criteria
- Demographic data
- Medical history and present status, including allergies, and previous and current medication
- General physical examination
- Vital signs
- Clinical laboratory parameters as listed in the inclusion criteria

1.5. Processing to obtain informed consent prior to the study commencement

- Potential healthy young volunteers were contacted by phone using the Chula Clinical Research Center's healthy volunteer database. Healthy elderly volunteers were recruited from out-patient of Comprehensive Geriatric Clinic of King Chulalongkorn Memorial Hospital by study team. Additionally, for healthy volunteers who are not on the Chula Clinical Research Center's healthy volunteer database and were interested in taking part into the study could contact Chula Clinical Research Center according to the detail on the website http://www.chulacrc.org/for-particpants-thai.htmL to get more information and leave their contact with the staff. The investigator would later on invite them to come to Chula Clinical Research Center to receive full information regarding the study.

- CKD patients were contacted at the OPD clinic of the general medicine and renal medicine, King Chulalongkorn Memorial Hospital by the nephrologist in the study team.

- All information (including objective, procedure, risk, benefits, restriction, and requirement of study) was presented to all subjects before the process of informed consent. The subjects were encouraged to ask questions, which would be fully answered. All subjects who would be signified there would be participated in this study be reading, signing, and dating the approved consent form. A copy of the information sheet and the signed consent form was provided to each subject.

- All study procedures were performed after subject had signed the informed consent. Neither the investigator, nor the trail staff, coerced or unduly influenced a subject to participate or to continue to participation in the study. The investigator or a person designated by the investigator fully informed the subject or the subject's legally representative of all patient aspects of the study including the written information. All pertinent aspects of the study were explained as non-technical as practical and understandable to the volunteer or the volunteer's legally acceptable representative the impartial witness, where acceptable. The investigator or person designated by the investigator provided the volunteer or the volunteer's legally acceptable representative ample time and opportunity to enquire about details of the study to decide whether or not

to participate in the trial. All questions about the study were answered to satisfaction of the volunteer or the volunteer's legally acceptable representative. The written informed consent forms were signed and personally dated both by the volunteer his/herself and by the staff who conducted the informed consent discussion.

1.6 Microdose cocktail preparation

Midazolam, pitavastatin, atorvastatin, rosuvastatin, dabigatran etexelate were diluted with lactose to subtherapeutic microdose by a qualified pharmacist of King Memorial Chulalongkorn Hospital. All drug used in preparation of the microdose are all innovator products. The process of preparation of the microdose cocktail was complied with Good Manufacturing Practice (GMP PIC/S) by Thai FDA.[166] In brief, all innovator drug products were separately weighed and geometric diluted with lactose monohydrate to obtain diluted powder of each drug at the strength as described in Table 11. The microdose cocktail powder was then prepared by mixing specified amount of each drug into 10 mL amber bottle. (Please see working formula in Appendix A)

Table 11 The microdose cocktail preparation

Study drugs	Dosage form	Diluent	Strength	Preparation
Midazolam (Dormicum®)	15 mg, tablet	Lactose monohydrate (1: 500)	30 µg	Powder
Dabigatran etexelate (Pradaxa®)	75 mg, capsule	Lactose monohydrate (1:100)	750 µg	Powder
Pitavastatin (Livalo®)	2 mg, tablet	Lactose monohydrate (1:200)	10 µg	Powder
Atorvastatin (Lipitor®)	10 mg, tablet	Lactose monohydrate (1: 100)	100 µg	Powder
Rosuvastatin (Crestor®)	10 mg, tablet	Lactose monohydrate (1: 200)	50 µg	Powder

According to Thai FDA announcement, Dormicum® tablet drug manufacturer in Mexico temporarily stopped the operation during the move production bases. Midazolam injection (5 mg/1 mL) diluted with sterile water for injection (SWII) was used instead in the last batch which given in 4 subjects of Group 3.

1.7 Administration of microdose cocktail

Subjects admitted to the study site 12 hours prior to the study day (around 7 p.m.). Prior to all dosing events, subjects were fasting overnight, starting from 9 pm. After at least 10 hours fasting state, a single dose of microdose cocktail was orally administered in fasting state with 240 mL of water. After the microdose administration, water and food was not permitted until 1 hour and 4 hours after dosing, respectively. Subjects were required to keep a supine position for 1 hour.

Phase II of study was performed in Group 1 (healthy young subjects) who took the second dose of microdose cocktail combined with rifampin 450 mg single dose as inhibitor of OATP1B1, BCRP, intestinal P-gp transporter with 240 mL of water in fasting state. The drug-free interval between two phases was at least 2 weeks.

1.8 Blood sample collection (figure 20)

18.1 Day 1 (all groups of subjects)

Blood samples (6 mL) were collected into vacutainer with EDTA at the following time points:

- Before dosing (predose): 6 mL EDTA tubes
- At 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 hours after a microdose cocktail administration: 6 mL of each time point

18.2 Day 15 (Group 1 healthy young subject)

Blood samples (6 mL) were collected before administration of rifampicin plus microdose cocktail and after receiving rifampicin plus microdose cocktail (6 mL in each time point) as Day 1.

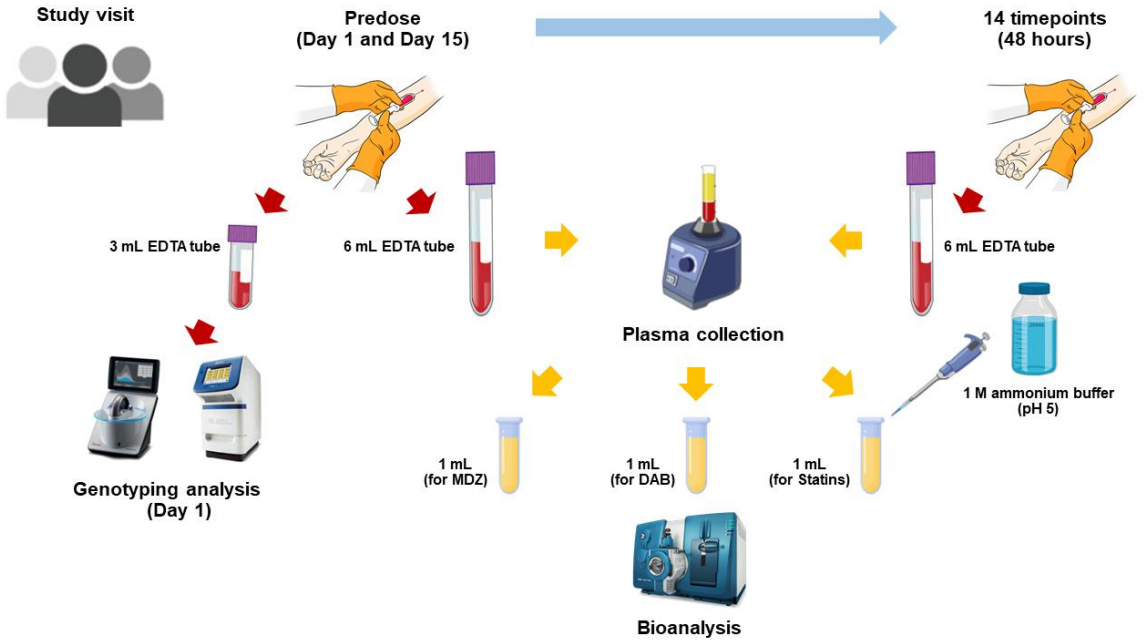


Figure 20 Blood sample collection diagram
At study visit, Blood samples were taken from an indwelling cannula which placed in a forearm vein of the subject and kept patent with normal saline. Whole blood sample was collected for 6 mL into EDTA tube at predose and 14 timepoints (0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 hours) after a microdose cocktail and rifampicin (in period 2 of Group 1) administration. 3 mL of whole blood sample for genotyping analysis were separately collected at predose of Day 1. Blood samples at timepoint of 24, 36 and 48 hours were taken directly by venipuncture. Within 30 minutes after blood collection, the samples were centrifuged for 10 minutes at approximately 3,200 g for plasma collection. 1 ml of plasma samples were then transferred into polypropylene tubes for three tubes. At predose, three tubes of plasma samples were used for predose of statins, midazolam, and dabigatran plasma analysis. At each time point, those were used for statins analysis, midazolam analysis and dabigatran analysis. Plasma samples used for statins analysis were treated immediately with 1 M ammonium buffer (pH 5) at a ratio 5/100 (buffer/plasma) to prevent interconversion before stored at -20C. Then samples were batched and transferred to be kept at -80C at the analytical laboratory site within 2 days after the last day of each study day. Each collection and storage tube were clearly and indelibly labeled. MDZ, midazolam, DAB, dabigatran.

18.3Urine sample collection (figure 21)

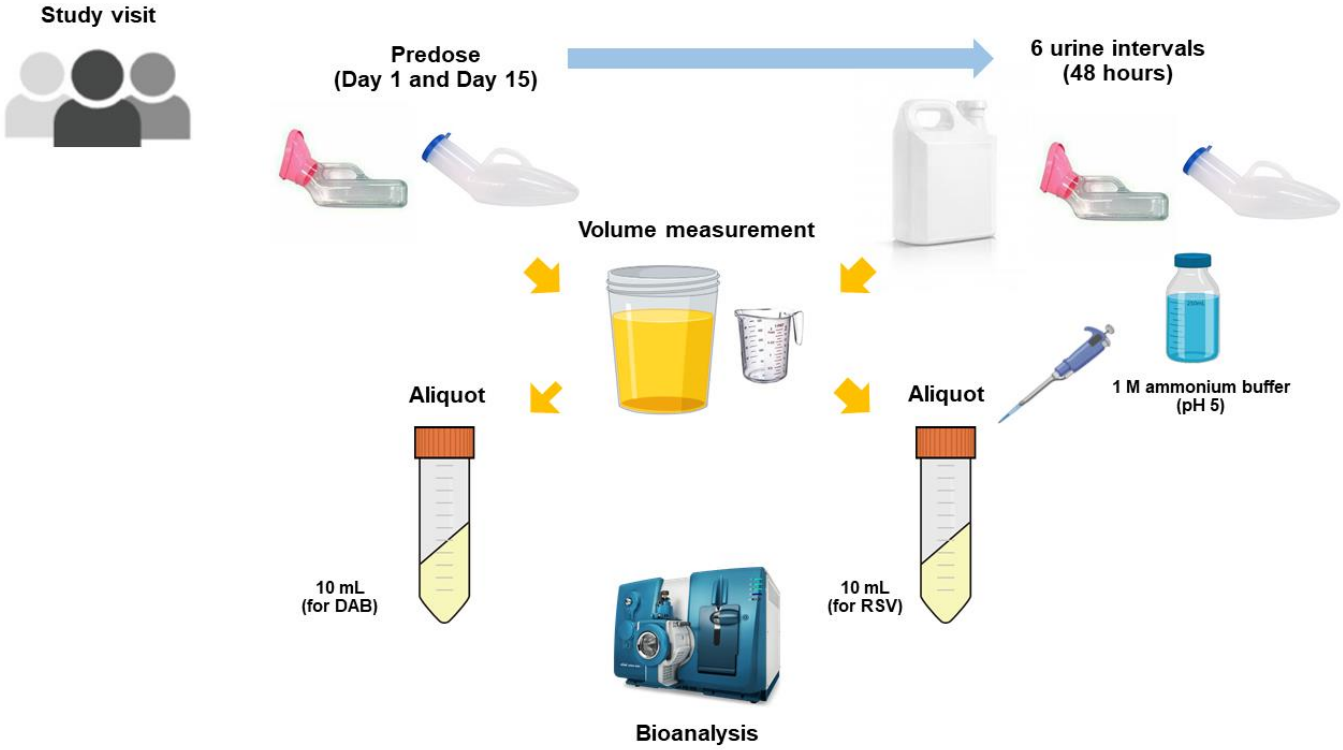


Figure 21 Urine sample collection diagram

At study visit, 24-hours urine samples and urine fraction were collected at predose and, 0-4 hours, 4-8 hours, 8-12 hours, 12-24 hours, 24-36 hours, and 36-48 hours after dosing of microdose cocktail and rifampicin (in period 2 of Group 1) administration by using portable urinal and gallon (3 Liters). Each urine sample was measured volume and aliquoted for 10 mL into polypropylene tubes (15 mL)**.** At predose and urine fraction for 2 tubes of urine sample (2 tubes for rosuvastatin analysis and 2 tubes for dabigatran analysis)**.** Urine samples used for rosuvastatin analysis were treated immediately with 1 M ammonium buffer (pH 5) at a ratio 5/100 (buffer/plasma) to prevent interconversion before stored at -20˚C. Then samples were batched and transferred to be kept at -80˚C at the analytical laboratory site within 2 days after the last day of each study day.DAB, dabigatran; RSV, rosuvastatin.

18.4 Dietary requirement

All subjects abstained from alcoholic preparations two weeks prior to and throughout the study. 72 hours prior to the first phase and throughout the study, xanthine (caffeine) containing beverages, citrus and their juice, herbal and dietary supplements, products containing St. John's wort (hypercum perforatum) were not allowed. On the study day, a standard meal was provided at 4 and 10 hours after drug intake.

18.5 Concomitant medication and treatment

Group 1 and 2 subjects abstained from other drugs intake 2 weeks prior to the study. During course of the study, no other medication was allowed. Group 3 subjects had continued their medication(s) as usual before and during the study. However, if subjects in Group 3 were on treatment with pitavastatin, atorvastatin or rosuvastatin, either discontinuation or changed to simvastatin at equivalent dose 2 weeks prior to the study would be considered depending on nephrologist's decision. Any treatment for adverse events was only administered after notifying the clinical investigator. If this became necessary, the medication(s) would be reported on the adverse event section of the Case Report Form

18.6 Subject's safety monitoring

Subjects were confined to the clinical study site for at least the first 12 hours post dose. Vital signs and medical examination would be performed immediately if subjects experienced any adverse events. The clinical investigator would be on site for dosing and remained on site of the study at least to the time to reach maximal plasma concentration of the study drugs. All possible adverse events were clearly explained to the subjects. The observation for any adverse drug effects was carefully monitored throughout the study. If any adverse events occurred, the appropriate treatments and further investigations would be acquired, and subjects could be considered to be withdrawn from the study for their own safety.

Possible adverse events are:

- Midazolam: over sedation
- Dabigatran etexilate: dyspepsia, gastritis, bleeding
- Rosuvastatin, Pitavastatin, Atorvastatin: muscle pain, tenderness

However, microdose cocktail was given in subtherapeutic dose, therefore, these adverse events were unlikely to occur.

18.7 Subject withdrawal pre-set to be

Subjects would be withdrawn from the study under one of these conditions: continuation with the study would be harmful to the subject's well-being as judged by the clinical investigator; serious adverse events and according to the subject's own request

All subjects were performed a post study early termination assessment including physical examination, vital signs, clinical laboratory testing and urinalysis. No subjects were recruited to replace the early withdraw subjects.

18.8Study Schedule (Table 12)

Table 12 Overview on activities of the study

Parameters	**Screening (wk-4 to 0)**	**Phase I**			**Washout 2 weeks (14 days)**	**Phase II (for Group 1)**			**Post study early termination assessment**
		Day 1	**Day 2**	**Day 3**		**Day 15**	**Day 16**	**Day 17**	
Consent									
Written informed consent	**x**								
Safety									
Medical history	**x**	**x**	**x**	**x**		**x**	**x**	**x**	**x**
Clinical laboratory test	**x**			**x**				**x**	**x**
Physical examination	**x**	**x**	**x**	**x**		**x**	**x**	**x**	**x**
Vital signs	**x**	**x**	**x**	**x**		**x**	**x**	**x**	**x**
Adverse events and concomitant medications	**x**	**x**	**x**	**x**		**x**	**x**	**x**	**x**
Urine pregnancy test (for female Group 1)		**x**				**x**			**x**
Pharmacokinetics									
Microdose cocktail administration		**x**				**x**			
Rifampicin administration (for Group 1)						**x**			
Blood sampling		**x**	**x**	**x**		**x**	**x**	**x**	
24 hours urine samples collection		**x**	**x**	**x**		**x**	**x**	**x**	

Subjects participated in the study at least 16 days and 4 days for healthy young subjects and elderly subjects with/without CKD, respectively..

18.9Data management, pharmacokinetics, and statistical analysis

18.9.1 Data management

Study data was recorded in the paper-based study source documents. Researcher transferred the data into online CRF via REDCap program that generated and verified by Chula Data Management Center (Chula DMC), faculty of medicine, Chulalongkorn University to capture all data listed the paper version of CRF which was approved by the Ethics committee

18.9.2 Pharmacokinetics parameters[167]

Noncompartmental analysis (NCA) was used. NCA is preferably used for PK analysis which aims to determine degree of drug exposure after administration and not require the assumption of specific compartment model. This NCA methodology provides the standard, efficient and efficiency for estimating PK parameters. The PK parameters generally computed from time course of measured drug concentration such as Area under the concentration-time curve (AUC), maximum concentration (C_{max}), time of maximum concentration (T_{max}), terminal elimination half-life ($T_{1/2}$), elimination rate constant (Kel), renal clearance (Cl_R).

Area under the concentration-time curve from time of drug administration "0" to time "t_{last}" ($AUC_{0\text{-}last}$) was calculated by the linear trapezoidal rule as following equation:

$$AUC_0^{t_{last}} = \sum_{i=1}^{n} \frac{C_i + C_{i+1}}{2} \times \Delta t$$

Note. Δt is time interval.

Area under the concentration-time curve from time of drug administration "0" to time "infinity" ($AUC_{0\text{-}inf}$) was used following equation:

$$AUC_{0-inf} = AUC_{0-last} + AUC_{last-inf}$$

which $AUC_{last\text{-}inf}$ was extrapolated by using following equation:

$$AUC_{last-inf} = \frac{C_{last}}{Ke}$$

Note. C_{last} is last quantifiable plasma concentration, Kel is elimination rate constant.

Normally, drugs are eliminated by first-order kinetics which elimination rate depends on drug concentration not of time. Kel was estimated by log-linear least square regression of the terminal part of the plasma concentration versus time curve.[168]

C_{max} and T_{max} were taken directly from the individual concentration versus time curve.

$T_{1/2}$ was calculated by using following equation:

$$T_{1/2} = \frac{ln2}{Ke}$$

Renal clearance (Cl_R) of dabigatran and rosuvastatin was calculated by following equation:[169]

$$Cl_R = \frac{Ae}{AUC_{0-t}}$$

where Ae in cumulative amount of drug recovered in urine during sampling period. The amount of drug in urine was calculated from urine concentration and urine volume by using following equation:

$$Amount\ (\mu g) = Concentration\ (\mu g/ml) \times volume\ (ml)$$

19 Statistical analysis:

STATA version 15.0 was used for statistical analysis. Statistical significance level was taken at $p < 0.05$.

19.1 Demographic data

Median and interquartile range (IQR) were calculated for continuous covariates (such as age, body mass index, laboratory results, etc.) and frequency, N (%) was computed for categorical covariate (such as gender). To limit the possibility errors from multiple testing, demographic and clinical continuous characteristics between groups were analyzed by using Kruskal-Wallis test, and if significant differences were found, pairwise comparisons between groups would made with a t-test (Wilcoxon rank-sum test) and a bonferronic correction was applied. Categorical characteristics were compared between groups first using a Chi-square or Fisher's exact test as appropriate, and if a significant difference were found between groups, pairwise comparisons would be made.

19.2 Pharmacokinetic parameters

The geometric mean (GM) with 95% confidential interval (CI) was computed for $AUC_{0\text{-}last}$, $AUC_{0\text{-}inf}$, C_{max}, Kel, $T_{1/2}$ and Clr. Median (IQR) was computed for T_{max}. The Geometric ratio (GMR) with 90% CI was calculated to compare $AUC_{0\text{-}last}$, $AUC_{0\text{-}inf}$, C_{max}, Kel, $T_{1/2}$ and Cl_R using an analysis of variance (ANOVA). When a significant difference was found in these models, pairwise comparisons of these parameters were made between study groups and a Bonferroni's correction was applied. Pairwise comparisons of the T_{max} was made between the three groups using a Wilcoxon test if a Kruskal-Wallis test indicated differences across the groups, and a bonferronic correction was applied.

The primary comparison of interest were the differences between elderly patients with CKD (Group 2), elderly patients with CKD (Group 3) and healthy young controls as a reference group (Group 1). These groups were surrogates for both age and renal function, so univariate comparison and multivariable analysis partially adjusted for these confounders.

Adjustment was made for other factors which influence the pharmacokinetic parameters – namely BMI, alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, total protein, albumin, if these variables showed some evidence for a relationship in univariable analysis at $P<0.1$. Due to genotypes of drug transporters are possible confounding factors, the impact of transporter activity on the drugs in the microdose cocktail between our 3 study groups was assessed. In univariable and multivariable analyses, associations between genotypes of transporters were

explored to understand how these influence pharmacokinetic parameters alone, and across the study groups.

Midazolam - CYP3A
Dabigratan – Intestinal P-gp
Pitavastatin – Hepatic OATP
Rosuvastatin – BCRP, Hepatic OATP, Intestinal P-gp
Atorvastatin – CYP3A, intestinal P-gp and BCRP, Hepatic OATP1B1

A sensitivity analysis explored the effect of age and renal function (eGFR) in study subjects, modelling these covariates as continuous, rather than as surrogates based on study group.

Bioanalysis [85]

The bioanalytical methods were adapted from Prueksaritanont, et al, 2017[85] and method validation was conformed to US FDA Guidance for Industry-Bioanalytical Method Validation.[170] Liquid chromatography coupled with an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system was used to determine plasma concentration of dabigatran, midazolam, pitavastatin, pitavastatin lactone, rosuvastatin, atorvastatin and its metabolites (2-hydroxy atorvastatin and 4-hydroxy atorvastatin), rifampicin and urine concentration of dabigatran and rosuvastatin.

Method validations and bioanalytical methods

LC-MS/MS was used for bioanalysis in this study. Method validation had to be conducted to ensure that the bioanalytical method using LC-MS/MS and its condition could provide the appropriate quantitative evaluation of containing drugs in microdose cocktail and their metabolites before drug administration.

Three different types of method validations:

- Full validation
- Partial validation
- Cross validation

Due to the analytical method of microdose cocktail in this study was validated in previous publication (Prueksaritanont, et al, 2017)[85], partial validation was performed including following demonstrations:[170]

1. Accuracy and precision
   1.1. Accuracy
   The replication of samples with known amount of analyte was used to determine the accuracy of bioanalytical methods. The number of replications was three determination and five determinations per concentration of drugs in microdose cocktail dosing and in plasma/urine, respectively. The quality control (QC) including three concentrations in determination range (High-QC, Medium-QC, Low-QC) and lower limit of quantification (LLOQ) were used. The accuracy indicates the closeness of mean values to actual value which accepted within 15% of the nominal value in High, Medium, Low-QC and within 20% in LLOQ
   1.2. Precision
   The determination of precision was done by repeatable analysis of single homogenous sample which provided the closeness of individual measures of an analyte. The number of replications was three determination and five determinations per concentration of drugs in microdose cocktail dosing and in plasma/urine, respectively. The quality control (QC) including three concentrations in determination range (High-QC, Medium-QC, Low-QC) and lower limit of quantification (LLOQ) were used. The percentage of relative standard deviation (%RSD) at each concentration was used to determine the precision. The acceptance was not more than 15% of %RSD except LLOQ (20% of RSD). Moreover, Intra-batch and inter-batch analysis were assessed for precision.

2. Calibration curve or standard curve
   The standard curve is the relationship between response (area ratio) and known concentration of analytes which should be continuous and reproducible. The concentration of standards was chosen within expected concentration range of each method. A standard curve consisted of single blank (matrix sample with internal standard), double blank (matrix sample without internal standard), and eight samples with differential standard concentrations, as described in each bioanalytical method below. The acceptance of standard curve would be considered: -
   - Linearity: The coefficient of determination (R^2) >0.99
   - Lower limit of quantification (LLOQ): %accuracy and %RSD (precision) should be within 20% of the nominal concentration.
   - Eight standard samples: %accuracy and %RSD (precision) should be within 15% of the nominal concentration.
3. Sensitivity
   LLOQ should achieve acceptable accuracy and precision.

The bioanalytical methods consisted of 2 major parts:

- Bioanalysis of micodose cocktail containing drugs in microdose cocktail dosing.
- Bioanalysis of microdose cocktail containing drugs and their metabolites in plasma and urine samples. In plasma samples, bioanalysis of statins and their metabolites were simultaneously analyzed which separated from midazolam and dabigatran.

The sample preparation for midazolam, rifampicin and statins were based on liquid-liquid extraction whereas protein precipitation was used for dabigatran etexelate. The analyte and stable isotope labeled internal standard were chromatographed using reversed phase liquid chromatography (midazolam, rifampicin, and statins) or hydrophilic interaction liquid chromatography (dabigatran) and detected with tandem mass spectrometric detection employing a turbo ionspray (TIS) interface in the positive ion mode. The biological methods including condition of LC-MS/MS were described in detail as below:

A. <u>Bioanalytical method for determination of microdose cocktail by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Midazolam
1.2. Dabigatran etexilate
1.3. Rosuvastatin calcium salt
1.4. Pitavastatin calcium salt
1.5. Atorvastatin calcium salt
1.6. Labetalol hydrochloride (Internal standard)
1.7. Acetonitrile
1.8. Formic acid
1.9. Dimethyl sulfoxide (DMSO)
1.10. Ultrapure water

2. Apparatus

2.1. Pipetted and pipette tips
2.2. HPLC-MS/MS ACE C18-300, 3 µm, 50x1.0 mm
2.3. Volumetric flask 100 mL
2.4. Stirling rod
2.5. Breaker 80 mL, 10 mL
2.6. Microcentrifuge tubes
2.7. Pasture pipette
2.8. Polypropylene syringe
2.9. Nylon membrane syringe filter 25 mm 0.45 µm

3. Sample preparation

3.1. A microdose cocktail was dissolved in 100 mL of 50%acetonitrile/water.
3.2. The solution was filtrated through nylon membrane syringe filter 0.45 µm into 100 mL volumetric flask.

4. Cocktail standard stock solution preparations

Weighed 5 compounds and dissolved in methanol (for midazolam) or DMSO (for statins) to prepare stock solutions. Then diluted with their own solvents for working stock solutions and mixed 25 µL of each working stock solution for cocktail stock solutions as described in table 13.

5. Internal standard preparation

To prepare 100 ng/mL labetalol in 100% acetonitrile, 100x diluted of 10 µg/mL labetalol stock in 100% acetonitrile.

6. Standard solutions preparation and quality control preparation

6.1. Standard solution preparation

Standard solutions were prepared in seven differential concentrations by using cocktail stock solutions and 10x diluted of cocktail stock solution as described in table 13.

6.2. Quality control preparation

High-quality control (High-QC) sample was prepared from cocktail stock solution whereas medium-quality control (Medium-QC) and low-quality control (Low-QC) sample were prepared from 10x diluted cocktail stock solution (Table 14).

The final concentration of standard and quality control of containing drugs in microdose cocktail was summarized in Table 15 and 16.

Table 13 Cocktail standard stock solutions

Compounds	**Stock solution**	**Solvent**	**Working stock solution (µg/mL)**	**Cocktail stock solution (µg/mL), 1 mL**
Midazolam	1mg/mL in methanol	50% acetonitrile	15	3
Dabigatran etexilate	-	50% acetonitrile + 0.5% formic acid	375	75
Rosuvastatin Calcium	0.5 mg/mL in DMSO	50% acetonitrile	25	5
Pitavastatin Calcium	0.5 mg/mL in DMSO	50% acetonitrile	5	1
Atorvastatin Calcium	0.5 mg/mL in DMSO	50% acetonitrile	50	10

Note: Dabigatran etexilate was directly weighed and prepared for working stock solution with 50% acetonitrile +0.5% formic acid

Table 14 Cocktail standard solution preparation

	Cocktail stock solution (µL)				10x Diluted cocktail stock solution (µL)		
	2.5x	3.33x	5x	10x	25x	33.33x	50x
Working cocktail	20	15	10	5	20	15	10
50% Acetonitrile +0.1% formic acid	30	35	40	45	30	35	40
Internal standard	25 µL						
100% Acetonitrile +0.1% formic acid	50 µL						
Water with 0.1% formic acid	875 µL						

Note: Working cocktail and internal standard in blank sample and working cocktail in double blank sample were replaced with 50 µL of 50% Acetonitrile + 0.1% formic acid.

Table 15 The final concentration of containing microdose cocktail drugs in standard solution samples.

Compound in Standard solution	Concentration (ng/mL)						
	2.5x	3.33x	5x	10x	25x	33.33x	50x
Midazolam	1200	900.90	600	300	120	90.01	60
Dabigatran etexelate	30000	22727.27	15000	7500	3000	2250.23	1500
Rosuvastatin	1923.96	1457.55	961.98	480.99	192.40	144.32	96.20
Pitavastatin	382.72	289.94	191.36	95.68	38.27	28.70	19.14
Atorvastatin	3698.12	2801.60	1849.06	924.53	369.81	277.39	184.91

Table 16 The final concentration of containing microdose cocktail drugs in quality control samples.

Compound in Quality control	Concentration (ng/mL)		
	High-QC (2.86x)	Medium-QC (20x)	Low-QC (40x)
Midazolam	1050	150	75
Dabigatran etexelate	26220	3750	1875
Rosuvastatin	1683.47	240.50	120.25
Pitavastatin	334.88	47.84	23.92
Atorvastatin	3235.85	462.26	231.13

7. Sample analysis

Prior to sample injection, 50 µL of sample was dissolved in 50% acetonitrile with 0.1% formic acid and then mixed with 25 µL of 100 µg/mL labetalol, 50 µL of 100% acetonitrile with 0.1% formic acid and 875 µL water with 0.1% formic acid.

8. Experimental conditions for LC-MS/MS

The following conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

8.1. M3 MicroLC autosampler with a 5 µL loop

Wash 1: 80% Acetonitrile with 0.1% formic acid
Wash 2: 20% Acetonitrile with 0.1% formic acid
Injection volume: 5 µL

8.2. M3 MicroLC pump

Mobile phase A: Water with 0.1% formic acid
Mobile phase B: 100% Acetonitrile with 0.1% formic acid
Flow rate: 50 µL/min

Table 17 Gradient (microdose cocktail)

Time (sec)	%A	%B
0	95	5
0.5	95	5
3	5	95
4	5	95
4.5	95	5
5	95	5

8.3. Chromatography

Column: ACE 3 C18-300, 50 x 1 mm, 3 µm, 300 Å
Temperature: 60°C

8.4. Mass spectrometer

AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain, and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 18 and 19.

Table 18 Mass spectrometry parameters (microdose cocktail)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 19 Multiple reaction monitoring transitions for the analytes and internal standard (microdose cocktail)

Substrates	Precursor (Q1, m/z)	Product Ion (Q3, m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Analytes						
Midazolam	326.1	291.2	41	10	34	8
		249.2	41	10	51	28
		223	41	10	49	22
Dabigatran etexilate	628.084	288.9	156	10	47	36
		332	156	10	35	24
		526.1	156	10	27	20
Rosuvastatin	482.072	257.8	156	10	47	34
		299.8	156	10	49	28
		271.9	156	10	43	22
Pitavastatin	422.019	289.9	120	10	37	20
		317.8	120	10	41	26
		273.8	120	10	59	28
Atorvastatin	559.078	440.1	136	10	31	14
		249.8	136	10	53	26
		466.1	136	10	23	16
Internal standard						
Labetalol	328.406	161.8	46	10	33	22
		90.9	46	10	63	14
		293.9	46	10	25	42

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

B. <u>Bioanalytical method for determination of Midazolam in plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Frozen human EDTA plasma samples
1.2. Midazolam
1.3. [$^2H_6$]-Midazolam (internal standard)
1.4. Sodium carbonate or sodium bicarbonate
1.5. Methyl tert-butyl ether (MTBE)
1.6. Nitrogen gas
1.7. 50% Methanol/water, 100% methanol
1.8. Ultrapure water

2. Apparatus

2.1. Pipetted and pipette tips
2.2. HPLC-MS/MS ACE C18-300, 3 µm, 50x1.0 mm
2.3. Ice bath
2.4. pH meter
2.5. Vortex mixer
2.6. Microcentrifuge tubes
2.7. Positive stepper
2.8. Nitrogen evaporator
2.9. 100 µL glass insert with bottom spring
2.10. Refrigerated centrifuge
2.11. 1.5 mL amber glass vial

3. Midazolam stock solution

- Diluted 10 µL of 1 mg/mL stock to a final volume of 1.0 mLwith 100% methanol.
  Stock solution: 10 µg/mL in 100% methanol.
- Diluted 10 µL of 10 µg/mL stock to a final volume of 1.0 mL with 100% methanol.
  Stock solution: 100 ng/mL in 100% methanol.
- Diluted 100 µL of 100 ng/mL stock to a final volume of 1.0 mL with 100% methanol.
  Stock solution: 10 ng/mL in 100% methanol.

4. Internal standard preparation

- Dissolved 1 mg of [$^2H_6$]-Midazolam in 50% methanol/water and mix well for 1 mg/mL internal standard concentration. Then diluted with 100% methanol to 10 ng/mL.
  Internal standard stock solution: 10 ng/mL of [$^2H_6$]-Midazolam in methanol
- On analyze date, diluted 10 ng/mL [$^2H_6$]-Midazolam in methanol with 100% methanol.
  Working internal standard stock solution: 1.25 ng/mL ^{2}[H_6]-Midazolam.

5. Working standard and quality control solution preparation

Working standard and quality control solutions were prepared from stock solution (10 ng/mL) diluted with 100% methanol in differential concentrations as described in Table 20 and 21.

Table 20 Working standard solution preparation (midazolam)
Stock solution: 10 ng/mL (A), diluent: 100% methanol

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 50 µL in 250 µL) (pg/mL)**
A	10000				
B	5000	60	of A	60	1000
C	4000	48	of A	72	800
D	2500	30	of A	90	500
E	500	20	of A	380	100
F	100	30	of E	120	20
G	25	20	of E	380	5
H	10	48	of G	72	2
I	5	30	of G	120	1

Table 21 Quality control solution preparation (midazolam)
Stock solution: 10 ng/mL (A'), diluent: 100% methanol

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 50 µL in 250 µL) (pg/mL)**
A'	10000				
High-QC	3750	75	of A'	125	750
Z	1000	20	of A'	180	
Medium-QC	300	90	of Z	210	60
Low-QC	12.5	30	of K	690	2.5
LLOQ	5	15	of K	885	1

6. Standard and quality control sample preparation

6.1. Thawed an aliquot of plasma sample (250 µL) on ice.

6.2. Added 50 µL of working standard stock solution B through I to successive tubes to yield nominal concentrations of 1000, 800, 500, 100, 20, 5, 2 and 1 pg/mL, respectively. And added 50 µL of quality control (High-QC, Medium-QC, Low-QC, and LLOQ) at concentrations of 750, 60, 2.5 and 1 pg/mL.

6.3. Designated two further tubes as "single" and "double" blank. Added 10 and 20 µL of 100% methanol, respectively.

6.4. Spiked the plasma with 50 µL of internal standard solution except for double blank.

7. Unknown plasma sample preparation

7.1. Aliquoted 250 µL of unknown samples into microcentrifuge tubes.

7.2. Added 50 µL of 100% methanol.

7.3. Added 50 µL of working internal standard to all samples.

8. Plasma sample extraction

Midazolam was isolated from plasma sample by liquid-liquid extraction.

8.1. Vortexed all prepared standard, quality control and unknown samples.

8.2. Adjusted pH by addition of 100 µL of carbonated buffer (pH 9.8), then mixed thoroughly.

8.3. Added 1,000 µL of MTBE (an extraction solvent) into sample and mixed for 15 minutes at room temperature.

8.4. Centrifuged all samples at 4,500 g for 10 minutes at 4°C

8.5. Transferred 850 µL of organic layer to new tube, then dried under nitrogen at 40°C

8.6. Reconstituted the dried sample in 100 µL of 50%methanol/water

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

10. M3 MicroLC autosampler with a 5 µL loop
Wash 1: Water with 0.1% formic acid
Wash 2: 100% Methanol with 0.1% formic acid
Injection volume: 5 µL

11. M3 MicroLC pump
Mobile phase A: Water with 0.1% formic acid
Mobile phase B: 100% Methanol with 0.1% formic acid
Flow rate: 50 µL/min

Table 22 Gradient (midazolam)

Time (sec)	%A	%B
0	65	35
0.7	65	35
1.2	5	95
2	5	95
2.3	65	35
2.8	65	35

12. Chromatography
Column: ACE 3 C18-300, 50 x 1 mm, 3 µm, 300 Å.
Temperature: 60°C

13. Mass spectrometer
AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain, and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 23 and 24.

Table 23 Mass spectrometry parameters (midazolam)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 24 Multiple reaction monitoring transitions for midazolam and [$^2H_6$]-midazolam

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Midazolam	326.1	291.2	150	46	10	34	8
Internal standard							
[$^2H_6$]-Midazolam	332.086	297	150	46	10	35	26

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

C. <u>Bioanalytical method for determination of Dabigatran in plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Frozen human EDTA plasma samples
1.2. Dabigatran
1.3. [$^{13}C_6$]-Dabigatran (internal standard)
1.4. Acetonitrile
1.5. Formic acid
1.6. Ultrapure water

2. Apparatus

2.1 Pipetted and pipette tips
2.2 HPLC-MS/MS TSKgel Amide-80, 50 x 1 mm, 5 µm.
2.3 Ice bath
2.4 Vortex mixer
2.5 Multitube vortex
2.6 Microcentrifuge tubes
2.7 250 µL glass flat bottom insert
2.8 1.5 mL amber glass vial

3. Dabigatran stock solution

- Weighed ~1 mg of dabigatran accurately into vial and dissolved with 1 mL of 50% acetonitrile/water, mixed well.
  Stock solution: 1 mg/mL dabigatran in 50% acetonitrile/water.
- Diluted 1 mg/mL stock solution to 1 µg/mL with 50% acetonitrile/water.
  Stock solution: 1 µg/mL dabigatran in 50% acetonitrile/water.

4. Internal standard preparation

- Dissolved ~1 mg of [$^{13}C_6$]-Dabigatran in 1 mL of 50% acetonitrile/water.
  Internal standard stock solution**:** 1 mg/mL of [$^{13}C_6$]-Dabigatran in 50% acetonitrile/water**.**
- On analyzed date, diluted 1 mg/mL internal standard stock solution with 50% acetonitrile/water.
  Working internal standard solution: 5 ng/mL [$^{13}C_6$]-Dabigatran in 50% acetonitrile/water.

5. Working standard and quality control solution preparation

Diluted 1 µg/mL dabigatran stock solution with 50% acetonitrile/water to 50 ng/mL dabigatran as working stock solution.

Working standard and quality control solution preparation was prepared in differential concentrations as described in Table 25 and 26

6. Standard and quality control sample preparation

6.1. Thawed an aliquot of plasma sample (250 µL) on ice.
6.2. Added 25 µL of working standard stock solution B through I to successive tubes to yield nominal concentrations of 5000, 4000, 2000, 1000, 400, 200, 100, 20 pg/mL, respectively. And add 25 µL of quality control solutions (High-QC, Medium-QC, Low-Q C, and LLOQ) at concentrations of 3750, 1200, 30, 20 pg/mL.
6.3. Designate two further tubes as "single" and "double" blank by adding 25 and 50 µL of 50 % acetonitrile/water, respectively.

6.4. Spike the plasma with 25 µL of internal standard solution except for double blank.

Table 25 Working standard solution preparation (dabigatran)
Stock solution: 50 ng/mL (A), diluent: 50% acetonitrile/water

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 25 µL in 100 µL) (pg/mL)**
A	50000				
B	20000	30	of A	45	5000
C	16000	24	of A	51	4000
D	8000	24	of A	126	2000
E	4000	24	of A	276	1000
F	1600	30	of E	45	400
G	800	30	of E	120	200
H	400	20	of E	180	100
I	80	20	of E	980	20

Table 26 Quality control solution preparation (dabigatran)
Stock solution: 50 ng/mL (A'), diluent: 50% acetonitrile/water

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 25 µL in 100 µL) (pg/mL)**
A'	50000				
High-QC	15000	30	of Z	70	3750
Medium-QC	4800	24	of Z	226	1200
K	500	10	of Z	990	
Low-QC	120	24	of Y	76	30
LLOQ	80	32	of Y	168	20

7. Unknown plasma sample preparation

7.1. Aliquoted 100 µL of unknown samples into microcentrifuge tubes.

7.2. Added 25 µL of 50% acetonitrile.

7.3. Added 25 µL of working internal standard to all samples.

8. Plasma sample extraction

Dabigatran was isolated from plasma sample by protein precipitation.

8.1. Added 300 µL of acetonitrile and mixed well.

8.2. Centrifuged at 12,000 rpm for 10 minutes at 4°C.

8.3. Collected 200 µL of supernatant.

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

9.1. M3 MicroLC autosampler with a 5 µL loop

Wash 1: 80% Acetonitrile/water containing 0.1% formic acid

Wash 2: 20% Acetonitrile/water containing 0.1% formic acid

Injection volume: 5 µL

9.2. M3 MicroLC pump

Mobile phase A: Water containing 0.1% formic acid

Mobile phase B: 100% acetonitrile, 2mM ammonium formate, pH 4.0

Flow rate: 50 µL/min

Table 27 Gradient (dabigatran)

Time (sec)	%A	%B
0	30	70
0.5	30	70
2	95	5
2.7	95	5
3	30	70
3.5	30	70

9.3. Chromatography

Column: TSKgel Amide-80, 50 x 1 mm, 5 µm.

Temperature: 60°C

9.4. Mass spectrometer

AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 28 and 29.

Table 28 Mass spectrometry parameters (dabigatran)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 29 Multiple reaction monitoring transitions for dabigatran and [^{13}C]-dabigatran

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Dabigatran	472.2	289.0	75	111	10	37	22
		330.0	75	111	10	27	12
Internal standard							
[^{13}C]-Dabigatran	478.1	295.0	75	101	10	37	12
			75	101	10	67	18

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

D. <u>Bioanalytical method for determination of Statins and their main metabolites in plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Frozen human EDTA plasma samples (pH adjusted by 1M acetate buffer, pH 5.0)
1.2. Pitavastatin calcium
1.3. Pitavastatin lactone
1.4. Rosuvastatin calcium salt
1.5. Atorvastatin calcium salt
1.6. 2-Hydroxyatovastatin calcium salt
1.7. 4-Hydroxyatorvastatin hemicalcium salt
1.8. [$^2H_5$]-Pitavastatin sodium salt (internal standard of pitavastatin)
1.9. [$^2H_5$]-Pitavastatin lactone (internal standard of pitavastatin lactone)
1.10. [$^2H_6$]-Rosuvastatin sodium salt (internal standard of rosuvastatin)
1.11. [$^2H_5$]-Atorvastatin calcium salt (internal standard of atorvastatin)
1.12. [$^2H_5$]-2-Hydroxyatovastatin (internal standard of 2-hydroxyatorvastatin)
1.13. [$^2H_5$]-4-Hydroxyatorvastatin (internal standard of 4-hydroxyatorvastatin)
1.14. Methyl tert-butyl ether (MTBE)
1.15. 1M Ammonium acetate pH 5.0
1.16. Nitrogen gas
1.17. Acetonitrile
1.18. Formic acid
1.19. Dimethyl sulfoxide (DMSO)
1.20. Ultrapure water

2. Apparatus

2.1. Pipetted and pipette tips
2.2. Positive strepper
2.3. Microcentrifuge tubes
2.4. HPLC-MS/MS ACE C18-300, 3 µm, 50x1.0 mm
2.5. Ice bath
2.6. pH meter
2.7. Vortex mixer
2.8. Multitube vortex mixer
2.9. Nitrogen evaporator
2.10. Refrigerated centrifuge
2.11. Multiwell sample plate

3. Mixed statin and pitavastatin lactone stock solution preparation

3.1. Prepared 50 ng/mL working stock solution of mixed statins (except pitavastatin lactone)

- Weighed ~0.5 mg of each standard statins (rosuvastatin, atorvastatin, 4-hydroxy atorvastatin, 2-hydroxy atorvastatin, pitavastatin) accurately into vials and diluted with DMSO separately. Then mixed well for 0.5 g mg/mL mixed statins stock solution. Then diluted 0.5 mg/mL to 0.1 mg/mL with DMSO.
  Stock solution: 0.1 mg/mL mixed statins stock solution.
- Using 0.1 mg/mL mixed statins stock solution diluted with 50% acetonitrile/water.

Stock solution: 50 ng/mL mixed statins stock solution.

3.2. Prepared 50 ng/mL working stock solution of pitavastatin lactone.

- Weighed ~0.5 mg of pitavastatin lactone diluted with DMSO for 0.5 mg/mL stock solution and then diluted with DMSO to 0.1 mg/mL
  Stock solution: 0.1 mg/mL pitavastatin lactone stock solution.
- Using 0.1 mg/mL stock solution of pitavastatin lactone diluted with 100% acetonitrile.
  Stock solution: 50 ng/mL pitavastatin lactone stock solution.

4. Internal standard preparation

4.1. Prepared 50 ng/mL of internal standard working stock solution
0.5 mg/mL internal standard of mixed statins and internal standard of pitavastatin lactone ($[^2H_5]$-Pitavastatin lactone) stock solution were prepared similar to standard of mixed statins and pitavastatin lactone preparation, respectively.

4.2. Working internal standard solution
On analyze date, diluted 50 ng/mL internal standard of mixed statins and $[^2H_5]$-Pitavastatin lactone stock solution with 50% acetonitrile/water and 100% acetonitrile, respectively. Working internal standard solution: 12.5 ng/mL.

5. Working standard and quality control solution preparation

Diluted 50 ng/mL of stock solutions with diluents which 100% acetonitrile for pitavastatin lactone and 50% acetonitrile/water for others to prepare working standard stock solution 10 ng/mL of standard and quality control solutions preparation as described in Table 30 and 31.

6. Standard and quality control sample preparation

6.1. Thawed an aliquot of plasma sample (250 µL) on ice, then spiked 1M ammonium acetate at pH 5.0 at 5/100 (buffer/plasma) into tubes.

6.2. Added 50 µL of each working standard stock solution B through I to successive tubes to yield nominal concentrations of 1000, 800, 500, 200, 100, 50, 20, 5 pg/mL, respectively. And add 50 µL of quality control solutions (High-QC, Medium-QC, Low-QC, and LLOQ) at concentrations of 750, 250, 40, 5 pg/mL.

6.3. Added 50 µL of each internal standard stock solution (mixed statins and pitavastatin lactone). For single blank, added 25 µL of 50% acetonitrile/water and 25 µL 100% acetonitrile instead.

6.4. For double blank, added 50 µL of 50/50 acetonitrile/water (%v/v) and 50 µL 100% acetonitrile (for pitavastatin lactone) instead.

Table 30 Working standard solution preparation (statins)
Stock solution: 10 ng/mL (A), diluent: 50% acetonitrile/water, 100% acetonitrile.

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 50 µL in 250 µL) (pg/mL)**
A	10000				
B	5000	60	of A	60	1000
C	4000	50	of A	75	800
D	2500	40	of A	120	500
E	1000	30	of A	270	200
F	500	60	of E	60	100
G	250	50	of E	150	50
H	100	20	of E	180	20
I	25	20	of E	780	5

Table 31 Quality control solution preparation (statins)
Stock solution: 10 ng/mL (A'), diluent: 50% acetonitrile/water, 100% acetonitrile.

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 50 µL in 250 µL) (pg/mL)**
A'	10000				
High-QC	3750	75	of A	125	750
Medium-QC	1250	25	of A	175	250
Z'	1000	20		180	
Low-QC	200	60	of Z'	240	40
LLOQ	25	20	of Z'	780	5

7. Unknown plasma sample preparation

7.1. Aliquoted 250 µL of unknown samples into microcentrifuge tubes.

7.2. Added 25 µL of 50% acetonitrile/water and 25 µL of 100% acetonitrile instead of working standard solution.

7.3. Added 50 µL of each internal standard solution.

8. Plasma sample extraction

Statins was isolated from plasma sample by liquid-liquid extraction.

8.1. Adjusted pH of plasma by addition of 100 µL 1M ammonium acetate at pH 5.0 buffer and mix thoroughly.

8.2. Added 1,000 µL of MTBE (extraction solvent) into the plasma and mix for 15 minutes at room temperature.

8.3. Centrifuged at 12,000 rpm for 5 minutes at 4°C

8.4. Transferred 850 µL of the organic layer to a new tube, and dry under nitrogen at 40°C.

8.5. Reconstituted the dried samples in 100 µL of 50% acetonitrile containing 0.05% acetic acid.

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

9.1. M3 MicroLC autosampler with a 5 µL loop
Wash 1: 80% Acetonitrile with 0.1% formic acid
Wash 2: 20% Acetonitrile with 0.1% formic acid
Injection volume: 5 µL

9.2. M3 MicroLC pump
Mobile phase A: Water with 0.1% formic acid
Mobile phase B: 100% acetonitrile/water with 0.1%formic acid
Flow rate: 50 µL/min

Table 32 Gradient (statins)

Time (sec)	%A	%B
0	50	50
1	50	50
2.5	5	95
3	5	95
3.2	50	50
5	50	50

9.3. Chromatography
Column: ACE 3 C18-300, 50 x 1 mm, 3 µm, 300 Å.
Temperature: 60°C

9.4. Mass spectrometer

AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain, and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 33 and 34

Table 33 Mass spectrometry parameters (statins)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 34 Multiple reaction monitoring transitions for statins and internal standards

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Pitavastatin	422.2	290.0	50	191	10	37	24
Rosuvastatin	482.2	257.9	20	196	10	45	18
Atorvastatin	559.2	440.2	20	156	10	29	18
2-OH Atorvastatin	575.2	440.1	20	136	10	31	16
4-OH Atorvastatin	575.2	440.1	20	131	10	29	16
Pitavastatin Lactone	404.1	290.0	20	191	10	33	12
Internal standard							
[$^{2}H^{5}$]-Pitavastatin	427.2	295.1	50	191	10	37	24
[$^{2}H^{6}$]-Rosuvastatin	488.2	263.9	20	201	10	45	20
[$^{2}H^{5}$]-Atorvastatin	564.2	445.2	20	186	10	29	18
[$^{2}H^{5}$]-2-OH-Atorvastatin	580.3	445.2	20	161	10	31	16
[$^{2}H^{5}$]-4-OH Atorvastatin	580.3	445.2	20	151	10	31	18
[$^{2}H^{5}$]-Pitavastatin Lactone	409.1	295.0	20	171	10	37	24

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

E. <u>Bioanalytical method for determination of Rifampicin in plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Frozen human EDTA plasma samples
1.2. Rifampicin
1.3. Rifabutin (internal standard)
1.4. Acetonitrile
1.5. Formic acid
1.6. MTBE
1.7. Nitrogen gas
1.8. Ultrapure water

2. Apparatus

2.1. Pipetted and pipette tips
2.2. HPLC-MS/MS TSKgel Amide-80, 50 x 1 mm, 5 µm.
2.3. Positive strepper
2.4. Microcentrifuge tubes
2.5. HPLC-MS/MS Kinetex C18, 1.7 µm, 50 x 0.5 mm
2.6. Ice bath
2.7. pH meter
2.8. Vortex mixer
2.9. Multitube vortex mixer
2.10. Nitrogen evaporator
2.11. Refrigerated centrifuge
2.12. Multiwell sample plate

3. Rifampicin stock solution

- Weighed ~1 mg of rifampicin accurately into vial and dissolved with 1 mL of 50% acetonitrile/water, mixed well.
  Stock solution: 1 mg/mL rifampicin in 50% acetonitrile/water.

4. Internal standard preparation

- Dissolved ~1 mg of rifabutin in 1 mL of 50% acetonitrile/water.
  Internal standard stock solution**:** 1 mg/mL of rifabutin in 50% acetonitrile/water**.**
- On analyze date, diluted 1 mg/mL rifabutin with 50% acetonitrile/water.
  Working internal standard solution: 0.2 ng/rifabutin in 50% acetonitrile/water

5. Working standard and quality control solution preparation

Diluted 1 mg/mL of stock solutions with 50% acetonitrile/water for 100 µg/mL rifampicin as working stock solution. Working standard and quality control solution preparation was prepared in differential concentrations as shown in Table 35 and 36.

6. Standard and quality control sample preparation

6.1 Thawed an aliquot of plasma sample (50 µL) on ice.
6.2 Added 10 µL of each working standard stock solution B through H to successive tubes to yield nominal concentrations of 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 µg/mL, respectively. And add 10 µL of quality control solutions (High-QC, Medium-QC, Low-QC, and LLOQ) at concentrations of 15, 4, 0.4, 0.1 µg/mL.

6.3 Designate two further tubes as "single" and "double" blanks. Add 10 and 50 µL of 50 % acetonitrile/water, respectively.

6.4 Spike the plasma with 10 µL of internal standard solution except for double blank.

Table 35 Working standard solution preparation (rifampicin)
Stock solution: 100 µg/mL (A), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (µg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 10 µL in 50 µL) (µg/mL)**
A	100				20
B	50	15	of A	15	10
C	25	10	of A	30	5
D	10	6	of A	54	2
E	5	5	of A	95	1
F	2.5	15	of E	15	0.5
G	1	6	of E	24	0.2
H	0.5	6	of E	54	0.1

Table 36 Quality control solution preparation (rifampicin)
Stock solution: 100 µg/mL (A'), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (pg/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final plasma concentration (add 10 µL in 50 µL) (µg/mL)**
A'	100				
High-QC	75	45	of A'	15	15
Medium-QC	20	12	of A'	48	4
Z'	5	5		95	
Low-QC	2	24	of Z'	36	0.4

7. Unknown plasma sample preparation

7.1. Aliquoted 50 µL of unknown samples into microcentrifuge tubes.

7.2. Added 10 µL of 50% acetonitrile/water instead of working standard solution.

7.3. Added 10 µL of working internal standard solution to all samples.

8. Plasma sample extraction

Rifampicin was isolated from plasma sample by liquid-liquid extraction.

8.1. Added 150 µL of 100% acetonitrile and mixed well.

8.2. Centrifuged at 12,000 rpm for 10 minutes at 4°C.

8.3. Collected supernatant.

8.4. Diluted supernatant to 50% acetonitrile/water before injection.

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

9.1. M3 MicroLC autosampler with a 5 µL loop

Wash 1: 80% Acetonitrile with 0.1% formic acid

Wash 2: 20% Acetonitrile with 0.1% formic acid

Injection volume: 1 µL

9.2. M3 MicroLC pump

Mobile phase A: Water with 0.1% formic acid

Mobile phase B: 100% acetonitrile/water with 0.1%formic acid

Flow rate: 100 µL/min

Table 37 Gradient (rifampicin)

Time (sec)	%A	%B
0	50	50
1	50	50
2.5	5	95
3	5	95
3.2	50	50
5	50	50

9.3. Chromatography

Column: Kinetex C18, 1.7 µm, 50 x 0.5 mm.

Temperature: 45°C

9.4. Mass spectrometer

AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain, and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 38 and 39

Table 38 Mass spectrometry parameters (rifampicin)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 39 Multiple reaction monitoring transitions for the rifampicin and rifabutin

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Rifampicin	823.3	791.2	85	126	10	23	28
		399.0	85	126	10	35	26
Internal standard							
Rifabutin	847.3	815.2	75	171	10	37	32
		755.1	75	171	10	43	20

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

F. <u>Bioanalytical method for determination of Dabigatran in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS)</u>

1. Reagents

1.1. Frozen human EDTA urine samples
1.2. Dabigatran
1.3. [$^{13}C_6$]-Dabigatran (internal standard)
1.4. Acetonitrile
1.5. Formic acid
1.6. Dimethyl sulfoxide (DMSO)
1.7. Ultrapure water

2. Apparatus

2.1. Pipettes and pipette tips
2.2. HPLC-MS/MS TSKgel Amide-80, 50 x 1 mm, 5 µm.
2.3. Microcentrifuge tubes
2.4. Ice bath
2.5. Multitube vortex
2.6. Refrigerated centrifuge
2.7. 1.5 mL Amber glass vial
2.8. 250 µL glass flat bottom insert

3. Dabigatran stock solution

- Weighed ~0.5 mg of dabigatran accurately into vial and dissolved with 1 mL of 50% acetonitrile/water, mixed well.
  Stock solution: 500 µg/mL dabigatran in 50% acetonitrile/water.

4. Internal standard preparation

- Dissolved ~0.5 mg of [$^{13}C_6$]-Dabigatran in 1 mL of 50% acetonitrile/water.
  Internal standard stock solution: 500 µg/mL of [$^{13}C_6$]-Dabigatran in 50% acetonitrile/water.
- On analyze date, diluted 500 µg/mL [$^{13}C_6$]-Dabigatran with 50% acetonitrile/water.
- Working internal standard solution: 20 ng/mL [$^{13}C_6$]-Dabigatran in 50% acetonitrile/water.

5. Working standard and quality control solution preparation

Diluted 500 µg/mL dabigatran with 50% acetonitrile/water to 500 ng/mL dabigatran as working stock solution. Working standard and quality control solution preparation was prepared in differential concentrations as described in Table 40 and 41**.**

6. Standard and quality control sample preparation

6.1. Thawed an aliquot of urine sample (100 µL) on ice.
6.2. Added 25 µL of working standard stock solution B through I to successive tubes to yield nominal concentrations of 50, 40, 25, 10, 5, 2, 1 and 0.5 ng/mL, respectively. And add 25 µL of quality control solutions (High-QC, Medium-QC, Low-QC, and LLOQ) at concentrations 40, 20, 1.5 and 0.8pg/mL, respectively.
6.3. Designated two further tubes as "single" and "double" blanks. Add 25 and 50 µL of 50 % acetonitrile/water, respectively.

6.4. Spiked the sample with 25 µL of internal standard solution except for double blank.

Table 40 Working standard solution preparation (dabigatran for urine sample)
Stock solution: 500 ng/mL (A), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (ng/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final urine concentration (add 25 µL in 100 µL) (ng/mL)**
A	500				
B	200	24	of A	36	50
C	160	24	of A	51	40
D	100	24	of A	96	25
E	40	16	of A	184	10
F	20	30	of E	30	5
G	8	20	of E	80	2
H	4	20	of E	180	1
I	2	15	of E	285	0.5

Table 41 Quality control solution preparation (dabigatran for urine sample)
Stock solution: 500 ng/mL (A'), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (ng/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final urine concentration (add 25 µL in 100 µL) (ng/mL)**
A'	500				
High-QC	160	48	of A'	102	40
Medium- QC	80	24	of A'	126	20
Z'	40	16	of A'	184	
Low-QC	6	24	of Z'	136	1.5
LLOQ	2	20	of Z'	380	0.8

7. Unknown urine sample preparation

7.1. Aliquoted 100 µL of unknown samples into microcentrifuge tubes.

7.2. Added 25 µL of 50% acetonitrile/water.

7.3. Added 25 µL of working internal standard to all samples.

8. Urine sample extraction

Dabigatran was isolated from urine sample by protein precipitation.

8.1. Added 300 µL of acetonitrile and mixed well.

8.2. Centrifuged at 12,000 rpm for 10 minutes at 4°C.

8.3. Collected 200 µL of supernatant.

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

9.1. M3 MicroLC autosampler with a 5 µL Loop

Wash 1: 20% acetonitrile/water containing 0.1% formic acid

Wash 2: 80% acetonitrile/water containing 0.1% formic acid

Injection volume: 5 µL

9.2. M3 MicroLC pump

Mobile phase A: 20% acetonitrile/water containing 0.1% formic acid

Mobile phase B: 100% acetonitrile, 2mM ammonium formate, pH 4.0.

Flow rate: 50 µL/min

Table 42 Gradient (dabigatran for urine sample)

Time (sec)	%A	%B
0	50	50
1	50	50
2.5	5	95
3	5	95
3.2	50	50
5	50	50

9.3. Chromatography

Column: TSKgel Amide-80, 50 x 1 mm, 5 µm.

Temperature: 45°C

9.4. Mass spectrometer

AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain, and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 43 and 44.

Table 43 Mass spectrometry parameters (dabigatran for urine sample)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 44 Multiple reaction monitoring transitions for the dabigatran and [^{13}C]-Dabigatran (urine sample)

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Dabigatran	472.2	289.0	75	111	10	37	22
		330.0	75	111	10	27	12
Internal standard							
[^{13}C]-Dabigatran	478.1	295.0	75	101	10	37	12
			75	101	10	67	18

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

G. Bioanalytical method for determination of Rosuvastatin in urine by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

1. Reagents

1.1. Frozen human EDTA plasma samples
1.2. Rosuvastatin calcium salt
1.3. [$^2H_6$]- Rosuvastatin calcium salt (internal standard)
1.4. Acetonitrile
1.5. Formic acid
1.6. Dimethyl sulfoxide (DMSO
1.7. Ultrapure water

2. Apparatus

2.1. Pipettes and pipette tips
2.2. HPLC-MS/MS ACE 3 C18-300, 50 x 1 mm, 3 µm, 300 Å.
2.3. Microcentrifuge tubes
2.4. Ice bath
2.5. Multitube vortex
2.6. Refrigerated centrifuge
2.7. 1.5 mL Amber glass vial
2.8. 250 µL glass flat bottom insert

3. Rosuvastatin stock solution

- Weighed ~0.5 mg of rosuvastatin calcium accurately into vial and diluted with 1040 µL DMSO and mixed well. The concentration of free base rosuvastatin was 500 µg/mL. Diluted 50 µL of 500 µg/mL stock to final volume 450 mL with DMSO. Then diluted with 50% acetonitrile/water to 1 µg/mL stock solution.
  Stock solution: 1 µg/mL rosuvastatin in 50% acetonitrile/water.
- Diluted 1 µg/mL stock solution with 50% acetonitrile/water.
  Stock solution: 100 ng/mL rosuvastatin in 50% acetonitrile/water.

4. Internal standard preparation

- Weighed ~0.5 mg of [$^2H_6$]-rosuvastatin calcium salt accurately into vial and diluted with 1045 µL DMSO and mixed well. The concentration of free base [$^2H_6$]-rosuvastatin was 500 µg/mL. Diluted 50 µL of 500 µg/mL stock to final volume 450 mL with DMSO. Then diluted with 50% acetonitrile/water to 1 µg/mL stock solution.
  Internal standard stock solution: 1 µg/mL [$^2H_6$]-rosuvastatin in 50% acetonitrile/water.
- Diluted 1 µg/mL internal standard stock solution with 50% acetonitrile/water.
  Internal standard stock solution: 100 ng/mL [$^2H_6$]-rosuvastatin in 50% acetonitrile/water**.**
- On analyze date, diluted 100 ng/mL internal standard stock solution.
  Working internal standard stock solution: 12 ng/mL [$^2H_6$]-rosuvastatin in 50% acetonitrile/water.

5. Working standard and quality control solution preparation

Diluted 1 µg/mL of rosuvastatin stock solution with 50% acetonitrile/water to prepare 100 ng/mL rosuvastatin working stock solution. Working standard and

quality control solution preparation was prepared in differential concentrations as described in Table 45 and 46.

Table 45 Working standard solution preparation (rosuvastatin in urine sample)
Stock solution: 100 ng/mL (A), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (ng/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final urine concentration (add 25 µL in 100 µL) (ng/mL)**
A	100				
B	40	40	of A	60	10
C	32	32	of A	68	8
D	20	20	of A	80	5
E	8	20	of A	230	2
F	4	50	of E	50	1
G	2	25	of E	75	0.5
H	0.4	20	of E	380	0.1
I	0.2	20	of E	780	0.05

Table 46 Quality control solution preparation (rosuvastatin in urine sample)
Stock solution: 100 ng/mL (A'), diluent: 50% acetonitrile/water.

Standards	**Standard concentration (ng/mL)**	**Volume of working stock (µL)**		**Volume of Diluent (µL)**	**Final urine concentration (add 25 µL in 100 µL) (ng/mL)**
A'	100				
High-QC	36	36	of A'	64	9
Medium- QC	16	16	of A'	84	4
Z'	4	16	of A'	384	
Low-QC	1.2	30	of Z'	70	0.3
LLOQ	0.32	32	of Z'	368	0.8

6. Standard and quality control sample preparation

6.1. Thawed an aliquot of urine sample (100 µL) on ice.

6.2. Added 25 µL of working standard stock solution B through I to successive tubes to yield nominal concentrations 10, 8, 5, 2, 1, 0.5, 0.1 and 0.05 ng/mL, respectively. And add 25 µL of quality control solutions (High-QC, Medium-QC, Low-QC, and LLOQ) at concentrations 9, 4, 0.3 and 0.08 ng/mL, respectively.

6.3. Designated two further tubes as "single" and "double" blanks. Add 25 and 50 µL of 50 % acetonitrile/water, respectively.

6.4. Spiked the sample with 25 µL of internal standard solution except for double blank.

7. Unknown urine sample preparation

Aliquoted 100 µL of unknown sample into microcentrifuge tubes. Added 25 µL of 50 % acetonitrile/water and 25 µL of working internal standard to all samples.

8. Urine sample extraction

Rosuvastatin was isolated from urine sample by protein precipitation.

8.1. Added 100 µL of acetonitrile and mixed well.

8.2. Centrifuged at 12,000 rpm for 10 minutes at 4°C.

8.3. Collected of supernatant.

9. Sample analysis

LC-MS/MS was used to analyze all samples including standard, quality control, and unknown samples by monitoring the precursor ion and the corresponding product fragment ion. The experimental conditions have been proposed based on the using of an AB Sciex QTRAP6500 Mass Spectrometer interfaced via the TurboV electrospray ionization source (ESI) to the M3 MicroLC system. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. All instruments were controlled by Analyst software version 1.6.

9.1. M3 MicroLC autosampler with a 5 µL loop
Wash 1: Acetonitrile with 0.1% formic acid
Wash 2: Water with 0.1% formic acid
Injection volume: 15 µL

9.2. M3 MicroLC pump
Mobile phase A: Acetonitrile with 0.1% formic acid
Mobile phase B: Water with 0.1% formic acid
Flow rate: 100 µL/min

Table 47 Gradient (rosuvastatin in urine sample)

Time (sec)	%A	%B
0	50	50
1	50	50
2.5	5	95
3	5	95
3.2	50	50
5	50	50

9.3. Chromatography
Column: ACE 3 C18-300, 50 x 1 mm, 3 µm, 300 Å.
Temperature: 45°C

9.4. Mass spectrometer
AB Sciex 6500 QTRAP Mass Spectrometer with electrospray (ESI) was used in the positive ionization mode. Nitrogen (99.999%) was used for nebulization, curtain and collision gas. Mass spectrometry parameters and multiple reaction monitoring (MRM) transitions are listed in Table 48 and 49

Table 48 Mass spectrometry parameters (rosuvastatin in urine sample)

Instrument parameters			
Curtain gas	40.0	**Heater setting (TEM)**	350
Collision gas	medium	**Gas 1**	30
IonSpray voltage (V)	4000	**Gas 2**	30

Table 49 Multiple reaction monitoring transitions for the rosuvastatin and [$^2H_6$]-rosuvastatin

Compounds	**Precursor (Q1, m/z)**	**Product Ion (Q3, m/z)**	**Dwell (ms)**	**DP (V)**	**EP (V)**	**CE (V)**	**CXP (V)**
Parent							
Rosuvastatin	482.2	257.9	20	196	10	45	18
Internal standard							
[$^2H_6$]-Rosuvastatin	488.2	263.9	25	201	10	45	20

Note: DP, Declustering potential; EP, Entrance potential); CE, Collision energy; CXP, Collision exit potential

Data analysis

Standard curve, as known as calibration curve, was constructed by plotting the peak area ratios against the concentrations of the analytes in sample. The coefficient of determination (R^2) was determined including the concentrations of each compound in microdose cocktail by using MultiQuant software version 2.1.

3. <u>In vitro experiments</u>

Pharmacogenetic analysis

To eliminate the effect of confounding factor to drug level, genotyping was done in *SLCO1B1* gene and *ABCG2* gene with following variants:

- *SLCO1B1* gene (encode OATP1B1 drug transporters)
  - c.521T>C (rs4149056)
  - c.388A>G (rs2306283)
  - g.-11187 (rs4149015)
- *ABCG2* gene (encode BCRP transports)
  - c.421C>A (rs2231142)

Genotype analysis RT-PCR

For each subject, 3 mL venous blood samples drawn with EDTA as anticoagulant was collected and stored at –80 °C before DNA extraction. According to PureLink™ Genomic DNA extraction protocol[171], DNA was extracted as following procedures.

A. Genomic DNA extraction

1. Reagent

1.1. PureLink™ Genomic DNA extraction mini kits.
- 1.1.1. RNase A
- 1.1.2. Proteinase K
- 1.1.3. Genomic lysis
- 1.1.4. Wash buffer 1
- 1.1.5. Wash buffer 2
- 1.1.6. Elution buffer
- 1.1.7. Ethanol

2. Apparatus

2.1. PureLink™ Spin columns in collection tube
2.2. Microcentrifuge tubes
2.3. Vortex mixer
2.4. Water bath

3. Blood lysate preparation

3.1. Set water bath at 55°C.
3.2. Added 200 µL of whole blood samples to Microcentrifuge tube.
3.3. Added 20 µL of Proteinase K to the sample.
3.4. Added 20 µL of RNase A to the sample and mixed well by vortexing.
3.5. Incubated the sample at 25°C for 2 minutes.
3.6. Added 200 µL of Genomic lysis and mixed well by vortexing to obtain homogenous solution.
3.7. Incubated at 55°C in water bath for 10 minutes for protein digestion.
3.8. Added 200 µL of 100% Ethanol to the lysate and mixed well by vortexing for 5 seconds.

4. Purification procedures

4.1. Added the prepared lysate to the PureLink™ Spin columns

4.2. Centrifuged at 10,000 g for 1 minute at 25°C.
4.3. Discarded the collection tube and placed the spin column into a new collection tube.

5. Washing DNA

5.1. Prepared Wash buffer 1 and Wash buffer 2 with ethanol 15 mL and 17.5 mL, respectively.
5.2. Added 500 µL of prepared Wash buffer 1 to the spin column.
5.3. Centrifuged at 10,000 g for 1 minute at 25°C.
5.4. Discarded the collection tube and placed the spin column into a new collection tube.
5.5. Added 500 µL of prepared Wash buffer 2 to the spin column.
5.6. Centrifuged at 14,000 g for 3 minutes at 25°C.
5.7. Discarded the collection tube.

6. Eluting DNA

6.1. Placed the spin column into sterile microcentrifuge tube.
6.2. Add 50 µL of elution buffer to the column.
6.3. Incubated at room temperature for 2 minutes.
6.4. Centrifuged at 13,000 g for 1 minutes at room temperature.
6.5. The tube contains purified genomic DNA.

B. Genotype analysis

1. Reagent

1.1. Purified genomic DNA samples.
1.2. Elution buffer
1.3. Taqman genotyping Master mix
1.4. Nuclease free water
1.5. TaqMan Drug Metabolism Genotyping Assays (containing TaqMan probe and Primers)
1.5.1. *SLCO1B1* rs4149056: Assay ID C__30633906_10
1.5.2. *SLCO1B1* rs2306283: Assay ID C__ 1901697_20
1.5.3. *SLCO1B1* rs4149015: Assay ID C__32325356_10
1.5.4. *ABCG2* rs2231142: Assay ID C__15854163_70

2. Apparatus

2.1. Nanodrop™ One/OneC Microvolume UV-Vis Spectrophotometer
2.2. Real Time PCR StepOnePlus
2.3. PureLink™ Spin columns in collection tube
2.4. Microcentrifuge tubes
2.5. 0.1 mL clear PCR strip of 8 tubes with 0.2 mL RT PCR caps
2.6. Lint-free laboratory wipe
2.7. Spin down
2.8. Centrifuge
2.9. Vortex mixer

3. DNA quantification

3.1. Cleaned the upper and lower pedestals with 2 µL of nuclease free water.
3.2. Pipetted 2 µL of elution buffer onto the lower measurement pedestal to set blank.
3.3. Pipetted 2 µL of purified genomic DNA sample onto the lower measurement pedestal for measurement.

3.4. Wiped the pedestals with lint-free laboratory wipe between samples and cleansed with nuclease free water after the measurement was completed.
3.5. DNA concentrations were provided.

4. Genotype analysis

4.1. Diluted DNA concentration to 10 ng/µL with nuclease free water
4.2. Prepared RT-PCR reaction mixture 20 µL in PCR strip tube contains 10 µL of Taqman genotyping Master mix, 1 µL of TaqMan Drug Metabolism Genotyping Assays, 6 µL of nuclease free water and 3 µL of diluted DNA (30 ng per tube).
4.3. Prepared negative control by using nuclease free water instead of diluted DNA.
4.4. Analyzed PCR assays by using universal thermal cycling conditions thermal cycling protocol[172]:
    4.4.1. Pre-PCR read (holding stage): 60°C for 30 seconds.
    4.4.2. Holding stage: 95°C for 10 minutes.
    4.4.3. Cycle stage: 95°C for 15 seconds (Denaturation)
    : 60°C for 1 minutes (Extension)
    4.4.4. Post-PCR read (holding stage): 60°C for 30 seconds

Chapter 4

Results

1. Bioanalytical method development and validations (Please see Appendix B).

2. Subjects, baseline characteristics, and genetic profile.

One hundred two subjects were enrolled for eligibility assessment. Of these, forty-nine subjects were excluded according to the inclusion and exclusion criteria (n=31) and subject's willingness (n=18). Fifty-three eligible subjects were allocated into three groups: healthy young subjects (Group 1, n=20), healthy elderly subjects (Group 2, n=16), elderly subjects with CKD (Group 3, n=17). All studied subjects completed the study per protocol. The study flow diagram is shown in Figure 22.

Baseline characteristics are shown in Table 50 (please see concomitant drug in Appendix C). The subject median age were 30, 64.5, and 74 years old in Group 1, Group 2, and Group 3, respectively. The median of Thai estimated glomerular filtration rate (eGFR) were 112.4, 94.8, 32.6 mL/min/1.73m^2 in Group 1, Group 2, and Group 3, respectively.

Besides age and eGFR, there were significant differences in red blood cell, hematocrit, platelet, blood urea nitrogen (BUN), serum creatinine, and parathyroid hormone between the groups due to renal deterioration in CKD. Although, the slight difference of alkaline phosphatase (ALP), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL) were also observed, there were no clinically significant difference.

The underlying diseases were found in elderly subjects, especially Group 3. These were hypertension (n=14), dyslipidemia (n=11), diabetes mellitus (n=6), gout (n=6), benign prostate hypertrophy (n=5), osteoarthritis (n=3), and coronary heart disease (n=2). Some subjects in Group 2 had osteoarthritis (n=2), gout (n=1), and benign prostate hypertrophy (n=1) (Table 50).

Table 51 presents genetic data. In *SLCO1B1* gene, homogenous wild type TT at c.521T>C and GG at -11187G>A were mostly found across three groups whereas homogenous variant GG was the highest frequency at c.388A>G. In *ABCG2* gene, homogenous wild type CC at c.421C>A was mostly observed.

Based on 3 SNPs consisted of c.521T>C, c.388A>G and g.-11187G>A, wild type haplotypes of *SLCO1B1* (**1A* and **1B*) were mostly found while variants haplotypes (**5, *15, *17, *21*) were less observed. All study groups had the similar highest prevalence of wild type diplotype **1B/*1B* (primary) and **1A/*1B*. Even though the sample size was small, the homozygous variant diplotype including **5/*15, *15/*15*, and **15/*17* were also found in our population as described in Table 52.

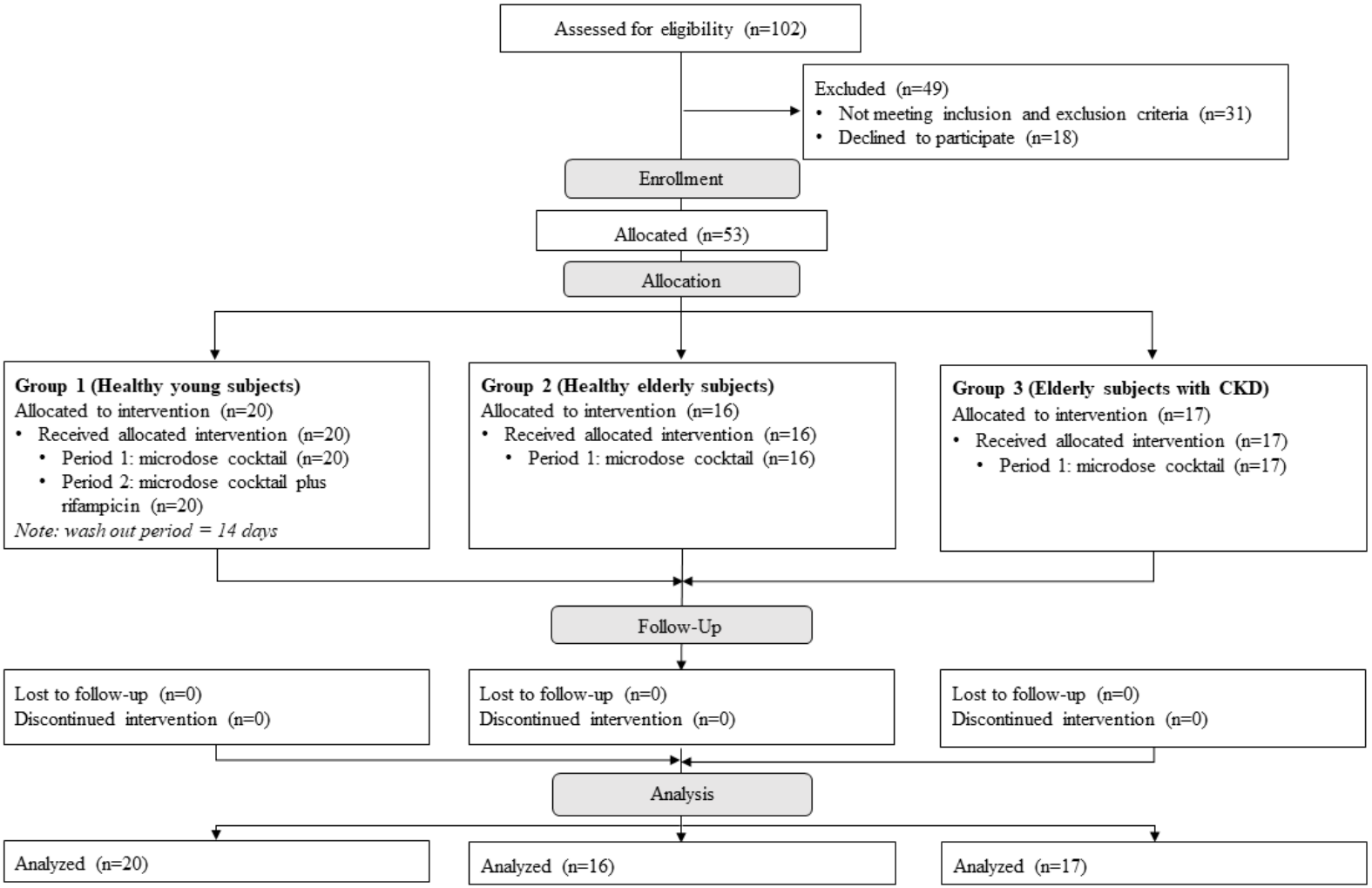


Figure 22 Study flow diagram

Table 50 Demographic data and baseline characteristics for three groups of subjects

Characteristics	**Group 1 (n=20)**	**Group 2 (n=16)**	**Group 3 (n=17)**
Gender (F/M) (n)	12/8	12/4	4/13
Age (yr)	30.0 (27.5-32.0)	64.5 (62.0-67.0)*	74.0 (67.0-77.0)*
Body weight	54.2 (46.8-65.5)	60.4 (52.4-65.0)	66.5 (60.8-70.3)
Body mass index (kg/m^2)	21.7 (19.8-23.5)	23.3 (21.3-26.1)	24.7 (23.2-26.7)
Complete blood count			
Red blood cell ($10^6/\mu L$)	5.0 (4.7-5.7)	4.7 (4.5-5.0)	4.2 (3.9-4.6)*
Hemoglobin($10^6/\mu L$)	13.4 (12.3-14.5)	13.2 (12.8-13.9)	12.0 (10.8-13.3)
Hematocrit (%)	41.0 (38.0-43.5)	40.1 (38.2-41.0)	36.1 (32.9-39.6)*
White blood cell ($10^3/\mu L$)	9.5 (5.2-7.6)	5.9 (5.5-6.8)	6.3 (5.3-8.0)
Neutrophil ($10^3/\mu L$)	3.7 (2.5-4.2)	3.0 (2.6-3.6)	3.6 (2.8-4.8)
Lymphocyte ($10^3/\mu L$)	2.2 (1.6-2.6)	2.2 (1.8-2.6)	2.1 (1.5-2.1)
Monocyte ($10^3/\mu L$)	0.4 (0.4-0.6)	0.4 (0.4-0.5)	0.5 (0.4-0.6)
Eosinophil ($10^3/\mu L$)	0.1 (0.1-0.2)	0.1 (0.1-0.4)	0.3 (0.2-0.3)
Basophil ($10^3/\mu L$)	0.03 (0.03-0.05)	0.03 (0.02-0.05)	0.03 (0.03-0.04)
Platelet count ($10^3/\mu L$)	265.5 (256.0-287.5)	251.0 (214.5-329.5)	224.0 (182.0-260.0)*
Blood chemistry tests			
Fasting blood sugar (mg/dL)	85.0 (80.5-93.0)	94.5 (90.0-102.0)	98.0 (88.0-105.0)
Total protein (g/dL)	7.8 (7.5-7.9)	7.7 (7.3-7.8)	7.6 (7.1-7.7)
Albumin (g/dL)	4.5 (4.3-4.6)	4.3 (4.3-4.5)	4.3 (4.2-4.4)
Total bilirubin (mg/dL)	0.6 (0.5-0.8)	0.6 (0.6-0.9)	0.6 (0.5-0.7)
Direct bilirubin (mg/dL)	0.2 (0.2-0.4)	0.3 (0.2-0.3)	0.3 (0.2-0.3)
AST (U/l)	16.0 (15.0-19.5)	21.5 (18.5-23.5)	18.0 (16.0-26.0)
ALT (U/l)	14.5 (13.0-20.0)	18.5 (16.0-22.0)	19.0 (16.0-25.0)
ALP (U/l)	54.0 (46.5-66.0)	66.5 (55.5-74.5)*	64.0 (53.0-73.0)*
TC (mg/dL)	202.5 (176.0-225.0)	241.5 (208.5-270.0)	167.0 (152.0-177.0)*,¶
TG (mg/dL)	87.0 (61.0-106.5)	129.5 (93.5-191.0)*	91.0 (87.0-141.0)

Characteristics	Group 1 (n=20)	Group 2 (n=16)	Group 3 (n=17)
LDL-C (mg/mL)	118.50 (108.5-137.0)	144.0 (123.1-170.0)	89.6 (82.0-101.4)
HDL-C (mg/mL)	60.0 (51.5-69.5)	52.0 (41.5-60.0)	45.0 (41.0-48.0)*
Electrolytes			
Sodium (mmol/L)	138.0 (137.5-139.0)	140.0 (138.5-141.0)	140.0 (137.0-142.0)
Chloride (mmol/L)	105.0 (103.5-106.0)	105.5 (104.0-107.0)	107.0 (104.0-108.0)
Potassium (mmol/L)	4.2 (4.1-4.5)	4.2 (3.9-4.3)	4.5 (4.3-4.9)
Phosphate (mg/dL)	3.3 (3.1-3.6)	3.6 (1.3-3.9)	3.4 (3.2-3.7)
Bicarbonate (mmol/L)	26.4 (24.6-29.0)	23.9 (23.1-25.5)	21.8 (20.3-23.5)*
Blood urea nitrogen (mg/dL)	10 (9.5-11.5)	11.0 (9.5-13.5)	26.0 (21.0-37.0)*,¶
Serum creatinine (g/dL)	0.7 (0.7-0.9)	0.7 (0.6-0.8)	2.2 (1.8-2.9)*,¶
Parathyroid hormone (pg/mL)	54.7 (45.2-60.4)	63.2 (48.8-75.6)	112.6 (75.7-167.5)*,¶
eGFR MDRD-Thai (mL/min/1.73m2)	112.4 (106.1-118.4)	94.8 (86.1-110.2)*	32.6 (24.0-39.8)*,¶
Underling disease (n)			
Hypertension	0	0	14
Diabetes mellitus	0	0	6
Dyslipidemia	0	0	11
Gout	0	1	6
Benign prostate hypertrophy	0	1	5
Osteoarthritis	0	2	3
Coronary heart disease	0	0	2

Data presented in median IQR unless otherwise stated

*significant level (p-value<0.05, healthy young subjects as reference group)

¶significant level (p-value<0.05, healthy elderly subjects as control group)

IQR, interquartile range; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate

Table 51 Genotype frequency of *SLCO1B1* and *ABCG2* gene in three groups of subjects

Genotype frequency (n)	**Group 1 (n=20)**	**Group 2 (n=16)**	**Group 3 (n=17)**	**Total (n=53)**
***SLCO1B1* gene (%), OATP1B1 transporter: influx transporter**				
rs4149056 (c.521T>C)				
521TT (wild type)	14	13	15	42
521TC	3	3	1	7
521CC	3	0	1	4
rs2306283 (c.388A>G)				
388AA (wild type)	1	1	0	2
388AG	5	7	7	19
388GG	14	8	10	32
rs4149015 (g.-11187G>A)				
11187GG (wild type)	15	12	13	40
11187GA	5	4	4	13
11187AA	0	0	0	0
***ABCG2* gene (%), BCRP transporter: efflux transporter**				
rs2231142 (421C>A)				
421CC (wild type)	13	8	9	30
421CA	5	8	7	20
421AA	2	0	1	3

SLCO1B1, Solute carrier organic anion transporter family member 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

Table 52 Haplotype and diplotype frequency of *SLCO1B1 ABCG2* gene in three groups of subjects

Frequency (n)	Group 1 (n=20)	Group 2 (n=16)	Group 3 (n=17)	Total (n=53)
Haplotype frequency				
**1A* (wild type)	6	9	7	22
**1B* (wild type)	24	18	22	64
**5*	1	0	0	1
**15*	5	1	1	7
**17*	3	2	2	7
**21*	1	2	2	5
Diplotype frequency				
**1A/*1A* (wild type)	1	1	0	2
**1A/*1B* (wild type)	2	4	6	12
**1B/*1B* (wild type)	10	5	7	23
**1A/*17*	1	1	0	2
**1A/*21*	1	2	1	4
**1B/*15*	0	1	0	1
**1B/*17*	2	1	1	4
**1B/*21*	0	0	1	1
**5/*15*	1	0	0	1
**15/*15*	2	0	0	2
**15/*17*	0	0	1	1

SLCO1B1, Solute carrier organic anion transporter family member 1B1

3. Safety monitoring

No serious adverse events observed (Table 53). Five Group 1, period 1 subjects in period 1 had diarrhea (n=1), dysmenorrhea (n=1), dyspepsia (n=1), migraine (n=1), and upper respiratory tract infection (n=1) which were unrelated to study medications. One subject in Group 3 had nasal congestion which was unlikely related to study drugs and was fully recovered after taking one dose of 60 mg of pseudoephedrine. All Group 1, period 2 subjects in period 2 had darken urine which was related to rifampicin. The darken urine was recovered in approximately 3-4 day.

Safety laboratory monitoring was also analyzed at baseline and the last visit. There were no significant differences in laboratory results between baseline and the last visit (Table 54).

Table 53 The summaries of adverse events and study drug related in three group of subjects

Safety parameters	**Group 1 (n=20)**		**Group 2 (n=16)**	**Group 3 (n=17)**
	Period 1	**Period 2**		
Adverse events (n)				
Diarrhea	1	0	0	0
Dysmenorrhea	1	0	0	0
Migraine	1	0	0	0
Upper tract infection	1	0	0	0
Darken urine	1	20	0	0
Nasal congestion	0	0	0	1
Study drug related (n)				
Unrelated	5	0	0	0
Unlikely	0	0	0	1
Definite	0	20	0	0

Table 54 The safety laboratory from baseline to last visit in each group of study

Characteristics	Group 1 (n=20)		Group 2 (n=16)		Group 3 (n=17)	
	Baseline	**Last visit**	**Baseline**	**Last visit**	**Baseline**	**Last visit**
Complete blood count						
Red blood cell (10^6/µL)	5.0 (4.7-5.7)	4.8 (4.5-5.6)	4.7 (4.5-5.0)	4.7 (4.3-5.2)	4.2 (3.9-4.6)	4.2 (3.9-4.7)
Hemoglobin (10^6/µL)	13.4 (12.3-14.5)	13.1 (11.8-14.3)	13.2 (12.8-13.9)	13.2 (12.8-14.9)	12.0 (10.8-13.3)	12.4 (10.5-13.2)
Hematocrit (%)	41.0 (38.0-43.5)	39.2 (37.1-42.5)	40.1 (38.2-41.0)	39.9 (38.6-44.6)	36.1 (32.9-39.6)	36.1 (33.0-40.2)
White blood cell (10^3/µL)	6.6 (5.2-7.6)	6.2 (5.1-7.4)	5.9 (5.5-6.8)	5.9 (4.8-6.9)	6.3 (5.3-8.0)	5.8 (5.3-7.2)
Neutrophil (10^3/µL)	3.7 (2.5-4.2)	3.2 (2.6-4.2)	3.0 (2.6-3.6)	2.9 (2.3-4.0)	3.6 (2.8-4.8)	3.5 (2.9-4.3)
Lymphocyte (10^3/µL)	2.2 (1.6-2.6)	2.2 (1.7-2.8)	2.2 (1.8-2.6)	2.1 (1.8-2.5)	2.1 (1.5-2.1)	1.8 (1.5-2.2)
Monocyte (10^3/µL)	0.4 (0.4-0.6)	0.5 (0.4-0.6)	0.4 (0.4-0.5)	0.4 (0.3-0.5)	0.5 (0.4-0.6)	0.5 (0.4-0.6)
Eosinophil (10^3/µL)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.1 (0.1-0.4)	0.1 (0.1-0.4)	0.3 (0.2-0.3)	0.3 (0.2-0.4)
Basophil (10^3/µL)	0.03 (0.03-0.05)	0.04 (0.03-0.05)	0.03 (0.02-0.05)	0.03 (0.02-0.04)	0.03 (0.03-0.04)	0.02 (0.02-0.04)
Platelet count (10^3/µL)	265.5 (256.0-287.5)	294.0 (263.5-318.5)	251.0 (214.5-329.5)	261.5 (209.0-320.0)	224.0 (182.0-260.0)	206.0 (185.0-230.0)
Blood chemistry tests						
FBS (mg/dL)	85.0 (80.5-93.0)	85.0 (79.5-88.0)	94.5 (90.0-102.0)	93.0 (88.5-101.5)	98.0 (88.0-105.0)	98.0 (83.0-118.0)
Total protein (g/dL)	7.8 (7.5-7.9)	7.5 (7.0-8.0)	7.7 (7.3-7.8)	7.4 (7.0-8.1)	7.6 (7.1-7.7)	7.6 (7.2-7.6)

Characteristics	Group 1 (n=20)		Group 2 (n=16)		Group 3 (n=17)	
	Baseline	**Last visit**	**Baseline**	**Last visit**	**Baseline**	**Last visit**
Albumin (g/dL)	4.5 (4.3-4.6)	4.5 (4.3-4.8)	4.3 (4.3-4.5)	4.4 (4.0-4.5)	4.3 (4.2-4.4)	4.2 (4.0-4.3)
Blood chemistry tests						
Total bilirubin (mg/dL)	0.6 (0.5-0.8)	0.4 (0.3-0.8)	0.6 (0.6-0.9)	0.6 (0.5-0.7)	0.6 (0.5-0.7)	0.6 (0.5-0.6)
Direct bilirubin (mg/dL)	0.2 (0.2-0.4)	0.2 (0.2-0.3)	0.3 (0.2-0.3)	0.2 (0.2-0.3)	0.3 (0.2-0.3)	0.2 (0.2-0.3)
AST (U/L)	16.0 (15.0-19.5)	15.0 (14.0-16.5)	21.5 (18.5-23.5)	19.5 (16.0-20.5)	18.0 (16.0-26.0)	17.0 (14.0-28.0)
ALT (U/L)	14.5 (13.0-20.0)	13.0 (9.5-17.5)	18.5 (16.0-22.0)	17.0 (13.5-20.0)	19.0 (16.0-25.0)	17.0 (13.0-27.0)
ALP (U/L)	54.0 (46.5-66.0)	53.5 (46.0-63.5)	66.5 (55.5-74.5)	65.0 (59.5-76.5)	64.0 (53.0-73.0)	63.0 (53.0-73.0)
TC (mg/dL)	202.5 (176.0-225.0)	195.5 (172.0-214.0)	241.5 (208.5-270.0)	227.5 (198.0-255.5)	167.0 (152.0-177.0)	174.0 (142.0-210.0)
TG (mg/dL)	87.0 (61.0-106.5)	94.0 (77.5-136.0)	129.5 (93.5-191.0)	112.5 (78.0-189.0)	91.0 (87.0-141.0)	103.0 (73.0-124.0)
LDL-C (mg/mL)	118.50 (108.5-137.0)	116.5 (105.0-138.0)	144.0 (123.1-170.0)	140.6 (117.0-159.2)	89.6 (82.0-101.4)	94.0 (85.0-134.2)
HDL-C (mg/mL)	60.0 (51.5-69.5)	56.0 (48.5-70.0)	52.0 (41.5-60.0)	52.5 (44.5-62.5)	45.0 (41.0-48.0)	47.0 (42.0-55.0)
Electrolytes						
Sodium (mmol/L)	138.0 (137.5-139.0)	138.0 (136.5-138.5)	140.0 (138.5-141.0)	140.0 (138.0-140.0)	140.0 (137.0-142.0)	139.0 (137.0-141.0)
Chloride (mmol/L)	105.0 (103.5-106.0)	103.5 (103.0-105.0)	105.5 (104.0-107.0)	103.5 (101.5-105.0)	107.0 (104.0-108.0)	103.5 (103.0-105.0)

Characteristics	Group 1 (n=20)		Group 2 (n=16)		Group 3 (n=17)	
	Baseline	**Last visit**	**Baseline**	**Last visit**	**Baseline**	**Last visit**
Potassium (mmol/L)	4.2 (4.1-4.5)	4.1 (3.9-4.4)	4.2 (3.9-4.3)	4.1 (4.0-4.3)	4.5 (4.3-4.9)	4.4 (4.0-4.8)
Phosphate (mg/dL)	3.3 (3.1-3.6)	3.5 (3.2-3.6)	3.6 (1.3-3.9)	3.4 (3.2-3.8)	3.4 (3.2-3.7)	3.4 (3.0-3.7)
Bicarbonate (mmol/L)	26.4 (24.6-29.0)	21.4 (19.0-22.6)	23.9 (23.1-25.5)	22.9 (21.0-26.9)	21.8 (20.3-23.5)	21.4 (19.0-22.6)
BUN (mg/dL)	10 (9.5-11.5)	11.0 (8.5-13.0)	11.0 (9.5-13.5)	12.0 (10.5-15.5)	26.0 (21.0-37.0)	28.0 (20.0-42.0)
Scr (g/dL)	0.7 (0.7-0.9)	0.7 (0.7-0.9)	0.7 (0.6-0.8)	0.6 (0.6-0.7)	2.2 (1.8-2.9)	2.0 (1.5-2.8)
PTH (pg/mL)	54.7 (45.2-60.4)	52.4 (46.4-58.8)	63.2 (48.8-75.6)	41.7 (35.4-65.9)	112.6 (75.7-167.5)	134.6 (101.1-165.9)
eGFR MDRD-Thai (mL/min/1.73m2)	112.4 (106.1-118.4)	116.0 (105.9-118.3)	94.8 (86.1-110.2)	110.0 (100.4-122.6)	32.6 (24.0-39.8)	37.6 (25.1-48.9)

Data presented in median IQR. Using Sign test to compare laboratory between two visits, p-value <0.05.

IQR, interquartile range; BMI, body mass index; FBS: fasting blood sugar; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BUN, blood urea nitrogen; Scr, serum creatinine; PTH, parathyroid hormone; eGFR, estimated glomerular filtration rate

4. Pharmacokinetic parameters estimation in microdose cocktail containing drugs.

The plasma concentration time curves and pharmacokinetic parameters were calculated from plasma drug level (midazolam, dabigatran, pitavastatin, pitavastatin lactone, atorvastatin, 4-hydroxy atorvastatin, rosuvastatin) and urine drug level (only dabigatran and rosuvastatin). Because plasma concentration was lower than LLOQ, 2-hydroxy atorvastatin could not be estimated.

Statins plasma concentration of one subject in Group 2 were considered outlier and were not included in the analyses. Some pharmacokinetic parameters ($AUC_{0\text{-}inf}$, $T_{1/2}$, and the Kel) of atorvastatin, 4-hydroxy atorvastatin, and rosuvastatin could not be estimated because last four timepoints were mostly lower than LLOQ.

Urine pharmacokinetic parameter represented by, renal clearance (CL_R), was estimated for dabigatran and rosuvastatin.

Please see timepoint plasma and urine concentration of each subject in Appendix D-E.

Midazolam (Figure 23 and Table 55)

The differences in plasma midazolam concentration among 3 groups were observed. $AUC_{0\text{-}last}$ and $AUC_{0\text{-}inf}$ in Group 2 and Group 3 had significantly higher than Group 1 (GMR~2.3 in Group 2, GMR~3 in Group 3). C_{max} of Group 2 and Group 3 were also higher than Group 1 (GMR~2 in both Group 2 and Group 3).

Half-life prolongation was found in Group 2 and Group 3 (GMR~2.3 in Group 2, GMR~2.5 in Group 3). Kel of Group 2 and Group 3 were slightly reduced (GMR~0.4 in both groups).

Pharmacokinetic parameters of midazolam co-administered with rifampicin in period 2 of Group 1 were comparable to that of period 1.

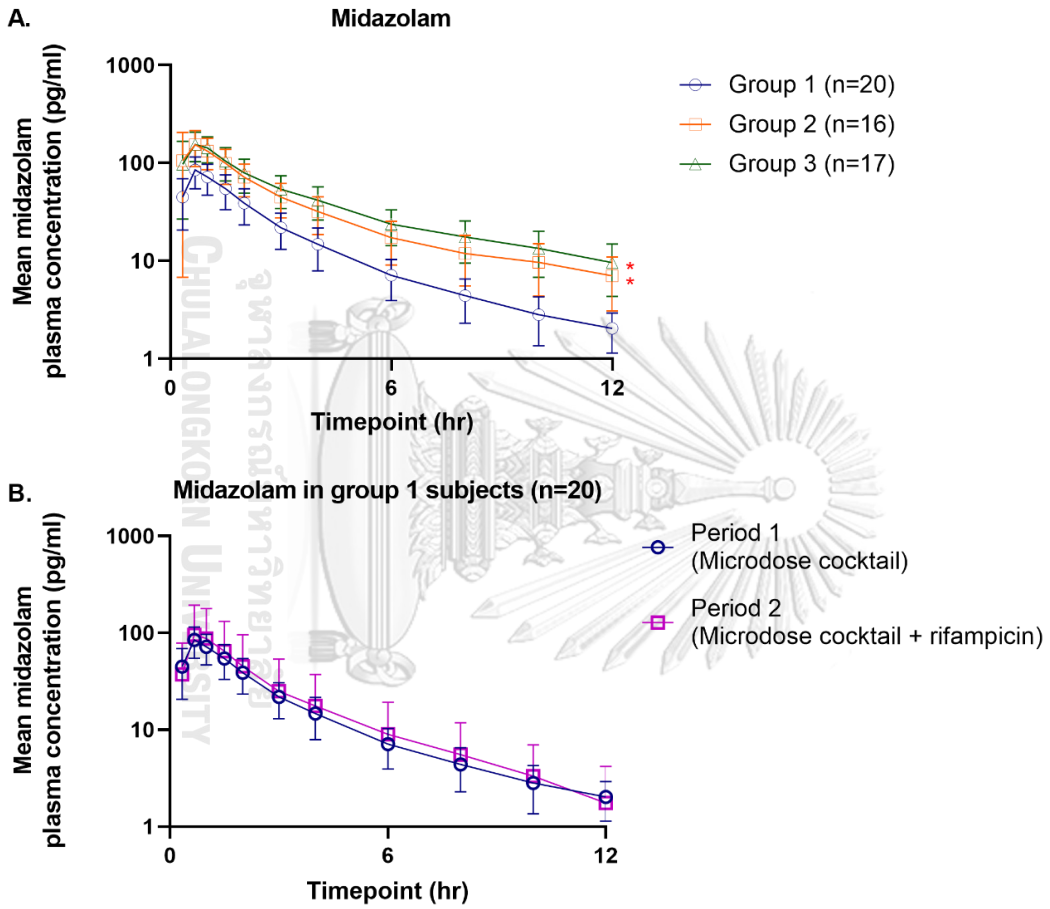


Figure 23The plasma concentration time-curve of midazolam
(A.) midazolam 30 µg in three groups of subjects and (B.) in period 1 and period 2
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease
*p-value<0.05, Group 1 as a reference group

Table 55 Pharmacokinetic parameters of midazolam in three groups of subjects

Drugs	**Group 1**	**Group 1 with rifampicin**		**Group 2**		**Group 3**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**
Midazolam							
$AUC_{0\text{-last}}$ (pg/mL.hr)	192 (155-237)	248 (198-309)	1.29 (0.96-1.74)	440 (342-567)	2.30 (1.70-3.09)*	556 (454-681)	2.90 (2.16-3.88)*
$AUC_{0\text{-inf}}$ (pg/mL.hr)	200 (162-247)	256 (206-318)	1.28 (0.96-1.72)	464 (365-590)	2.32 (1.74-3.10)*	579 (475-705)	2.90 (2.18-3.85)*
C_{max} (pg/mL)	80 (67-96)	78 (96-118)	1.20 (0.92-1.56)	151 (119-191)	1.88 (1.44-2.44)*	156 (132-185)	1.95 (1.51-2.52)*
T_{max} (hr)[a]	0.7 (0.7-0.7)	0.7 (0.7-0.8)	-	0.7 (0.7-1.0)	-	0.7 (0.7-1.0)	-
$T_{1/2}$ (hr)	2.6 (2.1-3.2)	2.6 (2.0-3.4)	0.99 (0.72-1.37)	6.0 (4.7-7.5)*	2.31 (1.72-3.10)*	6.5 (5.2-8.2)*	2.52 (1.89-3.36)*
Kel (hr^{-1})	0.268 (0.220-0.327)	0.278 (0.214-0.362)	1.04 (0.75-1.43)	0.116 (0.092-0.146)	0.43 (0.32-0.58)*	0.106 (0.084-0.134)	0.40 (0.30-0.53)*

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group.

GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance.

Dabigatran (Figure 24 and Table 56)

$AUC_{0\text{-last}}$ and $AUC_{0\text{-inf}}$ were higher in Group 2 (GMR~1.5) and Group 3 (GMR~4). Group 3 had significant higher in C_{max} than Group 1 (GMR~1.7).

CL_R of Group 2 and Group 3 were significantly decreased for GMR~0.61 and GMR~0.19, respectively. Half-life prolongation and reduced Kel were found only in Group 3.

Compared to Group 2, AUC, $T_{1/2}$, Kel, and CL_R in Group 3 also had significant differences.

Both advanced age and CKD might affect dabigatran plasma concentration in this study because the significant higher dabigatran level was found compared to either Group 1 or Group 2. The increased dabigatran plasma concentration was probably related to the observed lowering renal clearance in Group 2 and Group 3 whereas only Group 3 had half-life prolongation.

Results in period 2 of Group 1 showed that rifampicin, a intestine p-gp inhibitor, affected dabigatran pharmacokinetic process by increasing AUC and C_{max} compared to Group 1 (GMR~2 for AUC and C_{max}) and slightly increased in T_{max} whereas Kel and CL_R were decreased.

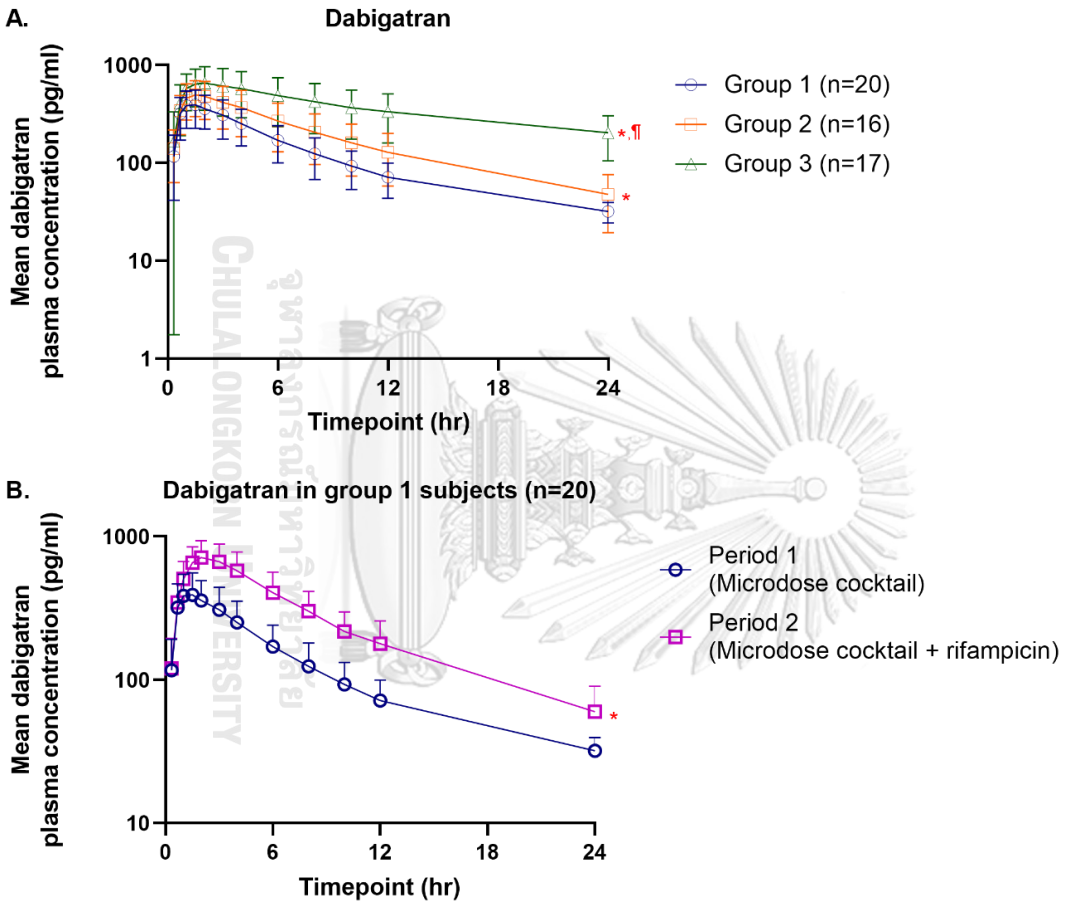


Figure 24 The plasma concentration time-curve of dabigatran
(A.) dabigatran 750 µg in three groups of subjects and (B.) in period 1 and period 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

Table 56 Pharmacokinetic parameters of dabigatran in three groups of subjects

Drugs	**Group 1**	**Group 1 with rifampicin**		**Group 2**		**Group 3**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**
Dabigatran							
$AUC_{0\text{-last}}$ (pg/mL.hr)	2,661 (2,147-3,298)	6,228 (5,165-7,511)	2.34 (1.78-3.08)*	4,118 (3,136-5,408)	1.55 (1.13-2.12)*	10,930 (8,714-13,708)	4.11 (3.01-5.61)*,¶
$AUC_{0\text{-inf}}$ (pg/mL.hr)	3,111 (2,577-3,757)	6,902 (5,849-8,146)	2.22 (1.74-2.83)*	4,557 (3,554-5,844)	1.46 (1.09-1.97)*	13,242 (10,480-16,732)	4.26 (3.18-5.70)*,¶
C_{max} (pg/mL)	375 (302-467)	699 (593-824)	1.86 (1.43-2.43)*	477 (378-601)	1.27 (0.94-1.73)	640 (506-808)	1.70 (1.26-2.30)*
T_{max} (hr)[a]	1.5 (1.0-1.5)	2.0 (2.0-2.5)*	-	1.5 (1.0-1.5)	-	1.5 (1.0-2.0)	-
$T_{1/2}$ (hr)	6.0 (5.0-7.2)	7.6 (6.4-8.9)	1.26 (0.99-1.60)	7.0 (6.1-8.0)	1.15 (0.93-1.44)	17.6 (15.0-20.7)	2.93 (2.35-3.64)*,¶
Kel (hr^{-1})	0.115 (0.096-0.138)	0.090 (0.077-0.105)	0.78 (0.62-0.99)*	0.100 (0.087-0.114)	0.87 (0.69-1.08)	0.039 (0.034-0.046)	0.34 (0.27-0.42) *,¶
CL_R, (mL/min)	78 (70-88)	59 (53-66)	0.76 (0.65-0.88)*	48 (39-59)	0.61 (0.48-0.78)*	15 (12-18)	0.19 (0.15-0.24)*,¶

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

Pitavastatin (Figure 25 and Table 57)

AUC and C_{max} were significantly increased in Group 3 with GMR~1.6 and GMR~1.5 compared to Group 1, respectively. And these differences were also found when compared to Group 2 (GMR~1.6 in AUC and GMR~1.4 in C_{max}). Hence, renal dysfunction could be a factor related to increase a pitavastatin plasma concentration.

There was no significant difference in pharmacokinetic parameters between Group 2 and Group 1. Thus, advanced age was possibly not associated with pitavastatin pharmacokinetic alteration in this study.

The coadministration of rifampicin acting as OATP1B1 and OATP1B3 inhibitors in Group 1 led to significantly increased in AUC (GMR~4.5), C_{max} (GMR~5.4), Kel (GMR~1.7), and a reduction in half-life (GMR~0.6).

Pitavastatin lactone (Figure 26 and Table 57)

Similar to pitavastatin, only Group 3 had a significant increase in AUC (GMR~1.8-1.9) and C_{max} (GMR~1.4) compared to Group 1. And these changes also held through when Group 2 was a reference group.

In period 2 of the study, pitavastatin lactone which is not a substrate for OATP showed that there was no difference in pharmacokinetic parameters of pitavastatin lactone except a slight increase in C_{max} with GMR~1.3.

There was no significant different in $AUC_{0\text{-}last}$ ratio of Pitavastatin lactone/Pitavastatin ratio among three groups. This $AUC_{0\text{-}last}$ ratio was significantly lower in Group 1 with rifampicin (GMR~0.4) versus without rifampicin which could be from OATP1B1/1B3 inhibitory effect of rifampicin resulting in increased a pitavastatin in systemic circulation and a reduction of the conversion of pitavastatin into pitavastatin lactone in hepatocyte.

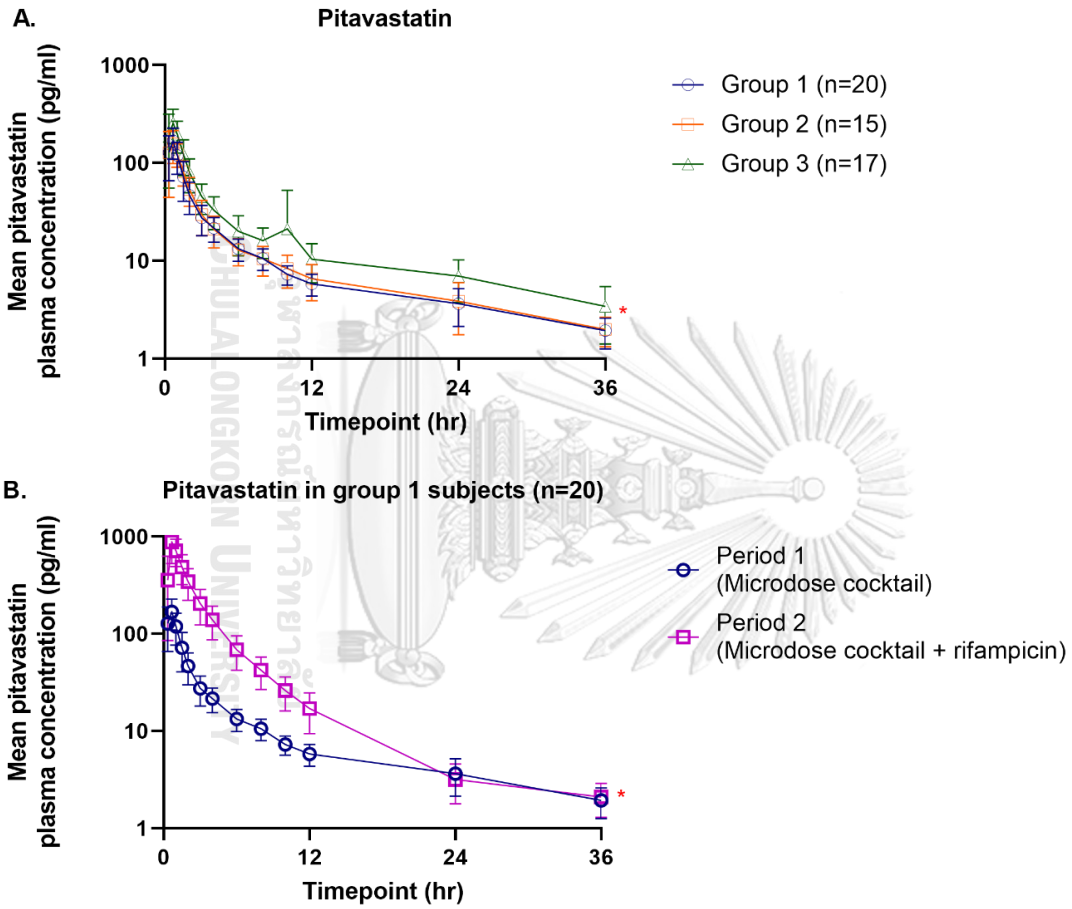


Figure 25 The plasma concentration time-curve of pitavastatin
(A.) pitavastatin 10 µg in three groups of subjects and (B.) in period 1 and period 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group.

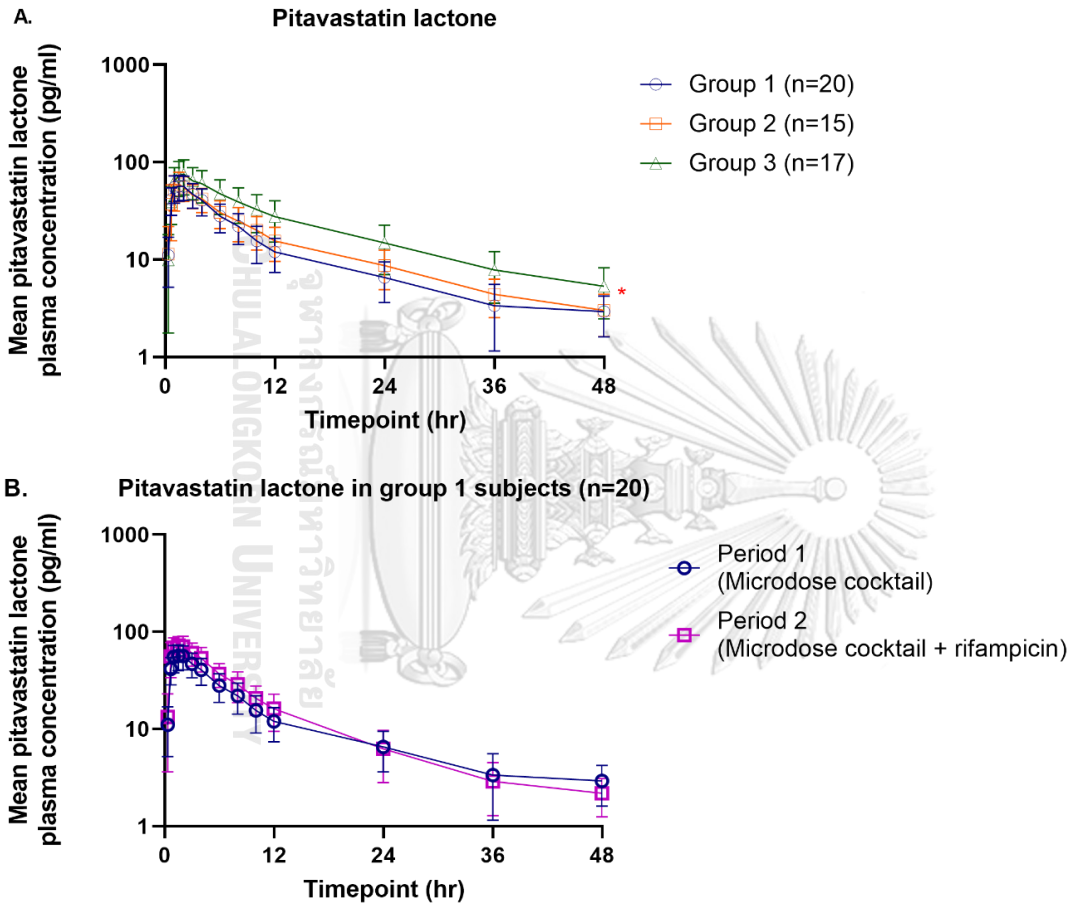


Figure 26 The plasma concentration time-curve of pitavastatin lactone
(A.) pitavastatin lactone in three groups of subjects and (B.) in period 1 and period 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group

Table 57 Pharmacokinetic parameters of pitavastatin, pitavastatin lactone, and pitavastatin lactone/pitavastatin ratio in three groups of subjects

Drugs	Group 1	Group 1 with rifampicin		Group 2		Group 3	
	GM (95% CI)	GM (95% CI)	GMR (95% CI)	GM (95% CI)	GM (95% CI)	GM (95% CI)	GMR (95% CI)
Pitavastatin							
$AUC_{0\text{-last}}$ (pg/mL.hr)	429 (375-491)	1,956 (1,656-2,312)	4.56 (3.71-5.61)*	446 (375-530)	1.04 (0.82-1.31)	715 (578-885)	1.67 (1.33-2.09)*,¶
$AUC_{0\text{-inf}}$ (pg/mL.hr)	468 (405-540)	1,980 (1,677-2,339)	4.24 (3.42-5.24)*	494 (417-585)	1.06 (0.83-1.34)	774 (624-959)	1.66 (1.32-2.08)*,¶
C_{max} (pg/mL)	159 (132-191)	857 (729-1,008)	5.40 (4.25-6.86)*	154 (123-192)	0.97 (0.73-1.28)	242 (193-303)	1.53 (1.16-2.00)*,¶
T_{max} (hr)	0.7 (0.7-0.7)	0.7 (0.7-0.7)	-	0.7 (0.7-0.7)	-	0.7 (0.7-0.7)	-
$T_{1/2}$ (hr)	11.8 (9.4-14.9)	7.5 (5.6-9.9)	0.63(0.44-0.90)*	13.6 (12.0-15.5)	1.15 (0.89-1.49)	14.2 (12.1-16.7)	1.20 (0.93-1.54)
Kel (hr^{-1})	0.059 (0.047-0.074)	0.097 (0.073-0.128)	1.65 (1.16-2.34)*	0.051 (0.045-0.058)	0.87 (0.67-1.12)	0.049 (0.042-0.057)	0.83 (0.65-1.07)
Pitavastatin lactone							
$AUC_{0\text{-last}}$ (pg/mL.hr)	524 (440-623)	649 (556-759)	1.24 (0.99-1.56)	604 (494-739)	1.15 (0.89-1.50)	950 (767-1177)	1.81 (1.41-2.34)*,¶
$AUC_{0\text{-inf}}$ (pg/mL.hr)	574 (475-693)	690 (589-809)	1.20 (0.95-1.53)	676 (558-819)	1.18 (0.90-1.54)	1,071 (861-1,332)	1.87 (1.44-2.42)*,¶
C_{max} (pg/mL)	59 (51-68)	76 (67-86)	1.29 (1.08-1.55)*	48 (58-69)	0.98 (0.78-1.22)	80 (66-97)	1.35(1.09-1.68)*,¶
T_{max} (hr)	1.5 (1.0-1.8)	1.5 (1.0-2.0)	-	1.5 (1.5-1.5)	-	1.5 (1.5-2.0)	-
$T_{1/2}$ (hr)	12.4 (9.9-15.6)	12.4 (9.9-15.6)	1.00 (0.73-1.36)	14.3 (12.4-16.5)	1.15 (0.89-1.49)	15.4 (13.1-18.1)	1.24 (0.96-1.59)
Kel (hr^{-1})	0.056 (0.044-0.070)	0.059 (0.048-0.074)	1.06 (0.78-1.44)	0.048 (0.042-0.056)	0.87 (0.67-1.13)	0.045 (0.038-0.053)	0.10 (0.63-1.04)
Pitavastatin lactone/Pitavastatin ratio							
$AUC_{0\text{-last}}$	1.22(1.08-1.38)	0.33 (0.30-0.37)	0.40 (0.34-0.47)*	1.35 (1.22-1.51)	1.13 (0.89-1.43)	1.33 (1.15-1.54)	1.13 (0.90-1.41)

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

Atorvastatin (Figure 27 and Table 58)

$AUC_{0\text{-last}}$ and C_{max} of subjects were increased in Group 2 (GMR~2) and Group 3 (GMR~4) subjects compared to Group 1. These were also observed in Group 3 compared to Group 2. Thus, both advanced age and CKD seemed to alter pharmacokinetics of atorvastatin.

The coadministration with rifampicin, an OATP1B1 and OATP1B3 inhibitor, in period 2 of Group 1 increased $AUC_{0\text{-last}}$ C_{max}, and T_{max} of atorvastatin.

4-Hydroxy atorvastatin (Figure 28 and Table 58)

Similar to atorvastatin, 4-hydroxy atorvastatin's $AUC_{0\text{-last}}$ and C_{max} in Group 3 were increased with GMR~3 and GMR~2, respectively. Group 2 showed significant change only in $AUC_{0\text{-last}}$ (GMR~2).

The comparison of Group 3 versus Group 2 showed significant difference in both $AUC_{0\text{-last}}$ and C_{max}.

4-Hydroxy atorvastatin is an OATP1B1 substrate. Therefore, the effect of rifampicin in Group 1 period 2 was found.

4-Hydroxy atorvastatin/Atorvastatin ratio

$AUC_{0\text{-last}}$ of the ratio was lower in Group 2 (GMR~0.8) and Group 3 (GMR~0.7) compared to Group 1. There was no changes seen on this ratio when rifampicin was added.

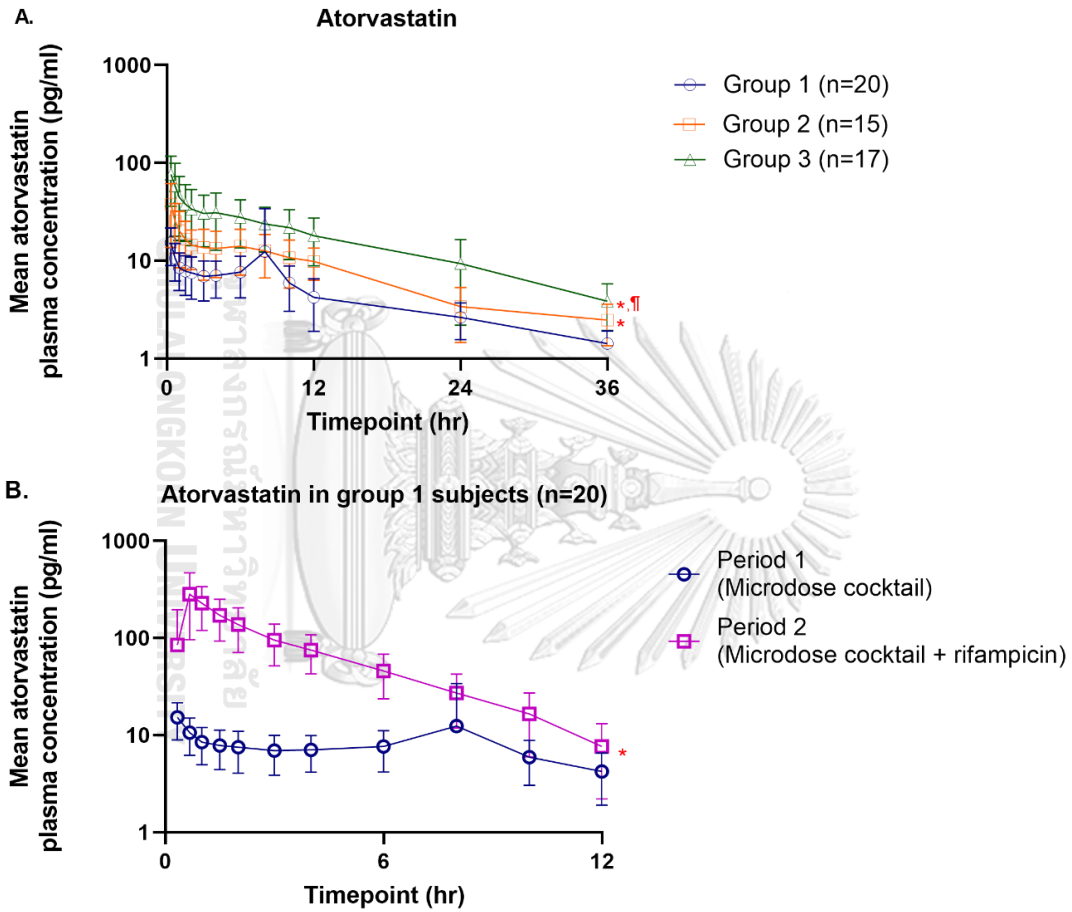


Figure 27 The plasma concentration time-curve of atorvastatin
(A.) atorvastatin 100 µg in three groups of subjects and (B.) in period 1 and period 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

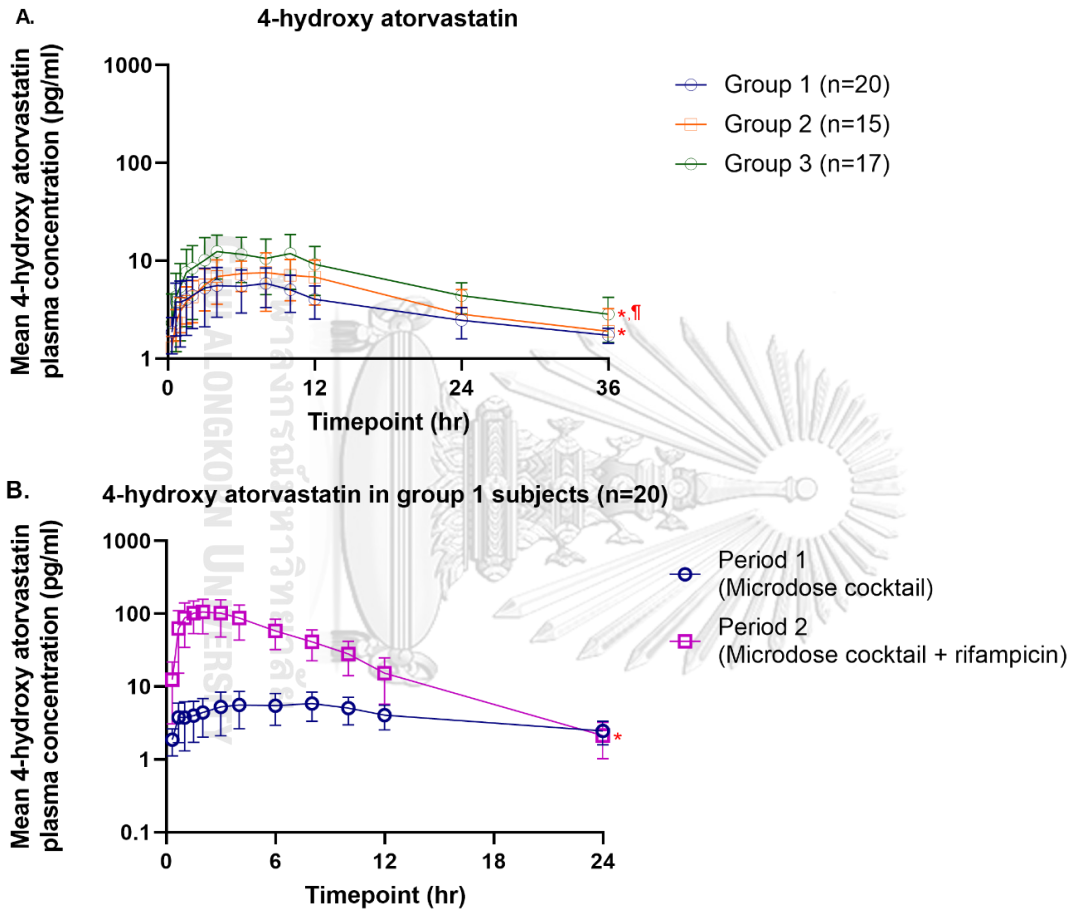


Figure 28 The plasma concentration time-curve of 4-hydroxy atorvastatin
(A.) 4-hydroxy atorvastatin in three groups of subjects and (B.) in period 1 and 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

Table 58 Pharmacokinetic parameters of atorvastatin and 4-hydroxy atorvastatin

Drugs	**Group 1**	**Group 1 with rifampicin**		**Group 2**		**Group 3**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GM (95% CI)**
Atorvastatin							
$AUC_{0\text{-last}}$ (pg/mL.hr)	131 (102-168)	767 (609-965)	5.84 (4.21-8.10)*	281 (228-347)	2.14 (1.52-3.02)*	545 (411-725)	4.15 (2.98-5.79)*,¶
C_{max} (pg/mL)	16 (12-21)	258 (196-339)	16.37 (11.25-23.81)*	35 (27-45)	2.22 (1.49-3.31)*	66 (47-92)	4.18 (2.85-6.14)*,¶
T_{max} (hr)[a]	0.3 (0.3-0.3)	0.7 (0.7-1.0)*	-	0.3 (0.3-0.3)	-	0.3 (0.3-0.3)	-
4-Hydroxy atorvastatin							
$AUC_{0\text{-last}}$ (pg/mL.hr)	94 (74-120)	729 (590-901)	7.75 (5.67-10.58)*	146 (113-187)	1.55 (1.12-2.14)*	243 (194-304)	2.5 (1.88-3.53)*,¶
C_{max} (pg/mL)	7 (6-8)	109 (89-134)	16.08 (12.40-20.86)*	9 (7-11)	1.27 (0.94-1.72)	12 (9-17)	1.84 (1.37-2.47)*,¶
T_{max} (hr)[a]	5.0 (4.0-8.0)	2.0 (1.0-2.5)*	-	8.0 (6.0-10.0)	-	6.0 (4.0-10.0)	-
4-Hydroxy atorvastatin/Atorvastatin ratio							
$AUC_{0\text{-last}}$	0.72 (0.57-0.90)	0.95 (0.84-1.07)	1.20 (0.96-1.50)	0.52 (0.42-0.64)	0.78 (0.64-0.96)*	0.45 (0.37-0.53)	0.72 (0.59-0.87)*

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

Rosuvastatin (Figure 29 and Table 59)

Group 3 had a significant increase of both $AUC_{0\text{-last}}$ and C_{max} compared to Group 1 (GMR~2 for both). Due to the nonsignificant differences results on $AUC_{0\text{-last}}$ and C_{max} of Group 2 versus Group 1 and Group 3 versus Group 2, the effects of either advanced age or CKD on rosuvastatin pharmacokinetic profiles cannot be estimated.

Renal clearance was significantly decreased in Group 2 (GMR~0.7) and Group 3 (GMR~0.2) compared to Group 1. This reduction was also found in between Group 3 vs Group 2 subjects.

Rifampicin, an OATP1B1 and OATP1B3 inhibitor, significantly increased $AUC_{0\text{-last}}$ (GMR~4) and C_{max} (GMR~7), in the same time, decreased T_{max} and CL_R (GMR~0.7) of plasma rosuvastatin.

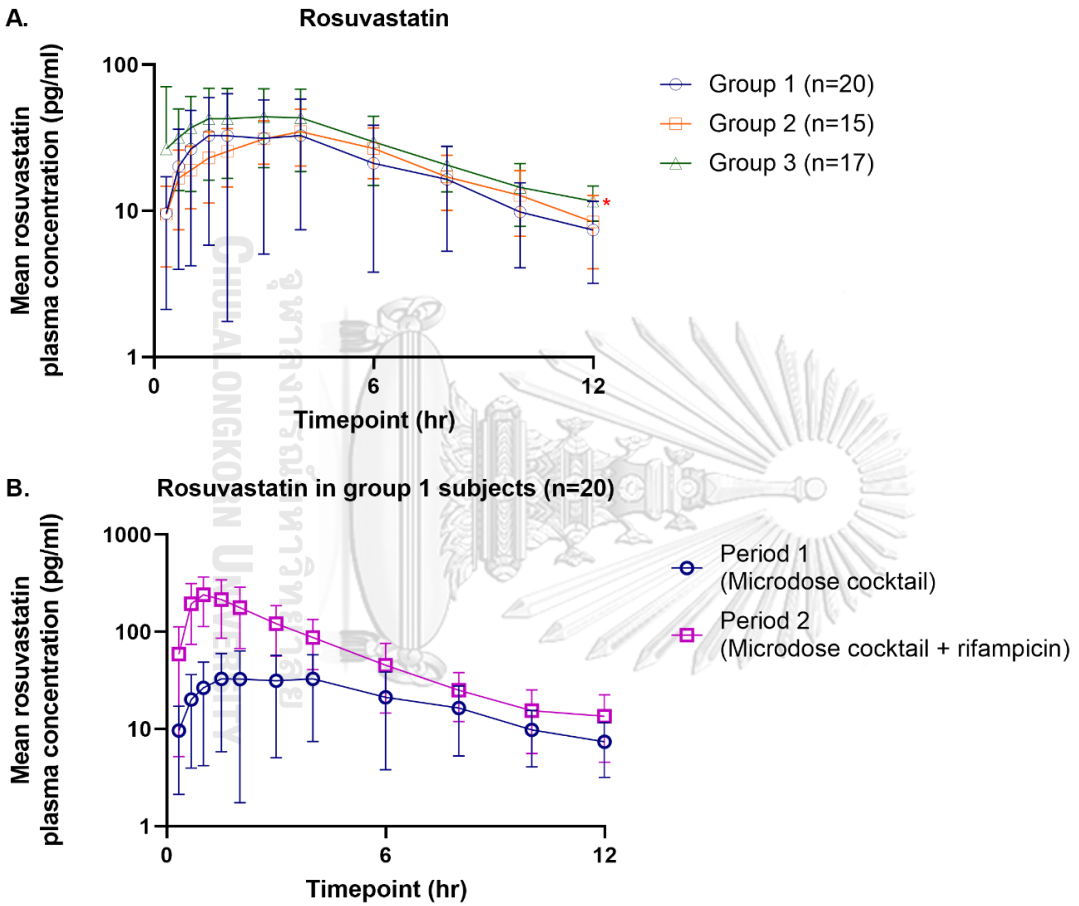


Figure 29 The plasma concentration time-curve of rosuvastatin
(A.) rosuvastatin 50 µg in three groups of subjects and (B.) in period 1 and period 2.
Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.
*p-value<0.05, Group 1 as a reference group.

Table 59 Pharmacokinetic parameters of rosuvastatin

Drugs	**Group 1**	**Group 1 with rifampicin**		**Group 2**		**Group 3**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GM (95% CI)**
Rosuvastatin							
$AUC_{0\text{-last}}$ (pg/mL.hr)	195 (133-288)	851 (690-1,050)	4.35 (2.84-6.67)*	290 (228-369)	1.48 (0.95-2.32)	372 (279-495)	1.90 (1.23-2.93)*
C_{max} (pg/mL)	32 (23-45)	232 (177-305)	7.21 (4.78-10.89)*	37 (31-45)	1.15 (0.78-1.69)	53 (40-70)	1.63 (1.12-2.37)*
T_{max} (hr)[a]	3.5 (1.8-4.0)	1.0 (1.0-1.5)*	-	4.0 (3.0-4.0)	-	2.0 (1.0-3.0)	-
CL_R (mL/min)	223 (192-259)	147 (131-164)	0.66 (0.55-0.79)*	157 (126-195)	0.70 (0.51-0.97)*	42 (30-60)	0.19 (0.14-0.26)*,¶

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group; ¶p-value<0.05, Group 2 as a reference group.

GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

5. Univariate and multivariable analysis.

Midazolam (Table 60 and Table 61)

$AUC_{0\text{-}last}$: After univariate analysis, albumin and AST were adjusted in multivariable analysis, $AUC_{0\text{-}last}$ was 2.19-fold higher (95%CI 1.61-2.98) in Group 2 vs Group 1 subjects and 2.68-fold higher (95%CI 1.95-3.68) in Group 3 vs Group 1. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.5248.

C_{max}: C_{max} was higher in Group 2 (1.83-fold, 95%CI 1.40-2.38) and Group 3 (1.86-fold, 95%CI 1.42-2.44) vs Group 1 after adjusting for AST. Adjusted R^2 of C_{max} multivariable model was 0.3889.

$T_{1/2}$ and Kel: After adjusting for BMI in multivariable model, $T_{1/2}$ of Group 2 and Group 3 were obviously prolonged by about 2.33-fold (95%CI 1.71-3.18) and 2.56 (95%CI 1.84-3.56) and Kel was reduced by 57% in Group 2 (95%CI 0.31-0.59) and 61% in Group 3 (95%CI 0.28-0.54) vs Group 1. Adjusted R^2 of $T_{1/2}$ and Kel multivariable models were both 0.4766.

Table 60 Univariate and multivariable regression models for geometric mean ratio of midazolam $AUC_{0\text{-last}}$ and C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.5248				
Group of subjects		<0.001*		
Group 2 vs Group 1	2.30 (1.70-3.09)	<0.001*	2.19 (1.61-2.98)	<0.001*
Group 3 vs Group 1	2.90 (2.16-3.88)	<0.001*	2.68 (1.95-3.68)	<0.001*
Group 3 vs Group 2	1.26 (0.93-1.72)	0.14	1.22 (0.89-1.67)	0.20
BMI (kg/m^2)	1.04 (0.98-1.11)	0.16		
Albumin (g/dL)	0.50 (0.22-1.14)	0.097*	0.88 (0.49-1.59)	0.67
Total bilirubin (mg/dL)	1.01 (0.68-1.53)	0.94		
Direct bilirubin (mg/dL)	1.14 (0.23-5.71)	0.87		
AST (U/L)	1.03 (1.01-1.05)	0.011*	1.01 (0.99-1.03)	0.21
ALT (U/L)	1.01 (1.00-1.03)	0.14		
C_{max} (pg/mL), Adjusted R^2 0.3889				
Group of subjects		<0.001*		
Group 2 vs Group 1	1.88 (1.44-2.44)	<0.001*	1.83 (1.40-2.38)	<0.001*
Group 3 vs Group 1	1.95 (1.51-2.52)	<0.001*	1.86 (1.42-2.44)	<0.001*
Group 3 vs Group 2	1.04 (0.79-1.36)	0.78	1.02 (0.77-1.34)	0.90
BMI (kg/m^2)	1.01 (0.96-1.06)	0.79		
Albumin (g/dL)	0.63 (0.33-1.18)	0.14		
Total bilirubin (mg/dL)	0.96 (0.70-1.32)	0.82		
Direct bilirubin (mg/dL)	1.06 (0.30-3.69)	0.93		
AST (U/L)	1.02 (1.00-1.03)	0.026*	1.01 (0.99-1.02)	0.27
ALT (U/L)	1.01 (1.00-1.02)	0.19		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

[a]Significant level, p-value <0.1, [b] Significant level, p-value <0.05

[*]p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 61 Univariate and multivariable regression models for geometric mean ratio of midazolam $T_{1/2}$ and Kel

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$T_{1/2}$ (hr), Adjusted R^2 0.4766				
Group of subjects		<0.001*		
Group 2 vs Group 1	2.31 (1.72-3.10)	<0.001*	2.33 (1.71-3.18)	<0.001*
Group 3 vs Group 1	2.52 (1.89-3.36)	<0.001*	2.56 (1.84-0.56)	<0.001*
Group 3 vs Group 2	1.09 (0.80-1.48)	0.57	1.10 (0.80-1.50)	0.55
BMI (kg/m^2)	1.07 (1.01-1.13)	0.026*	1.00 (0.95-1.04)	0.85
Albumin (g/dL)	0.53 (0.25-1.16)	0.11		
Total bilirubin (mg/dL)	1.00 (0.68-1.47)	0.99		
Direct bilirubin (mg/dL)	0.73 (0.16-3.34)	0.68		
AST (U/L)	1.02 (1.00-1.04)	0.11		
ALT (U/L)	1.01 (0.99-1.02)	0.21		
Kel (hr^{-1}), Adjusted R^2 0.4766				
Group of subjects		<0.001*		
Group 2 vs Group 1	0.43 (0.32-0.58)	<0.001*	0.43 (0.31-0.59)	<0.001*
Group 3 vs Group 1	0.40 (0.30-0.53)	<0.001*	0.39 (0.28-0.54)	<0.001*
Group 3 vs Group 2	0.92 (0.68-1.24)	0.57	0.91 (0.67-1.25)	0.55
BMI (kg/m^2)	0.94 (0.89-0.99)	0.026*	1.00 (0.96-1.05)	0.85
Albumin (g/dL)	1.87 (0.86-4.04)	0.11		
Total bilirubin (mg/dL)	1.00 (0.68-1.47)	0.99		
Direct bilirubin (mg/dL)	1.37 (0.30-6.32)	0.68		
AST (U/L)	0.98 (0.96-1.00)	0.11		
ALT (U/L)	0.99 (0.98-1.01)	0.21		

GMR, geometric mean ratio; CI, confidence interval; $T_{1/2}$, half-life; Kel, elimination rate constant; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

[a]Significant level, p-value <0.1, [b] Significant level, p-value <0.05

[*]p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Dabigatran (Table 62 and Table 63)

$AUC_{0\text{-}last}$: After adjusting for BMI, AST, and ALT in multivariable analysis, $AUC_{0\text{-}last}$ was 1.65-fold higher (95% CI 1.15-2.35) in Group 2 and 4.43-fold higher (95%CI 3.01-6.52) in Group 3 vs Group 1. Moreover, Group 3 had $AUC_{0\text{-}last}$ 2.69-fold (95%CI 1.93-3.75) higher than Group 2. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.6263.

C_{max}: Although Cmax of Group 2 seemed to be 23% higher than Group 1, there was no statistically significant difference. C_{max} was 61% increase in Group 3 vs Group 1 after adjusting for AST. Adjusted R^2 of C_{max} multivariable model was 0.1751.

$T_{1/2}$ and Kel: When BMI was adjusted, half-life was prolonged by 3.18-fold (95%CI 2.49-4.06) and Kel GM was reduced by 69% (95%CI 0.25-0.40) in Group 3 vs Group 1. The statistical difference between Group 3 vs Group 2 was also indicated, with 2.61-fold (95%CI 2.07-3.30) half-life prolongation and 62% (0.30-0.48) Kel reduction. Adjusted R^2 of $T_{1/2}$ and Kel multivariable models were both 0.6831.

Table 62 Univariate and multivariable regression models for geometric mean ratio of dabigatran $AUC_{0\text{-last}}$ and C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.6263				
Group of subjects		<0.001*		
Group 2 vs Group 1	1.55 (1.13-2.12)	0.008*	1.65 (1.15-2.35)	0.007*
Group 3 vs Group 1	4.11 (3.01-5.61)	<0.001*	4.43 (3.01-6.52)	<0.001*
Group 3 vs Group 2	2.65 (1.91-3.69)	<0.001*	2.69 (1.93-3.75)	<0.001*
BMI (kg/m^2)	1.07 (0.99-1.14)	0.070*	0.96 (0.91-1.01)	0.13
Albumin (g/dL)	0.68 (0.26-1.82)	0.44		
Total bilirubin (mg/dL)	0.88 (0.54-1.42)	0.59		
Direct bilirubin (mg/dL)	0.73 (0.11-4.88)	0.74		
AST (U/L)	1.03 (1.01-1.06)	0.009*	1.00 (0.97-1.04)	0.81
ALT (U/L)	1.02 (1.00-1.03)	0.071*	1.01 (0.98-1.03)	0.61
C_{max} (pg/mL), Adjusted R^2 0.1751				
Group of subjects		0.0035*		
Group 2 vs Group 1	1.27 (0.94-1.73)	0.12	1.23 (0.90-1.68)	0.19
Group 3 vs Group 1	1.70 (1.26-2.30)	0.001*	1.61 (1.17-2.21)	0.004*
Group 3 vs Group 2	1.34 (0.98-1.84)	0.07	1.31 (0.95-1.80)	0.10
BMI (kg/m^2)	1.01 (0.96-1.06)	0.75		
Albumin (g/dL)	0.81 (0.43-1.55)	0.52		
Total bilirubin (mg/dL)	0.82 (0.60-1.12)	0.22		
Direct bilirubin (mg/dL)	0.65 (0.19-2.25)	0.49		
AST (U/L)	1.02 (1.00-1.03)	0.045*	1.01 (0.99-1.02)	0.27
ALT (U/L)	1.01 (1.00-1.02)	0.16		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase

[a]Significant level, p-value <0.1, [b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 63 Univariate and multivariable regression models for geometric mean ratio of dabigatran $T_{1/2}$ and Kel

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$T_{1/2}$ (hr), Adjusted R^2 0.6831				
Group of subjects		<0.001*		
Group 2 vs Group 1	1.15 (0.93-1.44)	0.20	1.22 (0.97-1.53)	0.09
Group 3 vs Group 1	2.93 (2.35-3.64)	<0.001*	3.18 (2.49-4.06)	<0.001*
Group 3 vs Group 2	2.54 (2.01-3.19)	<0.001*	2.61 (2.07-3.30)	<0.001*
BMI (kg/m^2)	1.05 (0.99-1.11)	0.087*	0.98 (0.94-1.01)	0.15
Albumin (g/dL)	0.67 (0.32-1.42)	0.29		
Total bilirubin (mg/dL)	0.98 (0.68-1.41)	0.89		
Direct bilirubin (mg/dL)	0.90 (0.21-3.86)	0.89		
AST (U/L)	1.01 (0.99-1.03)	0.15		
ALT (U/L)	1.01 (0.99-1.02)	0.25		
Kel (hr^{-1}), Adjusted R^2 0.6831				
Group of subjects		<0.001*		
Group 2 vs Group 1	0.87 (0.69-1.08)	0.20	0.82 (0.65-1.04)	0.09
Group 3 vs Group 1	0.34 (0.27-0.42)	<0.001*	0.31 (0.25-0.40)	<0.001*
Group 3 vs Group 2	0.39 (0.31-0.50)	<0.001*	0.38 (0.30-0.48)	<0.001*
BMI (kg/m^2)	0.95 (0.90-1.01)	0.087*	1.03 (0.99-1.06)	0.15
Albumin (g/dL)	1.49 (0.71-3.12)	0.29		
Total bilirubin (mg/dL)	1.02 (0.71-1.48)	0.89		
Direct bilirubin (mg/dL)	1.11 (0.26-4.72)	0.89		
AST (U/L)	0.99 (0.97-1.01)	0.15		
ALT (U/L)	0.99 (0.98-1.01)	0.25		

GMR, geometric mean ratio; CI, confidence interval; $T_{1/2}$, half-life; Kel, elimination rate constant; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

[a]Significant level, p-value <0.1, [b] Significant level, p-value <0.05.

[*]p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Pitavastatin (Table 64 to Table 67)

$AUC_{0\text{-}last}$: Group 3 had 57% (95%CI 1.26-1.97) higher and 59% (95%CI 1.26-2.01) increased in $AUC_{0\text{-}last}$ than Group 1 and Groups 2, respectively, after adjusting for albumin, respectively. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.3440.

C_{max}: After adjusting for BMI and albumin in multivariable analysis, C_{max} GM was 90% (95% 1.42-2.54) higher in Group 3 vs Group 1 and 70% (95%CI, 1.31-2.20) higher in Group 3 vs Group 2. Moreover, C_{max} was about 7% decreased for an increase in BMI. Adjusted R^2 of C_{max} multivariable model was 0.3817.

$T_{1/2}$ and Kel: Both half-life and elimination rate constant did not show any significant association with variable risk factors.

Table 64 Univariate and multivariable regression models for geometric mean ratio of pitavastatin $AUC_{0\text{-last}}$.

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.3440				
Group of subjects		0.0001*		
Group 2 vs Group 1	1.04 (0.82-1.31)	0.75	0.99 (0.79-1.25)	0.93
Group 3 vs Group 1	1.67 (1.33-2.09)	<0.001*	1.57 (1.26-1.97)	<0.001*
Group 3 vs Group 2	1.60 (1.26-2.04)	<0.001*	1.59 (1.26-2.01)	<0.001*
BMI (kg/m^2)	0.99 (0.95-1.03)	0.50		
Albumin (g/dL)	0.54 (0.32-0.89)	0.017*	0.64 (0.41-.1.00)	0.05
Total bilirubin (mg/dL)	0.84 (0.65-1.09	0.18		
Direct bilirubin (mg/dL)	0.91 (0.32-2.55)	0.85		
AST (U/L)	1.01 (0.99-11.02)	0.23		
ALT (U/L)	1.00 (0.99-1.01)	0.99		
SLCO1B1 diplotype		0.34		
ABCG2 gene (rs2231142)		0.53		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 65 Univariate and multivariable regression model for geometric mean ratio of pitavastatin C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	*p*-value	GMR (95%CI)	*p*-value
C_{max} (pg/mL), Adjusted R^2 0.3817				
Group of subjects		0.003*		
Group 2 vs Group 1	0.97 (0.73-1.28)	0.82	1.12 (0.85-1.47)	0.41
Group 3 vs Group 1	1.53 (1.16-2.00)	0.003*	1.90 (1.42-2.54)	<0.001*
Group 3 vs Group 2	1.58 (1.18-2.11)	0.003*	1.70 (1.31-2.20)	<0.001*
BMI (kg/m^2)	0.96 (0.92-1.00)	0.078*	0.93 (0.89-0.97)	0.001*
Albumin (g/dL)	0.53 (0.30-0.94)	0.030*	0.81 (0.49-1.35)	0.42
Total bilirubin (mg/dL)	0.85 (0.64-1.13)	0.25		
Direct bilirubin (mg/dL)	0.99 (0.32-3.12)	0.99		
AST (U/L)	1.01 (0.99-1.02)	0.46		
ALT (U/L)	1.00 (0.99-1.01)	0.81		
SLCO1B1 diplotype		0.34		
ABCG2 gene (rs2231142)		0.53		

GMR, geometric mean ratio; CI, confidence interval; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 66 Univariate and multivariable regression model for geometric mean ratio of pitavastatin half-life

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$T_{1/2}$ (hr), Adjusted R^2 -				
Group of subjects		0.31		
Group 2 vs Group 1	1.15 (0.89-1.49)	0.28		
Group 3 vs Group 1	1.20 (0.93-1.54)	0.15		
Group 3 vs Group 2	1.04 (0.80-1.36)	0.76		
BMI (kg/m^2)	0.99 (0.96-1.03)	0.66		
Albumin (g/dL)	0.82 (0.50-1.35)	0.43		
Total bilirubin (mg/dL)	0.92 (0.73-1.77)	0.50		
Direct bilirubin (mg/dL)	0.72 (0.28-1.86)	0.50		
AST (U/L)	0.99 (0.98-1.00)	0.15		
ALT (U/L)	0.99 (0.99-1.0)	0.18		
SLCO1B1 diplotype		0.34		
ABCG2 gene (rs2231142)		0.33		

GMR, geometric mean ratio; CI, confidence interval; $T_{1/2}$, half-life; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

[*]p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 67 Univariate and multivariable regression model for geometric mean ratio of pitavastatin elimination rate constant

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
Kel (hr^{-1}), Adjusted R^2 -				
Group of subjects		0.31		
Group 2 vs Group 1	0.87 (0.67-1.12)	0.28		
Group 3 vs Group 1	0.83 (0.65-1.07)	0.15		
Group 3 vs Group 2	0.96 (0.74-1.25)	0.76		
BMI (kg/m^2)	1.01 (0.97-1.04)	0.66		
Albumin (g/dL)	1.21 (0.74-1.99)	0.43		
Total bilirubin (mg/dL)	1.09 (0.85-1.38)	0.50		
Direct bilirubin (mg/dL)	1.38 (0.54-3.55)	0.50		
AST (U/L)	1.01 (1.00-1.02)	0.15		
ALT (U/L)	1.01 (1.00-1.01)	0.18		
SLCO1B1 diplotype		0.34		
ABCG2 gene (rs2231142)		0.33		

GMR, geometric mean ratio; CI, confidence interval; Kel,elimination rate constant; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2.

[a]Significant level, p-value <0.1.

[b] Significant level, p-value <0.05.

Pitavastatin lactone (Table 68 to Table 71)

$AUC_{0\text{-}last}$: In multivariable analysis, $AUC_{0\text{-}last}$ was 80% (95%CI 1.40-2.31) higher in Group 3 vs Group 1 and 57% (95%CI 1.21-2.04) higher in Group 3 vs Group 2 after adjusting for albumin and total bilirubin. There was a minimal $AUC_{0\text{-}last}$ reduction by 26% (95%CI 0.59-0.97) for an increase total bilirubin. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.3669.

C_{max}: C_{max} was 36% (95%CI 1.10-1.68) higher in Group 3 vs Group 1 and 40% (95%CI 1.13-1.75) higher in Group 3 vs Group 2 after adjusting for albumin, total bilirubin, and direct bilirubin. Adjusted R^2 of C_{max} multivariable model was 0.3817.

$T_{1/2}$ and Kel: Both half-life and elimination rate constant did not have any significant association with variable risk factors.

Table 68 Univariate and multivariable regression models for geometric mean ratio of pitavastatin lactone $AUC_{0\text{-last}}$

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.3669				
Group of subjects		0.0001*		
Group 2 vs Group 1	1.15 (0.89-1.50)	0.28	1.14 (0.88-1.48)	0.31
Group 3 vs Group 1	1.81 (1.41-2.34)	<0.001*	1.80 (1.40-2.31)	<0.001*
Group 3 vs Group 2	1.57 (1.19-2.07)	0.002*	1.57 (1.21-2.04)	0.001*
BMI (kg/m^2)	0.99 (0.94-1.03)	0.51		
Albumin (g/dL)	0.53 (0.30-0.94)	0.030*	0.84 (0.49-1.45)	0.53
Total bilirubin (mg/dL)	0.75 (0.57-0.99)	0.045*	0.76 (0.59-0.97)	0.031*
Direct bilirubin (mg/dL)	0.48 (0.16-1.51)	0.21		
AST (U/L)	1.00 (0.99-1.02)	0.68		
ALT (U/L)	1.00 (0.99-1.01)	0.52		
SLCO1B1 diplotype		0.46		
ABCG2 gene (rs2231142)		0.74		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 69 Univariate and multivariable regression models for geometric mean ratio of pitavastatin lactone C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
C_{max} (pg/mL), Adjusted R^2 0.3817				
Group of subjects		0.009*		
Group 2 vs Group 1	0.98 (0.78-1.22)	0.83	0.97 (0.78-1.20)	0.77
Group 3 vs Group 1	1.35 (1.09-1.36)	0.008*	1.36 (1.10-1.68)	0.005*
Group 3 vs Group 2	1.39 (1.10-1.75)	0.007*	1.40 (1.13-1.75)	0.003*
BMI (kg/m^2)	0.97 (0.94-1.01)	0.12		
Albumin (g/dL)	0.67 (0.42-1.05)	0.078*	0.98 (0.62-1.55)	0.92
Total bilirubin (mg/dL)	0.75 (0.60-0.92)	0.008*	0.62 (0.40-0.94)	0.027*
Direct bilirubin (mg/dL)	0.45 (0.19-1.06)	0.068*	2.28 (0.46-11.19)	0.30
AST (U/L)	1.00 (0.99-1.02)	0.53		
ALT (U/L)	1.00 (0.99-1.01)	0.90		
SLCO1B1 diplotype		0.87		
ABCG2 gene (rs2231142)		0.51		

GMR, geometric mean ratio; CI, confidence interval; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 70 Univariate and multivariable regression models for geometric mean ratio of pitavastatin lactone half-life

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$T_{1/2}$ (hr), Adjusted R^2 -				
Group of subjects		0.22		
Group 2 vs Group 1	1.15 (0.89-1.49)	0.28		
Group 3 vs Group 1	1.24 (0.96-1.59)	0.09		
Group 3 vs Group 2	1.08 (0.82-1.41)	0.59		
BMI (kg/m^2)	1.01 (0.97-1.05)	0.61		
Albumin (g/dL)	0.76 (0.46-1.26)	0.28		
Total bilirubin (mg/dL)	1.05 (0.82-1.34)	0.69		
Direct bilirubin (mg/dL)	0.90 (0.34-2.35)	0.82		
AST (U/L)	1.00 (0.98-1.01)	0.57		
ALT (U/L)	1.00 (0.99-1.01)	0.48		
SLCO1B1 diplotype		0.08		
ABCG2 gene (rs2231142)		0.58		

GMR, geometric mean ratio; CI, confidence interval; $T_{1/2}$, half-life; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

Table 71 Univariate and multivariable regression models for geometric mean ratio of pitavastatin lactone elimination rate constant

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
Kel (hr^{-1}), Adjusted R^2 -				
Group of subjects		0.22		
Group 2 vs Group 1	0.87 (0.67-1.13)	0.28		
Group 3 vs Group 1	0.81 (0.63-1.04)	0.09		
Group 3 vs Group 2	0.93 (0.71-1.22)	0.59		
BMI (kg/m^2)	0.99 (0.96-1.03)	0.61		
Albumin (g/dL)	1.31 (0.80-2.15)	0.28		
Total bilirubin (mg/dL)	0.95 (0.75-1.22)	0.69		
Direct bilirubin (mg/dL)	1.12 (0.43-2.92)	0.82		
AST (U/L)	1.00 (0.99-1.02)	0.57		
ALT (U/L)	1.00 (0.99-1.01)	0.48		
SLCO1B1 diplotype		0.08		
ABCG2 gene (rs2231142)		0.58		

GMR, geometric mean ratio; CI, confidence interval; Kel, elimination rate constant; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

Atorvastatin (Table 72 and Table 73)

$AUC_{0\text{-}last}$: After adjusting for BMI, AST, and ALT in multivariable model, $AUC_{0\text{-}last}$ of Group 2 subjects was higher by 2.19-fold (95%CI 1.48-3.22) compared to Group 1. And Group 3 subjects had dramatically higher $AUC_{0\text{-}last}$ by 4.20-fold (95%CI 2.78-6.35) and 1.92-fold (1.34-2.76) than Group 1 and Group 2. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.5972.

C_{max}: The magnitude of changes in C_{max} was comparable to $AUC_{0\text{-}last}$. After adjusting for BMI, albumin, and AST in multivariable analysis, C_{max} was 2.28-fold (95%CI 1.56-3.33) higher in Group 2 vs Group 1 and 4.42-fold (95%CI 2.93-6.65) higher in Group 3 vs Group 1. And C_{max} in Group 3 was also 1.94-fold (95%CI 1.35-2.78) higher than Group 2. Adjusted R^2 of C_{max} multivariable model was 0.6002.

Table 72 Univariate and multivariable regression models for geometric mean ratio of atorvastatin $AUC_{0\text{-last}}$

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.5972				
Group of subjects		<0.001*		
Group 2 vs Group 1	2.14 (1.52-3.02)	<0.001*	2.19 (1.48-3.22)	<0.001*
Group 3 vs Group 1	4.15 (2.98-5.79)	<0.001*	4.20 (2.78-6.35)	<0.001*
Group 3 vs Group 2	1.94 (1.36-2.77)	<0.001*	1.92 (1.34-2.76)	0.001*
BMI (kg/m^2)	1.08 (1.00-1.16)	0.044*	0.97 (0.91-1.03)	0.28
Albumin (g/dL)	0.50 (0.18-1.37)	0.17		
Total bilirubin (mg/dL)	0.99 (0.60-1.63)	0.96		
Direct bilirubin (mg/dL)	1.93 ().27-13.69)	0.50		
AST (U/L)	1.04 (1.01-1.06)	0.005*	1.02 (0.98-1.06)	0.40
ALT (U/L)	1.02 (1.00-1.03)	0.091*	1.00 (0.97-1.03)	0.92
SLCO1B1 diplotype		0.55		
ABCG2 gene (rs2231142)		0.12		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 73 Univariate and multivariable regression models for geometric mean ratio of atorvastatin C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
C_{max} (pg/mL), Adjusted R^2 0.6002				
Group of subjects		<0.001*		
Group 2 vs Group 1	2.22 (1.49-3.31)	<0.001*	2.28 (1.56-3.33)	<0.001*
Group 3 vs Group 1	4.18 (2.85-6.14)	<0.001*	4.42 (2.93-6.65)	<0.001*
Group 3 vs Group 2	1.88 (1.25-2.84)	0.003*	1.94 (1.35-2.78)	<0.001*
BMI (kg/m^2)	1.07 (0.99-1.15)	0.092*	0.96 (0.91-1.02)	0.17
Albumin (g/dL)	0.40 (0.14-1.17)	0.092*	1.23 (0.61-2.51)	0.55
Total bilirubin (mg/dL)	1.02 (0.60-1.74)	0.94		
Direct bilirubin (mg/dL)	2.19 (0.27-17.73)	0.46		
AST (U/L)	1.03 (1.00-1.06)	0.051*	1.01 (1.00-1.03)	0.10
ALT (U/L)	1.01 (0.99-1.03)	0.29		
SLCO1B1 diplotype		0.63		
ABCG2 gene (rs2231142)		0.17		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-}last}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

4-hydroxy Atorvastatin (Table 74 and Table 75)

$AUC_{0\text{-last}}$: After adjusting for AST, Group 2 and Group 3 had higher $AUC_{0\text{-last}}$ by 1.54-fold (95%CI 1.10-2.14) and 2.55-fold (95%CI 1.82-3.56) vs Group 1. And Group 3 had 1.66-fold (95%CI 1.17-2.34) higher than Group 2. Adjusted R^2 of $AUC_{0\text{-last}}$ multivariable model was 0.3931.

C_{max}: After adjusting for *SLCO1B1* diplotype, C_{max} GM was 71% higher in Group 3 vs Group 1. There was only one subject with *15/*17 diplotype (subject number 36 in Group 3) showed slightly significance of 2.49-fold higher in C_{max} GM vs subjects with wild type. Adjusted R^2 of C_{max} multivariable model was 0.2840.

Table 74 Univariate and multivariable regression models for geometric mean ratio of 4-hydroxy atorvastatin $AUC_{0\text{-last}}$

	Univariate		Multivariable	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.3931				
Group of subjects		<0.001*		
Group 2 vs Group 1	1.55 (1.12-2.14)	0.010*	1.54 (1.10-2.14)	0.013*
Group 3 vs Group 1	2.58 (1.88-3.53)	<0.001*	2.55 (1.82-3.56)	<0.001*
Group 3 vs Group 2	1.67 (1.19-2.34)	0.004*	1.66 (1.17-2.34)	0.005*
BMI (kg/m^2)	1.04 (0.99-1.11)	0.14		
Albumin (g/dL)	0.72 (0.32-1.61)	0.42		
Total bilirubin (mg/dL)	1.05 (0.71-1.55)	0.82		
Direct bilirubin (mg/dL)	1.30 (0.28-6.11)	0.74		
AST (U/L)	1.02 (1.00-1.04)	0.097*	1.00 (0.99-1.02)	0.82
ALT (U/L)	1.01 (0.99-1.02)	0.32		
SLCO1B1 diplotype		0.23		
ABCG2 gene (rs2231142)		0.25		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 75 Univariate and multivariable regression models for geometric mean ratio of 4-hydroxy atorvastatin C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
C_{max} (pg/mL), Adjusted R^2 0.2840				
Group of subjects		0.001*		
Group 2 vs Group 1	1.27 (0.94-1.73)	0.12	1.32 (0.97-1.79)	0.08
Group 3 vs Group 1	1.84 (1.37-2.47)	<0.001*	1.71 (1.26-2.33)	0.001*
Group 3 vs Group 2	1.44 (1.05-1.98)	0.024*	1.30 (0.94-1.79)	0.10
BMI (kg/m^2)	1.01 (0.96-1.06)	0.71		
Albumin (g/dL)	0.71 (0.37-1.38)	0.30		
Total bilirubin (mg/dL)	1.02 (0.74-1.41)	0.90		
Direct bilirubin (mg/dL)	1.73 (0.49-6.15)	0.39		
AST (U/L)	1.01 (0.99-1.03)	0.27		
ALT (U/L)	1.00 (0.99-1.01)	0.70		
SLCO1B1 diplotype		0.084*		
wild type	1.00 (reference)			
*15/*15	1.27 (0.63-2.54)		1.66 (0.87-3.18)	0.12
*15/*17	3.25 (1.23-8.58)		2.49 (1.02-6.08)	0.046*
*1A/*17	0.81 (0.41-1.63)		0.93 (0.49-1.76)	0.82
*1A/*21	1.02 (0.61-1.69)		1.02 (0.64-1.61)	0.94
*1B/*17	0.83 (0.50-1.38)		0.89 (0.56-1.41)	0.61
*1B/*21	2.84 (1.07-7.50)		2.17 (0.89-5.51)	0.09
*5/*15	0.58 (0.22-1.52)		0.75 (0.31-1.84)	0.53
ABCG2 gene (rs2231142)		0.89		

GMR, geometric mean ratio; CI, confidence interval; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Rosuvastatin (Table 76 and Table 77)

$AUC_{0\text{-}last}$: $AUC_{0\text{-}last}$ GM was 83% (95%CI 1.25-2.69) higher in Group 3 vs Group 1 subjects after adjusting for rs2231142 *ABCG2* (c.421C>A) genotype. Moreover, Subjects who carried CA and AA genotype had higher $AUC_{0\text{-}last}$ by 1.67-fold (95%CI 1.51-6.18) and 3.06-fold (95%CI 1.18-2.35) vs subjects with CC (wild type) genotype. Adjusted R^2 of $AUC_{0\text{-}last}$ multivariable model was 0.3241.

C_{max}: Consistent with $AUC_{0\text{-}last}$, C_{max} was 59% (95%CI 1.12-2.26) higher in Group 3 vs Group 1 and subjects with CA and AA genotype had increased C_{max} GM by 2.18-fold (95%CI 1.14-4.16) and 1.43-fold (95%CI 1.04-1.96) compared to subjects with wild type genotype. Adjusted R^2 of C_{max} multivariable model was 0.2119.

Table 76 Univariate and multivariable regression model for geometric mean ratio of rosuvastatin $AUC_{0\text{-last}}$

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.3241				
Group of subjects		0.015*		
Group 2 vs Group 1	1.48 (0.95-2.32)	0.083*	1.43 (0.95-2.16)	0.08
Group 3 vs Group 1	1.90 (1.23-2.93)	0.004*	1.83 (1.25-2.69)	0.003*
Group 3 vs Group 2	3.00 (0.30-1.08)	1.28	1.28 (0.85-1.93)	0.24
BMI (kg/m^2)	1.02 (0.96-1.09)	0.50		
Albumin (g/dL)	1.28 (0.51-3.19)	0.59		
Total bilirubin (mg/dL)	1.07 (0.69-1.67)	0.75		
Direct bilirubin (mg/dL)	1.17 (0.20-6.78)	0.86		
AST (U/L)	1.01 (0.99-1.04)	0.22		
ALT (U/L)	1.00 (0.99-1.02)	0.59		
SLCO1B1 diplotype		0.63		
ABCG2 gene (rs2231142)		0.001*		
421CC (wild type)	1.00 (reference)			
421AA	2.84 (1.34-6.03)		3.06 (1.51-6.18)	0.003*
421CA	1.81 (1.26-2.59)		1.67 (1.18-2.35)	0.004*

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

[*]p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

Table 77 Univariate and multivariable regression models for geometric mean ratio of rosuvastatin C_{max}

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
C_{max} (pg/mL), Adjusted R^2 0.2119				
Group of subjects		0.036*		
Group 2 vs Group 1	1.15 (0.78-1.69)	0.47	1.12 (0.77-1.64)	0.54
Group 3 vs Group 1	1.63 (2.37-1.59)	0.011*	1.59 (1.12-2.26)	0.011*
Group 3 vs Group 2	1.42 (0.95-2.12)	0.087*	1.42 (0.97-2.07)	0.07
BMI (kg/m^2)	0.98 (0.93-1.04)	0.59		
Albumin (g/dL)	1.21 (0.55-2.63)	0.63		
Total bilirubin (mg/dL)	1.11 (0.76-1.62)	0.58		
Direct bilirubin (mg/dL)	2.69 (0.62-11.68)	0.18		
AST (U/L)	1.01 (0.99-1.03)	0.59		
ALT (U/L)	1.00 (0.98-1.01)	0.63		
SLCO1B1 diplotype		0.54		
ABCG2 gene (rs2231142)		0.014*		
421CC (wild type)	1.00 (reference)			
421AA	2.14 (1.09-4.21)		2.18 (1.14-4.16)	0.020*
421CA	1.48 (1.07-2.05)		1.43 (1.04-1.96)	0.027*

GMR, geometric mean ratio; CI, confidence interval; C_{max}, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B1; *ABCG2*, ATP Binding Cassette Subfamily G 2

[a]Significant level, p-value <0.1

[b] Significant level, p-value <0.05

*p-value<0.1 for univariate analysis, <0.05 for multivariable analysis

6. Bioanalysis of rosuvastatin in wild type (rs2231142) *ABCG2* genotype subjects and multivariable analysis

Due to rosuvastatin multivariable analysis, c.421C>A variant genotype was a confounding factor in this study. The bioanalysis data of rosuvastatin was re-analyzed with data of subjects with CA (n=20) and AA (n=3) genotype were excluded.

Subjects who carried 421CA genotype was excluded for 5 subjects of Group 1, 8 subjects of Group 2, and 7 subjects of Group3. Subjects with 421AA genotype including 2 subjects in Group 1 and 1 subject in Group 2 were also excluded.

Interestingly, the results showed that the significant difference in $AUC_{0\text{-last}}$ was observed by GMR 2.02 (95%CI 1.16-3.51) in Group 2 vs Group 1 but not in overall subject comparison. Moreover, the higher magnitude of difference was shown from GMR 1.09 (1.23-2.93) to GMR 2.09 (95%CI 1.26-3.49) between Group 3 and Group 1.

The pharmacokinetic parameters of rosuvastatin in wild type subjects are described in Table 78. Plasma concentration time profile of rosuvastatin in three groups of subjects with and without variant c.421C>A genotype are shown in Figure 30.

Univariate and multivariable analysis in rosuvastatin (wild type rs2231142) was carried out. There were no other variables associated with $AUC_{0\text{-last}}$ and C_{max} except subjects in Group 2 and Group 3. From univariate analysis, $AUC_{0\text{-last}}$ was higher in Group 2 (2.02-fold, 95%CI 1.16-3.51) and Group 3 (2.09-fold, 95%CI 1.26-3.49) vs Group 1. C_{max} was increased only found in Group 3 (1.57-fold, 95%CI 1.01-2.44) compared to Group 1 subjects.

The univariate regression model for rosuvastatin (wild type rs2231142) $AUC_{0\text{-last}}$ and C_{max}, are shown in Table 79-80.

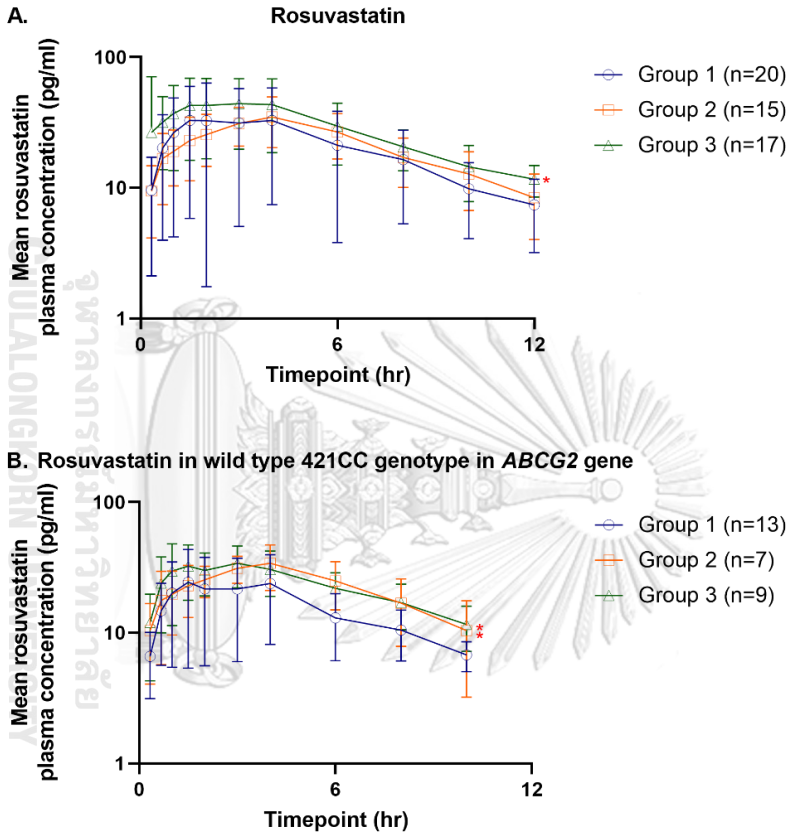


Figure 30 Plasma concentration time profile of rosuvastatin in all subjects and wild type of *ABCG2* gene subjects
(A.) total subjects including all c.421C>A genotype, (B.) subjects with specific wild type 421CC genotype of *ABCG2* gene.
Group 1, healthy young subjects, Group 2, healthy elderly subjects., Group 3, elderly subjects with chronic kidney disease; *ABCG2*, ATP Binding Cassette Subfamily G 2
*p-value<0.05, Group 1 as a reference group.

Table 78 Pharmacokinetic parameters of rosuvastatin with wild-type *ABCG2* gene (rs2231142)

Drug	**Group 1**	**Group 1 with rifampicin**		**Group 2**		**Group 3**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GM (95% CI)**
Rosuvastatin							
$AUC_{0\text{-last}}$ (pg/mL.hr)	136 (91-203)	700 (570-860)	5.15 (3.36-7.89)*	274 (180-419)	2.02 (1.16-3.51)*	285 (193-419)	2.0 (1.26-3.49)*
C_{max} (pg/mL)	25 (17-37)	203 (143-290)	8.04 (4.87-13.28)*	39 (33-47)	1.56 (0.97-2.50)	40 (30-52)	1.57 (1.01-2.44)*

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{last}}$, area under the concentration-time curve of time zero to the last time point; C_{max},, maximum plasma concentration

Table 79 Univariate and multivariable regression for geometric mean ratio of rosuvastatin $AUC_{0\text{-last}}$ wild type rs2231142 subjects

	Univariate[a]		Multivariable[b]	
	GMR (95%CI)	p-value	GMR (95%CI)	p-value
$AUC_{0\text{-last}}$ (pg/mL.hr), Adjusted R^2 0.3241				
Group of subjects		0.009*		
Group 2 vs Group 1	2.02 (1.16-3.51)	0.015*		
Group 3 vs Group 1	2.09 (1.26-3.49)	0.006*		
Group 3 vs Group 2	1.04 (0.57-1.88)	0.90		
BMI (kg/m^2)	1.05 (0.96-1.13)	0.27		
Albumin (g/dL)	0.82 (0.25-2.75)	0.74		
Total bilirubin (mg/dL)	1.18 (0.74-1.88)	0.47		
Direct bilirubin (mg/dL)	1.39 (0.18-10.50)	0.74		
AST (U/L)	1.05 (0.99-1.11)	0.13		
ALT (U/L)	1.01 (0.97-1.05)	0.75		
SLCO1B1 diplotype		0.54		

GMR, geometric mean ratio; CI, confidence interval; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1*, Solute Carrier Organic Anion Transporter 1B

[a]Significant level, p-value <0.1; [b]Significant level, p-value <0.05

*p-value<0.05

Table 80 Univariate and multivariable regression for geometric mean ratio of rosuvastatin C_{max} in wild type rs2231142 subjects

C_{max} (pg/mL), Adjusted R^2 0.2119				
Group of subjects		0.069*		
Group 2 vs Group 1	1.56 (0.97-2.50)	0.07		
Group 3 vs Group 1	1.57 (1.01-2.44)	0.043*		
Group 3 vs Group 2	1.01 (0.61-1.68)	0.97		
BMI (kg/m^2)	0.99 (0.92-1.05)	0.67		
Albumin (g/dL)	0.72 (0.28-1.85)	0.48		
Total bilirubin (mg/dL)	1.16 (0.81-1.67)	0.41		
Direct bilirubin (mg/dL)	3.33 (0.72-15.40)	0.12		
AST (U/L)	1.03 (0.98-1.08)	0.25		
ALT (U/L)	0.99 (0.96-1.02)	0.42		
SLCO1B1 diplotype		0.58		

GMR, geometric mean ratio; CI, confidence interval; C_{max},, maximum plasma concentration; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; *SLCO1B1,* Solute Carrier Organic Anion Transporter 1B

[a]Significant level, p-value <0.1; [b]Significant level, p-value <0.05

*p-value<0.05

Chapter 5

Discussion and Conclusion

Discussion

In this study, the activity of CYP3A and drug transporters were indirectly determined by pharmacokinetic profile of microdose cocktail containing probe substrates. These microdose cocktail contained 30 µg of midazolam (a specific and sensitive probe substrate of CYP3A), 750 µg of dabigatran etexelate (a selective and sensitive probe substrate of intestinal P-gp), 10 µg of pitavastatin (a relatively selective probe substrate of OATP1B), 50 µg of rosuvastatin (a probe substrate of BCRP, OATP), and 100 µg of atorvastatin (a probe substrate of OATP, BCRP, P-gp, CYP3A) and had approximately 1/100th subtherapeutic dose due to safety concern. This study showed that the microdose cocktail were efficient to represent their own probe substrates activity and CYP3A and rug transporters activities were altered by aging and CKD.[85, 173]

Midazolam

The results showed that CYP3A activity was obviously decreased in advanced age by increased in AUC (~2-fold in Group 2, ~3-fold in Group 3) and C_{max} (~2-fold in both Groups) of midazolam plasma concentration. The elimination rate constant was reduced ~60% and half-life was prolonged by ~2-3-fold in both groups vs Group 1, but not found in Group 3 vs Group 2.

CKD seemed not to influence on midazolam pharmacokinetics because there were no significant differences in pharmacokinetic parameters between Group 3 and Group 2.

Midazolam is metabolized by liver using CYP3A (mainly CYP3A4 and CYP3A5), and then conjugated with UGT (UGT1A4, UGT2B4, and UGT2B7).[115, 174] The metabolites including 1'-hydroxy midazolam glucuronide (primary metabolite), 4-hydroxy midazolam glucuronide, and 1',4 dihydroxy midazolam glucuronide, are rapidly excreted via urine within 24 hour.[119, 175]

There are several reasons supporting the results of this study in relation to the physiological change in the elderly. First, midazolam is an intermediate hepatic extraction ratio drug (extraction ratio, ER 0.3-0.6) which generally depends on hepatic blood flow, unbound fraction, and enzyme capacity.[110, 111] As a reduction of cardiac output in elderly causes a reduction in hepatic blood flow, hepatic clearance of intermediate hepatic extraction ratio drug then depend on enzyme capacity and unbound fraction, resulting in drug hepatic clearance reduction.[110, 111, 176, 177] Once the hepatic clearance decreases, the elevation of midazolam plasma level and half-life prolongation might occur.[176-178]

Second, although the main excretion of midazolam is via the kidney, renal dysfunction does not affect midazolam plasma level because there is less than 1% of midazolam unchanged form excreted by urine.[110] Besides, midazolam has very high plasma protein binding (96%), especially albumin. And the reduced plasma protein binding is commonly found in advancing age and CKD leading to prolonged midazolam half-

life.[108, 179, 180] However, plasma albumin in our subjects was within normal range and no differences were seen between the patients groups. These might explain the results of this study which showed that renal dysfunction in CKD did not alter pharmacokinetic profiles of midazolam.

The effect of CKD on midazolam in this study was consistent with Tatosian et al.[107] they evaluated the effect of renal impairment on drug interactions using the same microdose cocktail as the present study. They also found that neither AUC nor C_{max} of midazolam, a CYP3A probe, was affected by renal impairment.[107]
Moreover, atorvastatin which is mainly metabolized by CYP3A4 was markedly increased in AUC and C_{max} when combined with itraconazole, a strong CYP3A4 inhibitor[85, 181] Atorvastatin, therefore, is also another effective probe substrate for CYP3A4 including several drug transports (such as BCRP, OATP1B, ang P-gp).[85, 157] The significance difference approximately 2-fold higher of $AUC_{0\text{-last}}$ and C_{max} in Group 2 vs Group 1 confirmed age-related CYP3A4 activity change.

Rifampicin is a either CYP3A4 inducer or inhibitor depending on period of time after the last dose. The earlier the time after rifampicin dose favor an inhibitory effect. Then later the time after rifampicin dose suggests an induction effect.[182] In this study, there were a slight increase in midazolam plasma concentration by GMR~1.2-1.3 (both AUC and C_{max}) when coadministration with 450 mg of rifampicin (intestinal-CYP3A inhibitor) but no statistically significant difference was found. This is consistent with previous study where coadministration of rifampicin 600 mg and microdose cocktail containing midazolam 10 µg had no significance change in AUC and T_{max} but slightly affected midazolam pharmacokinetics by 30% increase in C_{max}[85]

Genetic polymorphism of CYP3A may be a confounder in this study. CYP3A4 polymorphism is rarely found in Asian population whereas CYP3A5*3 is predominantly found in many population, especially 65-85% in Asians.[183, 184]Although some studies indicated polymorphism of CYP3A4/5 may affect their substrate clearance such as tacrolimus, CYP3A genetic variation has no significant correlation with *in vivo* midazolam metabolism and disposition.[185-187] To ensure the influence of CYP3A genetic variation on midazolam pharmacokinetics in this study, CYP3A5*3 polymorphism genotyping and pharmacokinetic parameters analysis were also conducted. The genotype frequency of CYP3A5 showed no wild type CYP3A5 in Group 2 subject was not found (Table 109 in Appendix F). The midazolam pharmacokinetic comparison in wild type subjects was analyzed to exclude possible confounding effect of the genetic variation. The results showed that wild type subjects in Group 3 (n=5) still had higher midazolam plasma concentration by GMR~2.0-3.0 in AUC and Cmax than wild type subjects in Group 1 (n=5) (Table 110 in Appendix F). To exclude age effect, the comparison between CYP3A5 genotype within Group 1 subjects was analyzed. There were no significant differences between CYP3A5 genotypes (Table 111 in Appendix F).

Hence, the pharmacokinetic alteration of midazolam shown here can be concluded that it was associated mainly with advanced age by reducing midazolam elimination resulting in half-life prolongation and increased midazolam plasma level.

Dabigatran

Dabigatran etexelate is a prodrug that is metabolized by carboxylesterase (CES) to an active metabolite, dabigatran, at the intestine (major pathway), plasma, and liver.[127] The substrate of intestinal P-gp is dabigatran etexelate, not the active metabolite. Furthermore, dabigatran etexelate is not transported by other transporters at the intestine and is higher sensitive to intestinal P-gp at the microdose than the therapeutic dose due to unsaturated intestinal P-gp at microdose.[85, 131, 188-190]

Digoxin, another P-gp probe substrate, has been previously studied.[103, 104] However, digoxin has low sensitivity to P-gp and has no selectivity for P-gp *in vitro* and *in vivo* used for studying intestinal P-gp. Additionally, digoxin also involved in P-gp located at urinary and bile efflux. Hence, dabigatran etexilate was preferred to be used as an intestinal P-gp probe substrate than digoxin.[86, 87]

In the present study, AUC of dabigatran was elevated with approximately 2-fold in Group 2, 4-fold in Group 3 compared to Group 1 and 3-fold in Group 3 vs Group 2. However, increased in C_{max} was only observed in Group 3 vs Group 1 by ~2-fold. It seems that advanced age and renal dysfunction influence the change in dabigatran plasma level.

The elimination rate constant and half-life of dabigatran were changed in Group 3 with a decreased Kel (~70% of Group 1, ~60% of Group 2) and an increased in $T_{1/2}$ (~3-fold vs Group 1, ~2.6-fold vs Group 2).

Furthermore, renal clearance of dabigatran showed a significant reduction by approximately 40% (Group 2 vs Group 1), 80% (Group 3 vs Group 1), and 70% (Group 3 vs Group 2). These might be related to their significant different eGFR.

Although, advanced age seems to affect intestinal P-gp activity in this study, the previous studies showed P-gp expression and activity changes in several organs but not in human intestinal P-gp.[191-193] Larsen et al. reported AUC elevation of digoxin, another probe of intestinal P-gp, in the elderly compared to adults but no significant different was found.[191]

Dabigatran is mainly 80% excreted by glomerular filtration at the kidneys.[194] Renal function is generally reduced by 8 mL/min/1.73 m^2 per decade from age of 30 years old. Evidence confirmed the significant lower eGFR in healthy elderly compared to younger subjects.[195, 196] Although insignificant, a slight decreased in eGFR was observed in Group 2 compared to Group 1. These could explain the findings which showed a higher dabigatran plasma level in healthy elderly than healthy young subjects.[131, 197]

The comparison between elderly with CKD subjects and healthy young subjects showed a higher magnitude of increased in dabigatran plasma level than those compared to healthy elderly subjects due to the effect of both aged-related renal function reduction and CKD.

The previous studies in rats reported that CKD affected intestinal P-gp activity by downregulation of intestinal P-gp leading to an increase in drug absorption.[198, 199]. The latest clinical study by Tatosian et al. was conducted in renal impairment patients using dabigatran etexilate as intestinal p-gp probe. They found the progressive increased in dabigatran $AUC_{0\text{-}inf}$ and C_{max} with severity of renal impairment up to 4.9 and 1.7-fold, respectively.[107] Comparable to the present study, $AUC_{0\text{-}inf}$ of subjects with severe and moderate renal impairment were higher by 4.3 and 3.1-fold compared to normal renal function subjects but had no difference was seen in moderate vs severe renal function (Table 112 in Appendix G).

Tatosian et al. also found the lower magnitude of drug-drug interaction with rifampicin in higher severity of renal impairment. These confirm their conclusion that renal impairment reduced intestinal P-gp activity.[107] Unfortunately, there was no such data in this study as rifampicin was administered only in Group 1.

Therefore, the results in the present study might be concluded that was mainly associated with their renal function. Although, the effect of renal impairment on intestinal P-gp might not be definitely concluded in this study, it should not be excluded.

To determine human intestinal P-gp activity in advance age population and/or CKD population, confirmation by rifampicin coadministration in further study is necessary.

Rifampicin is an intestinal P-gp inhibitor when given acutely.[200]. Prueksaritanont et al. proved the intestinal P-gp inhibitory effect of rifampicin confirming dabigatran etexilate as P-gp substrate.[85] In this study, rifampicin slightly increased dabigatran level by GMR~2.3-fold of AUC, GMR~1.9-fold of C_{max}, and increased T_{max} from 1.5 hour to 2.0 hour consistent with previous the microdose cocktail study.[85]

Pitavastatin

Pitavastatin is an OATP1B1 substrate. Although, OATP1B1 transporter locates at several organs including apical site of intestine and sinusoidal membrane of liver, pitavastatin is more selective and sensitive to hepatic OATP1B1 influx transporter.[142]

Rifampicin, a potent OATP1B1 inhibitor, has been used to confirm the function of pitavastatin as an OATP1B1 probe substrate. The coadministration of microdose cocktail and rifampicin significantly increased AUC (GMR~4) and C_{max} (GMR~5.4) of pitavastatin but there were not seen in pitavastatin lactone. Although there was a slight increase in C_{max} (GMR~1.3) of pitavastatin lactone, AUC of pitavastatin lactone showed no change. The AUC ratio of pitavastatin lactone to pitavastatin was decreased (GMR~0.4) in rifampicin coadministration confirming that hepatic OATP1B1 influx transporter was inhibited by rifampicin and the subsequence metabolism for pitavastatin lactone mediated by UGTs was therefore prevented.[142] The effect of rifampicin in the present study was consistent with previous Prueksaritanont et al microdose cocktail study[85] where an increased in AUC and C_{max} of pitavastatin with

GMR~4.0 and a decreased in AUC ratio for pitavastatin lactone to pitavastatin with GMR~0.2 were observed.

In the present study, pitavastatin level in the elderly subject with CKD was affected by an increased in AUC (~1.6-fold compared to both healthy young and healthy elderly subjects) and C_{max} (~2-fold of healthy young subjects and ~1.7-fold of healthy elderly subjects). It seems that renal dysfunction plays a role in pitavastatin pharmacokinetics. Advanced age has a lesser role as there was a minimal change only ~10% higher in C_{max} between healthy elderly and healthy young subjects. These are consistent with the pharmacokinetic study which demonstrated an increased in ~10% C_{max} and ~30% AUC of pitavastatin in the elderly.[132]

Although pitavastatin is mainly excreted via biliary excretion after glucuronidation into pitavastatin lactone (major inactive metabolite) and urinary excretion in its unchanged form is relatively small (less than 15% of dose), the renal impairment might influence pitavastatin exposure as reported in several previous studies.[132, 137, 201] It was reported that patients with moderate renal impairment (eGFR 30-59 mL/min/1.73 m^2) had 102% and 86% increase in $AUC_{0\text{-}inf}$ and C_{max}, respectively, than healthy subjects.[132] In case of patients with severe renal impairment (eGFR 15-29 mL/min/1.73 m^2) without hemodialysis in another pharmacokinetic studies, 36% and 18% higher in $AUC_{0\text{-}inf}$ and C_{max}, respectively, compared with healthy subjects were shown.[132] In rat model, Naud et al. incubated rat hepatocytes with serum from chronic renal impairment subjects. Hepatocytes incubated with chronic renal impairment serum had a decreased in OATP1B1 expression.[202] Those results led to a hypothesizing uremic toxin accumulation in renal impairment patients which alters nonrenal enzymes and drug transporters including OATPs transporters.[203]

In this study, pharmacokinetic parameters comparison between normal, moderate, and severe renal function was analyzed. Similar to the previous studies, the effect of renal impairment on pitavastatin plasma level was observed.[107, 132] Tatosian et al[107] used microdose pitavastatin as OATP1B probe substrate in renal impairment patients found an increased pitavastatin level by approximately 2-fold in both $AUC_{0\text{-}inf}$ and C_{max} without association with renal impairment severity comparable to the results of this study where a trend $AUC_{o\text{-}inf}$ and C_{max} were higher by 1.5 to 1.6-fold in moderate and severe renal impairment than normal subjects (Table 113 in Appendix H).

Of note, the increased in pitavastatin plasma level without a trend on severity of renal impairment does not support the uremic toxin hypothesis.[107]

Interestingly, the recent Physiologically Based Pharmacokinetic Modeling (PBPK) study also reported the changes in the activity of hepatic OATP1B1 transporter up to 60% in patients with severe CKD.[204]

The effect of renal dysfunction on OATP1B1 activity in the present study was still inconclusive. Because pitavastatin lactone, which is not an OATP1B1 substrate, also had an increased in AUC (~2-fold) and C_{max} (~1.4-fold) in CKD elderly subjects compared to both healthy elderly and healthy young subjects. Besides, if OATP1B1

activity change was the major cause of an increased in pitavastatin exposure, the difference in AUC ratio of pitavastatin lactone to pitavastatin between groups should also be observed.

Therefore, changes in pitavastatin in drug disposition may also be affected by other drug metabolism enzymes, uptake and efflux transporters such as UGTs, BCRP, P-gp, NTCP which also involved in metabolism and excretion of pitavastatin and pitavastatin lactone.[134, 138, 204]

Moreover, the association of increasing BMI and pitavastatin C_{max} was observed by approximately a decreased 7% in C_{max}. Due to high lipophilicity of pitavastatin, it could be affected by higher fat in increased BMI resulting in higher volume of distribution. The physiological change of the elderly in higher fat and BMI is usually occurred. Although, BMI was not significant different among the three groups, there was a trend of higher BMI in the elderly subjects.

Rosuvastatin

Rosuvastatin is a probe substrate of BCRP and hepatic OATP1B1 transporter. [85, 104, 142] The plasma level of rosuvastatin was only higher in elderly subjects with CKD for approximately 2-fold and 1.6-fold in AUC and C_{max}, respectively, than healthy young subjects.

Interestingly, after adjustment for confounding factors in multivariable analysis, *ABCG2* polymorphisms showed the impact on both AUC and C_{max} of rosuvastatin. Subjects with homozygous variant 421AA had dramatically increased by 3-fold in AUC and 2-fold in C_{max} and heterozygous variant 421CA also had an increased in AUC and C_{max}, 1.7-fold and 1.4-fold, respectively, compared to 421CC wild type. Consistent with previous studies, they reported the association of C.421A allele with higher rosuvastatin plasma level by increase in both AUC and C_{max}.[71, 205, 206]

To evaluate rosuvastatin exposure without confounding factor, the data of c.421CA and c.421AA subjects were excluded. The significant 2-fold higher of AUC in healthy elderly subjects and more in CKD (from 1.90 to 2.09-fold), were shown after re-analyzing data whereas no significant difference between elderly with or without CKD was found. Therefore, rosuvastatin exposure might be affected only by advanced age, not CKD.

Although rosuvastatin is mainly excreted by biliary excretion, 28% of rosuvastatin is also significantly eliminated by renal tubular secretion via OAT3, BCRP, P-gp, and MRP2 efflux transporter.[145, 207] Renal clearance was also examined. The significantly reduction was found in healthy elderly and elderly with CKD compared to healthy young subjects. A significant difference between renal clearance of elderly with CKD and healthy elderly subjects was also found. These results were not related to rosuvastatin AUC, thus the reduction of renal clearance was caused mainly by their own renal function and probably not associated with rosuvastatin exposure in this study.

Rosuvastatin package insert suggests that mild/moderate renal impairment does not affect rosuvastatin level but dosage adjustment is recommended in severe renal impairment.[140] Consistent with the present study, Tatosian et al. with the same microdose cocktail also reported the nonsignificant difference of rosuvastatin among mild, moderate, and severe renal impairment patients, thus renal impairment does not affect rosuvastatin level.[107]

Rifampicin, an intestinal BCRP efflux and hepatic OATP influx transporter[208], markedly affected rosuvastatin exposure as evidenced by 5-fold increased in AUC and 8-fold increased in C_{max}. Consistent with Prueksaritanont et al[85] and Tatosian et al[107], their rosuvastatin level was increased in 5-fold (AUC) and 11-fold (C_{max}), 3.5-fold (AUC) and 5-fold (C_{max}), respectively. The greater impact on C_{max} suggest that rifampicin has strong intestinal BCRP inhibitory effect leading to increase rate of absorption more than increase the extent of rosuvastatin absorption.

The cause of advanced age increasing rosuvastatin level was still unclear in this study. If the significant change of rosuvastatin level was associated with age-related renal dysfunction, there would be significant difference between elderly with CKD and healthy elderly subjects. Furthermore, the significant change of rosuvastatin exposure have not been reported in the elderly.[209-211]

Taking these into consideration, in this study, the cause of change in rosuvastatin plasma level was inconclusive. Further study on drug transporters involved in rosuvastatin elimination and the larger sample size in the elderly with or without CKD is warranted.

Atorvastatin

The last probe substrate in microdose cocktail, atorvastatin is a substrate of CYP3A4, OATP1B1, BCRP, and P-gp.[212, 213] Atorvastatin had a significant increase in both AUC and C_{max}.compared to healthy young subjects. There was also a significant difference between the elderly with CKD and healthy elderly by an increased approximately 2-fold in both AUC and C_{max}. These imply that both advanced age and CKD affect atorvastatin exposure.

A previous study reported the effect of age on atorvastatin pharmacokinetics in the elderly. Both AUC and C_{max} was increased by 27.3% and 42.5% in elderly participants (66-92 years) than young participants (19-35 years) which might be caused by CYP3A4 metabolizing enzyme activity reduction.[214] The association of advanced age and atorvastatin level in this study could be related to a decrease in CYP3A4 activity comparable to findings observed in midazolam.

The effect of renal dysfunction to atorvastatin and its metabolite level alteration, it could not be definitely concluded. There was less than 2% of atorvastatin and its metaboites excreted via urine.[153] The reported renal impairment effect on atorvastatin pharmacokinetics in previous studies were inconsistent. Stern et al. reported the unchanged in atorvastatin pharmacokinetics in renal dysfunction whereas some previous studies reported the decreased in *in vivo* CYP3A4 activity by 28% in the end-

stage renal disease group.[215, 216] Tatosian et al. found the significant effect of renal impairment on atorvastatin level. Their results also showed the higher AUC and C_{max} of atorvastatin as the severity of renal impairment increased.[107] Conversely, 4-hydroxy atorvastatin , another OATP1B substrate, showed similar findings to atorvastatin in this study but no data reported in Tatosian et al.[107]

Rifampicin, a potent OATP, BCRP, and P-gp inhibitor, had a larger impacted on C_{max} than AUC of atorvastatin and 4-hydroxy atorvastatin and had no significant change in 4-hydroxy atorvastatin to atorvastatin ratio consistent with previous studies.[85, 217] These confirm that the effect of rifampicin causing higher AUC and C_{max} in atorvastatin and its metabolite were due to inhibition of hepatic OATP1B uptake transporter resulting in a reduction in hepatobiliary elimination.[217]

Interestingly, Tatosian et al. also found the greater increase in C_{max} of atorvastatin in renal impairment patients with rifampicin, thus intestinal BCRP and P-gp could be additional factors involved in atorvastatin pathway in CKD.[107] However, BCRP and intestinal P-gp are substrate to atorvastatin, its effect was still unexplained in this study due to inconclusive result of dabigatran and rosuvastatin.

Because of the conversion of atorvastatin into 4-hydroxy atorvastatin is mediated by CYP3A4, the significant reduction in 4-hydroxy atorvastatin to atorvastatin ratio in both healthy elderly and elderly with CKD subjects compared to healthy young subjects in this study were associated with CYP3A4 activity reduction more than effect on hepatic OATP1B activity. Unfortunately, pitavastatin, another potent OATP1B substrate, was unable to determine OATP1B activity in this study.

Therefore, the differences in atorvastatin pharmacokinetic parameters could be determined by the association of decrease CYP3A4 activity with advanced age as mentioned in midazolam.

The strength of this study is there was the novel application of microdose cocktail approach to determine CYP enzyme and drug transporters in special population, especially in the elderly. Although some drug transporter activity cannot be concluded yet, the study results could provide a trend of their changes. The further study with larger sample sizes, especially elderly with CKD subjects, rifampicin coadministration in all study population, using internal biomarkers for result confirmation will be of benefit.

Conclusion

Microdose cocktail approach indicated that CYP3A4 activity was obviously reduced in elderly subjects, especially Thai population. Although the association of OATP1B1, BCRP, and P-gp transporter with special population in this study might be inconclusive, there was probably a trend in these transporter activity alterations. Elderly with CKD in this study was a preliminary study, to obtain more explicit impact, the larger sample size should be considered. It is very important to determine mechanistic changes in PK and PD specifically for Thai elderly population. This would help adjusted the optimal dosage regimen for Thai patients.

Clinical application

The study of CYP enzymes and drug transporters activity dose not only provide more understand in pharmacokinetic changes in special population, but it is also important and useful for medical professional. CYP3A4 is a metabolizing enzyme which mostly involved in metabolism of severe drugs. The study results indicated CYP3A4 activity change by advanced age lead to enhance awareness of possible drug interaction in the elderly, predict drug efficacy and promote more concern about adverse drug reaction.

Appendix

A. Microdose cocktail preparation

Materials

- Midazolam 15 mg, tablet (Dormicum®)
- Dabigatran etexelate 75 mg, capsule (Pradaxa®)
- Pitavastatin 2 mg, tablet (Livalo®)
- Atorvastatin 10 mg, tablet (Lipitor®)
- Rosuvastatin 10 mg, tablet (Crestor®)
- Lactose monohydrate, USP
- Sterile water for injection
- Mortar and pestle
- Spoon
- Stainless spatula
- Analytical balance
- Weighing paper
- Volumetric flask, 10 mL
- Cylinder, 10 mL
- Amber bottle, 10 mL

<u>Midazolam 30 µg</u> (Powder) **Prep by…….**
Date……...
Compound:

- Active ingredient: Midazolam (Dormicum®) 15 mg (15,000 µg)/tablet
- Diluent: Lactose monohydrate

For midazolam 30 µg/dose, 35 doses: dilute 1:500
Preparation:

1. Weighed midazolam (15,000 µg) tablet: <u>X</u> g
2. Added lactose q.s. to <u>10-fold of X</u> g

↓ Mixed (geometric dilution)

3. Weigh mixing compound in step 2 equal to <u>2-fold of X g</u>

↓

Midazolam strength **3,000 µg**

4. Added lactose q.s. to <u>20-fold of X</u> g

↓ Mixed (geometric dilution)

5. Weigh mixing compound in step 4 equal to <u>7-fold of X</u> g (A)

↓

Midazolam 1,050 µg (for 35 doses)

<u>Midazolam 30 µg</u> (Solution)*
Compound:

- Active ingredient: Midazolam injection 5 mg/1 mL (5,000 µg)/vial
- Diluent: Sterile water for injection

For midazolam 30 µg/10mL/dose, 20 doses
Preparation:

1. Pipetted midazolam injection (5,000 µg/mL): 120 µL into volumetric flask, 200 mL
2. Added sterile water for injection q.s. to 200 mL (for 20 doses)
3. Measured to cylinder for volume 10 mL
4. Filled into amber bottle and label

*For last 4 subjects due to Dormicum® tablet was temporarily stopped operation.

<u>Dabigatran etexilate 750 µg</u> **Prep by……. Date……...**

Compound:

- Active ingredient: Dabigatran etexilate (Pradaxa®) 75 mg (75,000 µg)/capsule
- Diluent: Lactose monohydrate

For dabigatran etexilate 750 µg/dose, 35 doses: dilute 1:100

Preparation:

1. Weighed dabigatran etexilate (75,000 µg) 2 capsules): <u>X</u> g
2. Added lactose q.s. to <u>10-fold of X</u> g

Mixed (geometric dilution)

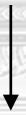
3. Weigh mixing compound in step 2 equal to <u>2-fold of X</u> g

Dabigatran etexilate strength **30,000 µg**

4. Added lactose q.s. to <u>20-fold of X</u> g

Mixed (geometric dilution)

5. Weigh mixing compound in step 4 equal to <u>17.5-fold of X</u> g (B)



Dabigatran etexilate 26,250 µg (for 35 doses)

Pitavastatin 10 µg **Prep by…….**

Date……...

Compound:

- Active ingredient: Pitavastatin (Livalo®) 2 mg (2,000 µg)/tablet
- Diluent: Lactose monohydrate

For pitavastatin 10 µg/dose, 35 doses: dilute 1:200

Preparation:

1. Weighed pitavastatin 1 tablet (2,000 µg): X g
2. Added lactose q.s. to 10-fold of X g

↓ Mixed (geometric dilution)

3. Weigh mixing compound in step 2 equal to 2-fold of X g

↓

Pitavastatin strength **400 µg**

4. Added lactose q.s. to 10-fold of X g

↓ Mixed (geometric dilution)

5. Weigh mixing compound in step 4 equal to 8.75-fold of X g (C)

↓

Pitavastatin 350 µg (for 35 doses)

<u>Rosuvastatin 50 µg</u> **Prep by……. Date……...**

Compound:

- Active ingredient: Rosuvastatin (Crestor®) 10 mg (10,000 µg)/tablet
- Diluent: Lactose monohydrate

For rosuvastatin 50 µg/dose, 35 doses: dilute 1:200

Preparation:

1. Weighed rosuvastatin 1 tablet (10,000 µg): <u>X</u> g
2. Added lactose q.s. to <u>10-fold of X</u> g

↓ Mixed (geometric dilution)

3. Weigh mixing compound in step 2 equal to <u>2-fold of X</u> g

↓

Rosuvastatin strength **2,000 µg**

4. Added lactose q.s. to <u>10-fold of X</u> g

↓ Mixed (geometric dilution)

5. Weigh mixing compound in step 4 equal to <u>8.75-fold of X</u> g (D)

↓

Rosuvastatin 1,750 µg (for 35 doses)

Atorvastatin 100 µg **Prep by……. Date……...**

Compound:

- Active ingredient: Atorvastatin (Lipitor®) 10 mg (10,000 µg)/tablet
- Diluent: Lactose

For atorvastatin 100 µg/dose, 35 doses: dilute 1:100

Preparation:

1. Weighed atorvastatin (10,000 µg) 2 tablets: X g
2. Added lactose q.s. to 10-fold of X g

↓ Mixed (geometric dilution)

3. Weigh mixing compound in step 2 equal to 2-fold of X g

↓

Atorvastatin strength **4,000 µg**

4. Added lactose q.s. to 20-fold of X g

↓ Mixed (geometric dilution)

5. Weigh mixing compound in step 4 equal to 17.5-fold of X g (E)

↓

Atorvastatin 3,500 µg (for 35 doses)

Microdose cocktail

Component:

- Active ingredients
  1. Midazolam (Dormicum®) 30 µg
  2. Dabigatran etexilate (Pradaxa®) 750 µg
  3. Pitavastatin (Livalo®) 10 µg
  4. Rosuvastatin (Crestor®) 50 µg
  5. Atorvastatin (Lipitor®) 100 µg

- Working formular
  1. Midazolam (Dormicum®) A µg
  2. Dabigatran etexilate (Pradaxa®) B µg
  3. Pitavastatin (Livalo®) C µg
  4. Rosuvastatin (Crestor®) D µg
  5. Atorvastatin (Lipitor®) E µg

- Preparation*
  1. Mixed A+B+C+D+E by geometric dilution technique. (for 35 doses)
  2. Weighed 1/35 of total mixing compound in step 1.
  3. Filled into amber bottle and labeled.

*For last 4 subjects, B+C+D+E mixing and midazolam solution were prepared separately. B+C+D+E mixing was administered to subjects followed by midazolam solution.

B. Results of method validation

Developed LC-MS/MS method for quality control dosing of microdose cocktail

Reagent: Midazolam, Dabigatran etexelate, Rosuvastatin Calcium, Pitavastatin Calcium, Atorvastatin Calcium, Labetarol hydrochloride

Diluent: 50% acetonitrile with 0.1% formic acid

Drugs label amount in microdose cocktail, standard concentration range and retention time are shown in Table 81 (including internal standard). The standard curves (a plot of area ratio vs concentration ratio) of each drug in microdose cocktail with R^2 was met linearity and in acceptance criteria (Figure 31 to Figure 35). The chromatogram contains 6 nominal concentration of 6 standard points, 3 nominal concentrations of QC, and internal standard are presented in Figure 36. The partial validations for dosing of microdose cocktail were conducted both intra-day (three replications of QC, n=3) and inter-day (two days of three replications of QC, n=6) assays. These partial validation results corresponded to acceptance criteria in both precision and accuracy as presented in Table 82.

Table 81. The amounts of drugs in microdose cocktail, standard curve range and their retention time

	Label amount (µg)	**Standard curve range (µg/mL)**		**Retention time (min)**
		Min	**Max**	
Midazolam	30	6	120	2.14
Dabigatran etexilate	750	150	3000	2.42
Rosuvastatin calcium	50	10	200	2.41
Pitavastatin calcium	10	2	40	2.35
Atorvastatin calcium	30	20	400	2.75
Labetalol (internal standard)				1.94

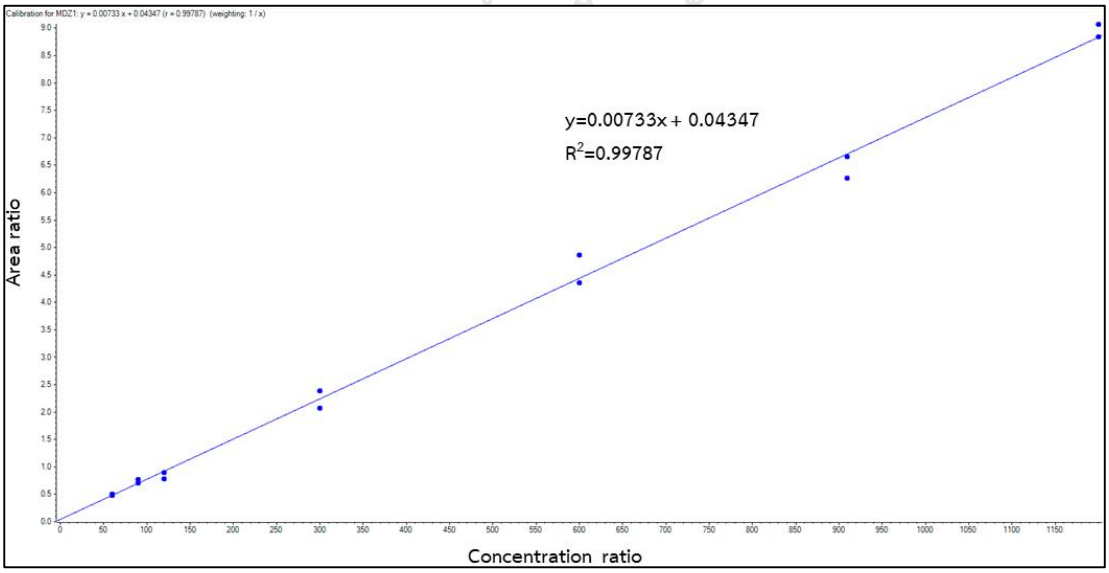


Figure 31. The standard curve and R^2 of midazolam in microdose cocktail dosing

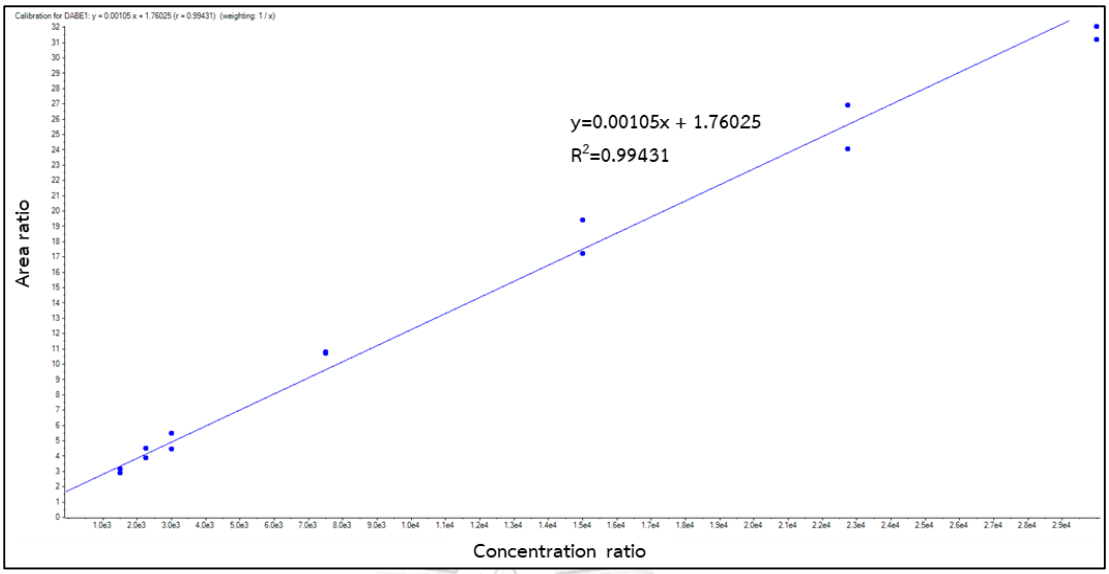


Figure 32. The standard curve and R^2 of dabigatran in microdose cocktail dosing

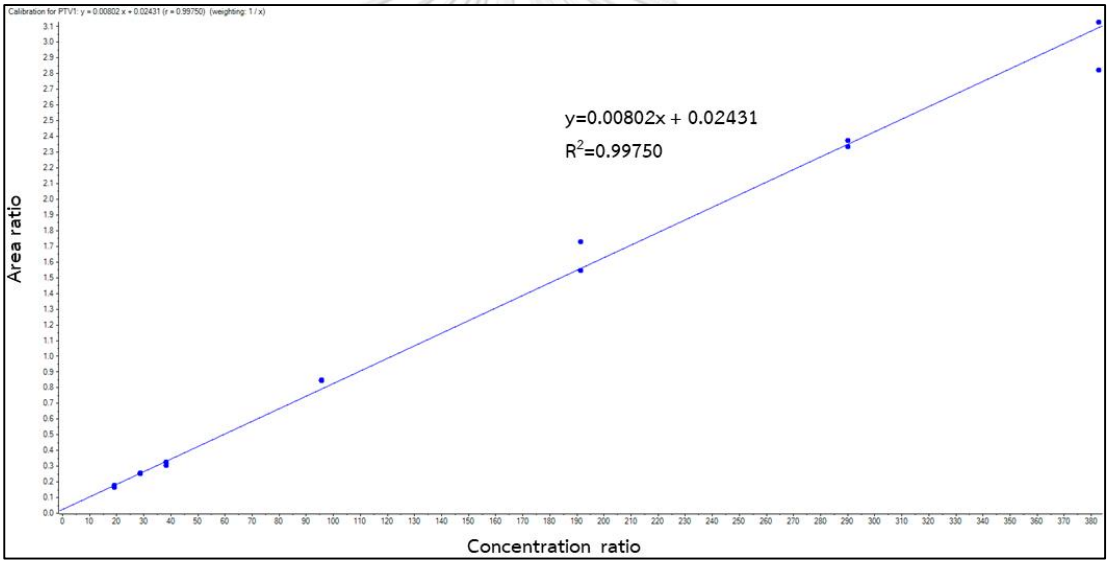


Figure 33. The standard curve and R^2 of pitavastatin in microdose cocktail dosing

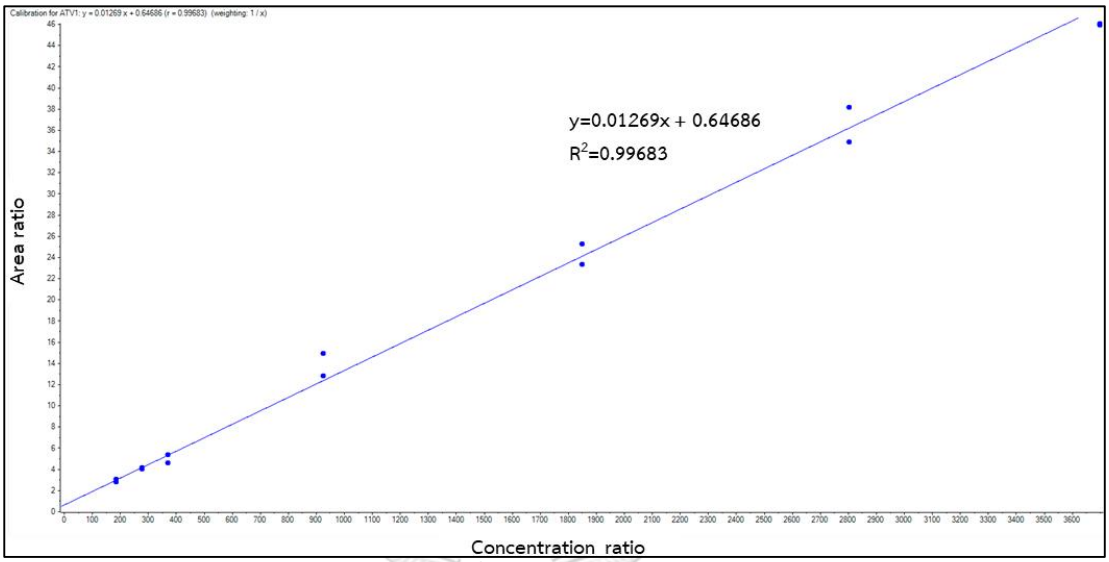


Figure 34. The standard curve and R^2 of atorvastatin in microdose cocktail dosing

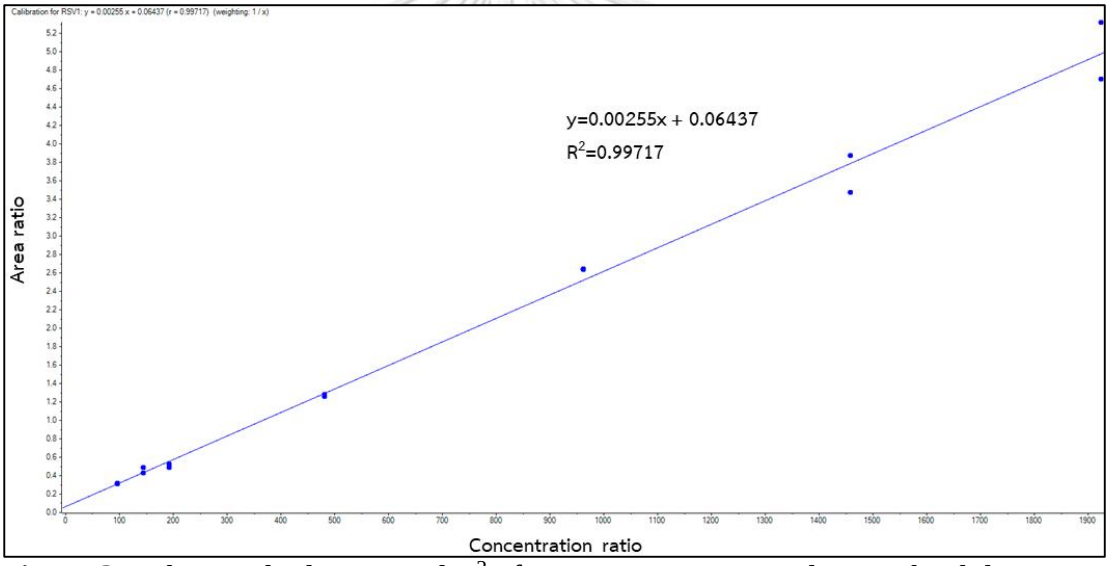


Figure 35. The standard curve and R^2 of rosuvastatin in microdose cocktail dosing

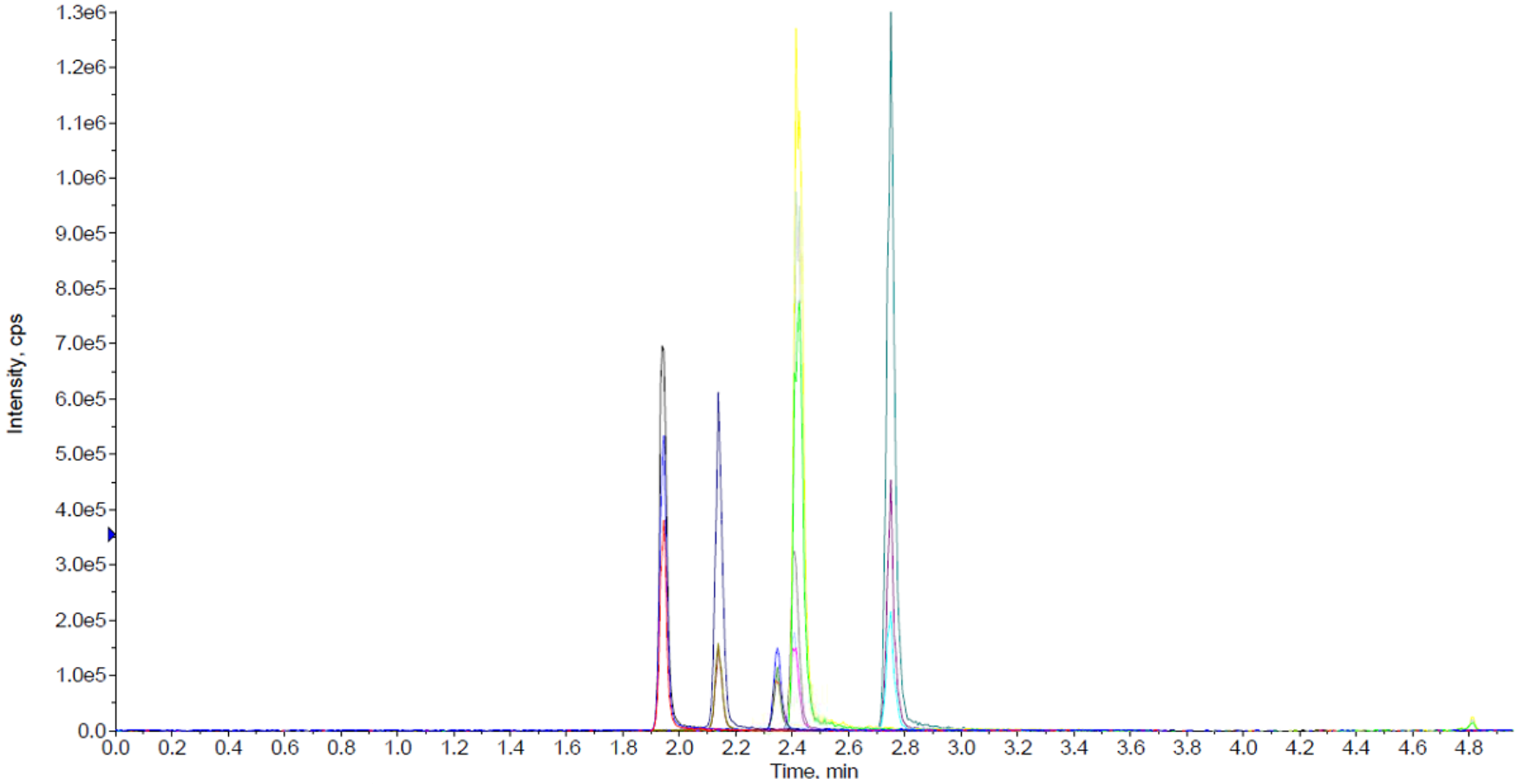


Figure 36. The chromatogram of labetalol, midazolam, pitavastatin, rosuvastatin, dabigatran etexilate and atorvastatin, respectively

Table 82. The partial validation results of microdose cocktail dosing including precision and accuracy.

Compound	**Target concentration (µg/mL)**	**Intra-day assay (n=3)**				**Inter-day assay (n=6)**			
		Mean concentration (SD) (µg/mL)		**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (µg/mL)**		**Precision (% RSD)**	**Accuracy (%)**
Midazolam	Low (7.5)	8.22	0.80	9.70	109.58	6.95	0.30	4.28	92.71
	Medium (15)	16.58	1.01	6.11	110.53	14.37	0.89	6.18	95.82
	High (105)	114.57	4.12	3.59	109.11	104.11	5.31	5.10	99.15
Dabigatran etexilate	Low (187.5)	196.39	9.63	4.90	104.74	162.92	19.95	12.24	86.89
	Medium (375)	426.79	20.23	4.74	113.81	405.71	51.08	12.59	108.19
	High (2622)	2508.20	236.28	9.42	95.66	2671.93	226.26	8.47	101.90
Pitavastatin	Low (2.39)	2.24	0.25	11.35	93.61	2.18	0.09	3.97	91.08
	Medium (4.78)	4.48	0.21	4.64	93.65	4.42	0.20	4.42	92.35
	High (33.49)	31.77	1.95	6.15	94.87	32.88	1.62	4.92	98.18
Atorvastatin	Low (23.11)	24.43	1.00	4.08	105.72	22.91	1.32	5.74	99.14
	Medium (46.23)	50.56	3.12	6.18	109.38	48.60	2.61	5.37	105.14
	High (323.59)	341.38	20.05	5.87	105.50	343.26	10.68	3.11	106.08
Rosuvastatin	Low (12.03)	11.36	0.54	4.73	94.48	10.23	0.56	5.51	85.09
	Medium (24.05)	21.91	1.12	5.10	91.10	22.59	1.25	5.54	93.93
	High (168.347)	153.83	8.38	5.45	91.38	159.40	4.35	2.73	94.69

SD, standard deviation; LLOQ, lower of quantification

Developed LC-MS/MS method for determine the concentration of cocktail in plasma and urine

Midazolam

Reagent: Midazolam, [$^2H_6$]-Midazolam (internal standard)
Sample matrix: Plasma
Standard calibration range: 1-1000 pg/mL
Retention time: 1.54 min
The standard curve with R^2 was accepted for linearity. The standard curve with R^2 and chromatogram of midazolam and [$^2H_6$]-Midazolam with retention time are shown in Figure 37 and Figure 38, respectively. Inter-day (five replications of QC in two days, n=10) and intra-day (five replications of QC, n=5) assays were performed to assess partial validation. The partial validation was accepted for precision and accuracy as described in Table 83.

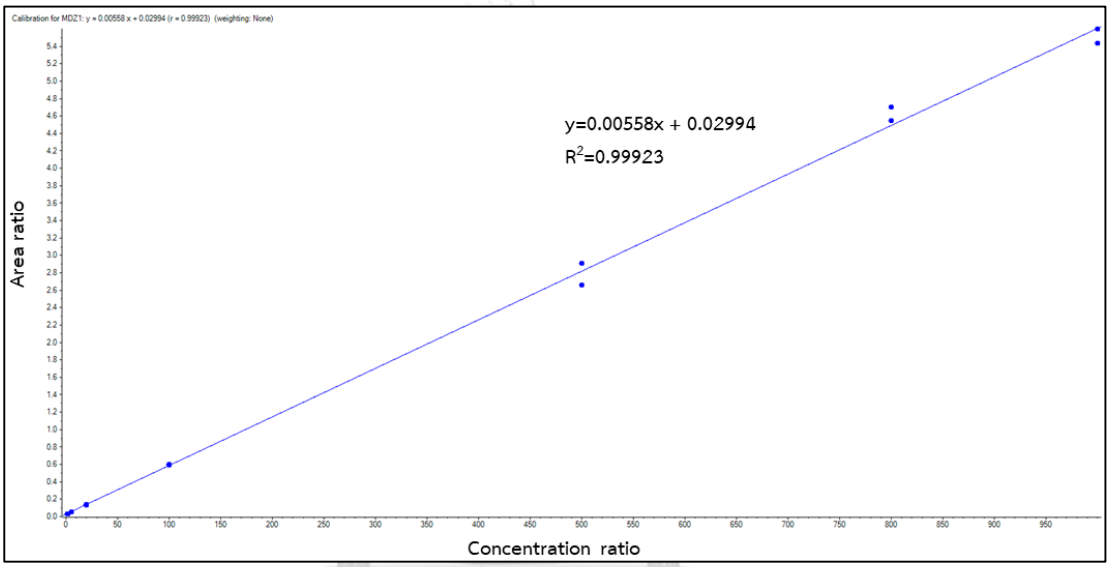


Figure 37. The standard curve and R^2 of midazolam

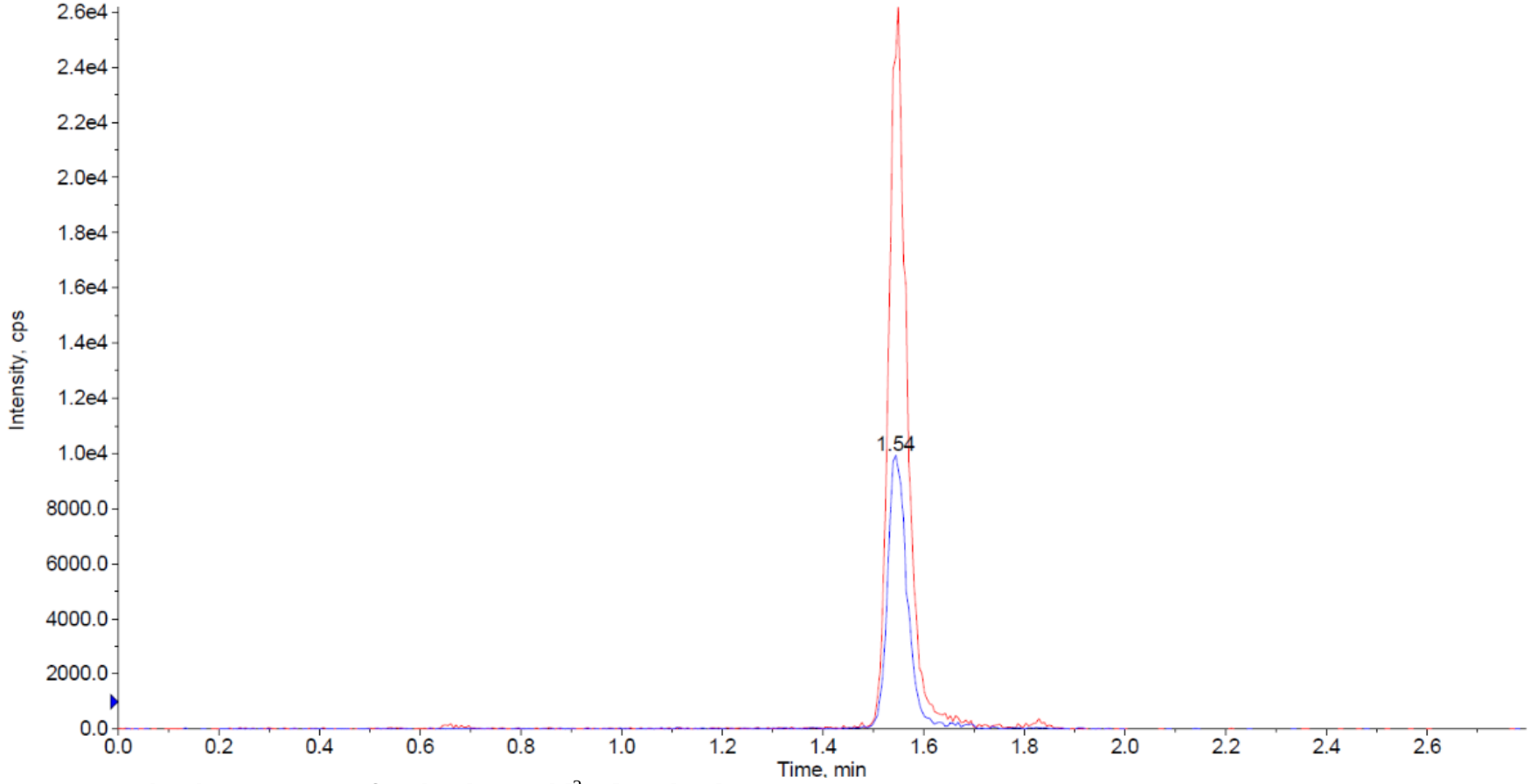


Figure 38. The chromatogram of midazolam and [$^{2}H_{6}$]-Midazolam.

Table 83. The partial validation results of midazolam including precision and accuracy

Target concentration (pg/mL)	**Intra-day assay (n=5)**			**Inter-day assay (n=10)**		
	Mean concentration (SD) (pg/mL)	**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (pg/mL)**	**Precision (% RSD)**	**Accuracy (%)**
LLOQ (1.00)	1.20 (0.42)	35.25	119.53	1.08 (0.08)	7.56	107.85
Low (2.50)	2.40 (0.17)	7.07	96.04	2.69 (0.20)	7.47	107.52
Medium (60)	65.10 (4.00)	6.14	108.50	62.15 (2.21)	3.55	103.58
High (750)	699.77 (16.80)	2.40	93.30	684.40 (16.88)	2.47	91.25

SD, standard deviation; LLOQ, lower of quantification

Dabigatran

Reagent: Dabigatran, [$^{13}C_6$]-Dabigatran (internal standard)
Sample matrix: Plasma
Standard calibration range: 20 - 5000 pg/mL
Retention time: 1.09 min
The standard curve with R^2 and the chromatogram of Dabigatran and [$^{13}C_6$]-Dabigatran are shown in Figure 39 and Figure 40. Inter-day (five replications of QC in two days, n=10) and intra-day (five replications of QC, n=5) assays were performed to assess partial validation. The partial validation results corresponded to the acceptance of linearity, precision, and accuracy (Table 84).

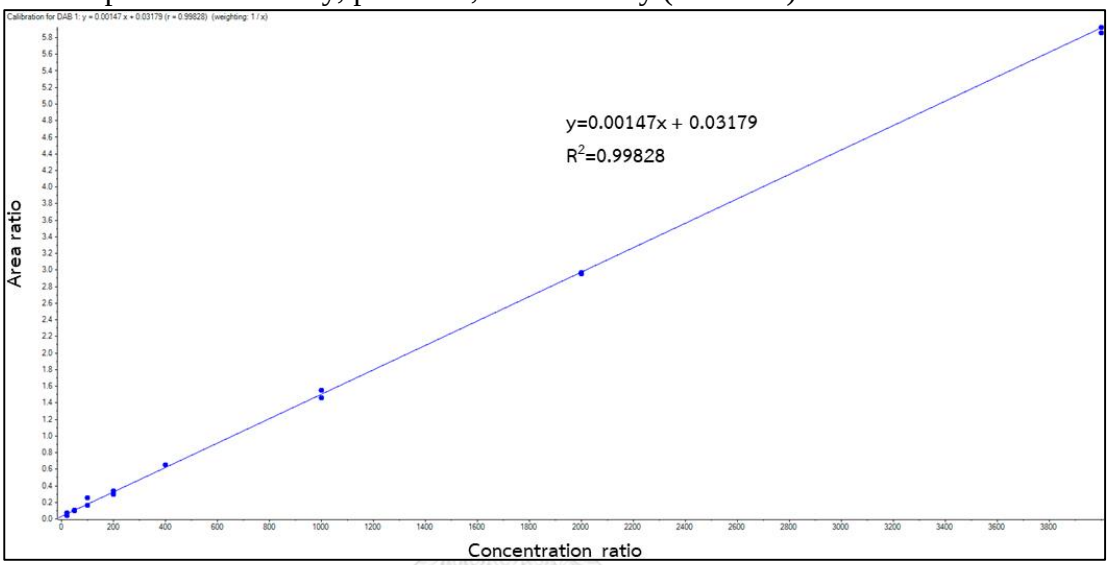


Figure 39. The standard curve and R^2 of dabigatran.

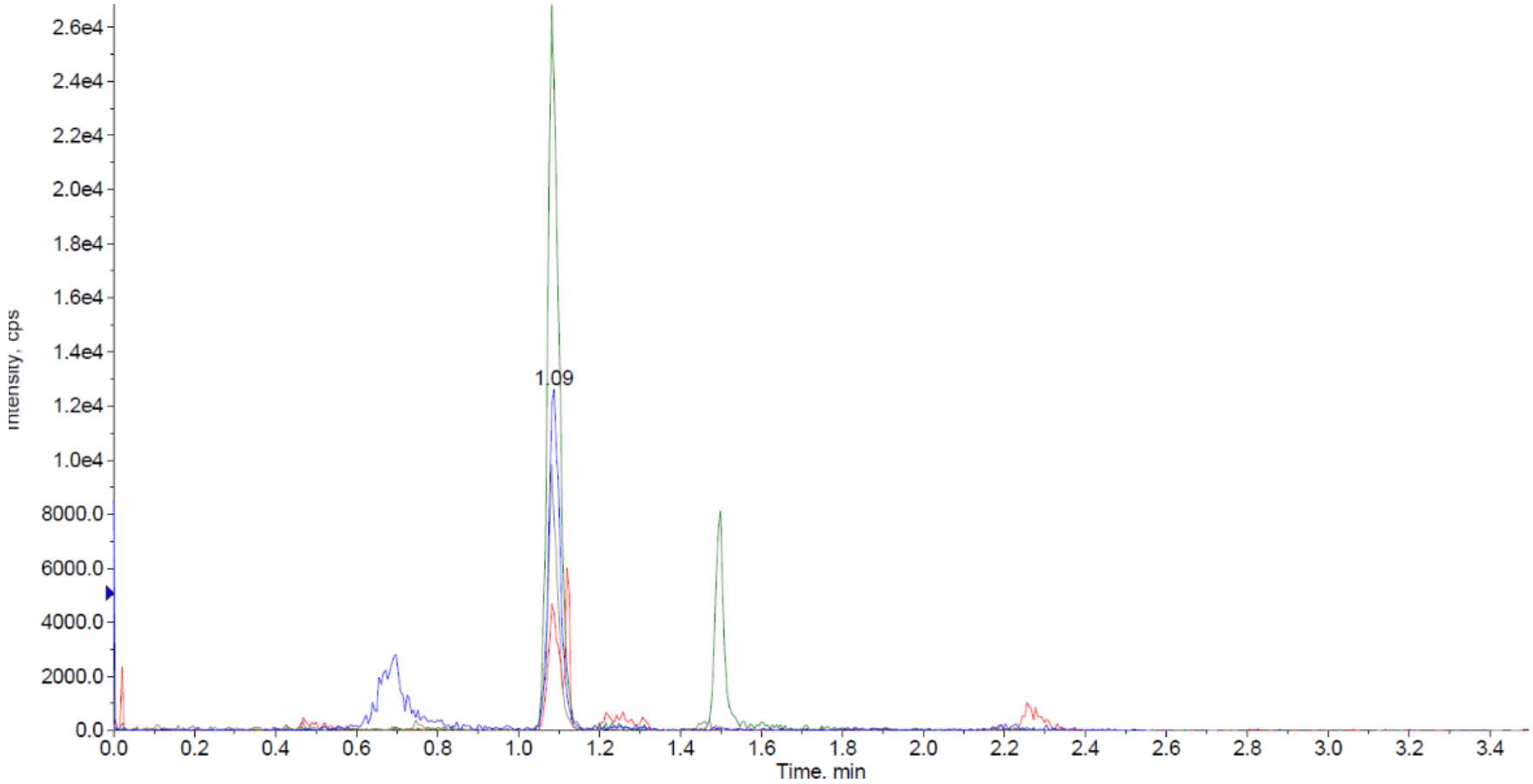
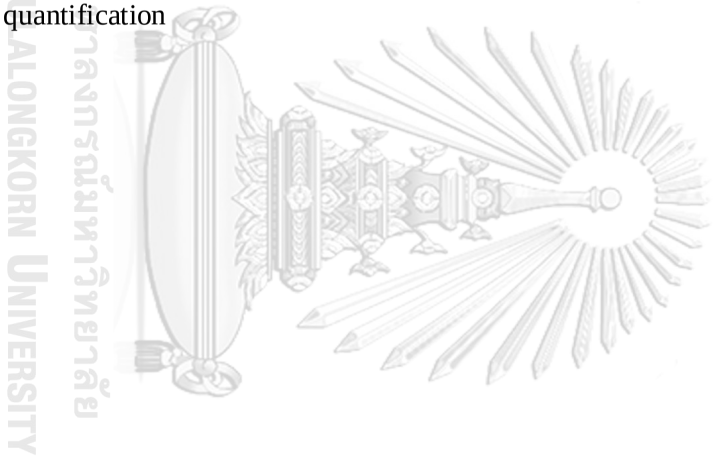


Figure 40. The chromatogram of dabigatran and [$^{13}C_6$]-Dabigatran

Table 84. The partial validation results of dabigatran including precision and accuracy.

Target concentration (pg/mL)	**Intra-day assay (n=5)**			**Inter-day assay (n=10)**		
	Mean concentration (SD) (pg/mL)	**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (pg/mL)**	**Precision (% RSD)**	**Accuracy (%)**
LLOQ (20)	12.68 (1.91)	15.05	63.41	18.03 (2.86)	15.86	90.17
Low (50)	43.74 (3.00)	6.87	87.48	48.02 (4.84)	10.09	96.05
Medium (1200)	1175.55 (29.42)	2.50	97.96	1216.78 (20.64)	1.70	101.40
High (3750)	3625.52 (206.28)	5.69	96.68	3924.37 (140.99)	3.59	104.65

SD, standard deviation; LLOQ, lower of quantification



Statins

Reagent:

1. Pitavastatin calcium
2. Pitavastatin lactone
3. Rosuvastatin calcium salt
4. Atorvastatin calcium salt
5. 2-Hydroxy atovastatin calcium salt
6. 4-Hydroxy atorvastatin hemicalcium salt
7. [$^2H_5$]-Pitavastatin sodium salt (internal standard of pitavastatin)
8. [$^2H_5$]-Pitavastatin lactone (internal standard of pitavastatin lactone)
9. [$^2H_6$]-Rosuvastatin sodium salt (internal standard of rosuvastatin)
10. [$^2H_5$]-Atorvastatin calcium salt (internal standard of atorvastatin)
11. [$^2H_5$]-2-Hydroxy atovastatin (internal standard of 2-hydroxyatorvastatin)
12. [$^2H_5$]-4-Hydroxy atorvastatin (internal standard of 4-hydroxyatorvastatin)

Sample matrix: Plasma

Standard calibration range: 5 - 1000 pg/mL

The standard curves of pitavastatin, pitavastatin lactone, atorvastatin, 2- Hydroxy atorvastatin, 4- Hydroxy atovastatin with R^2 are shown in Figure 41 to Figure 45. The chromatogram and their retention time are presented in Figure 46 and Table 85, respectively. Inter-day (three replications of QC in two days, n=6) and intra-day (three replications of QC, n=5) assays were performed to assess partial validation as shown in Table 85. The results of partial validation including linearity, precision, and accuracy were accepted.

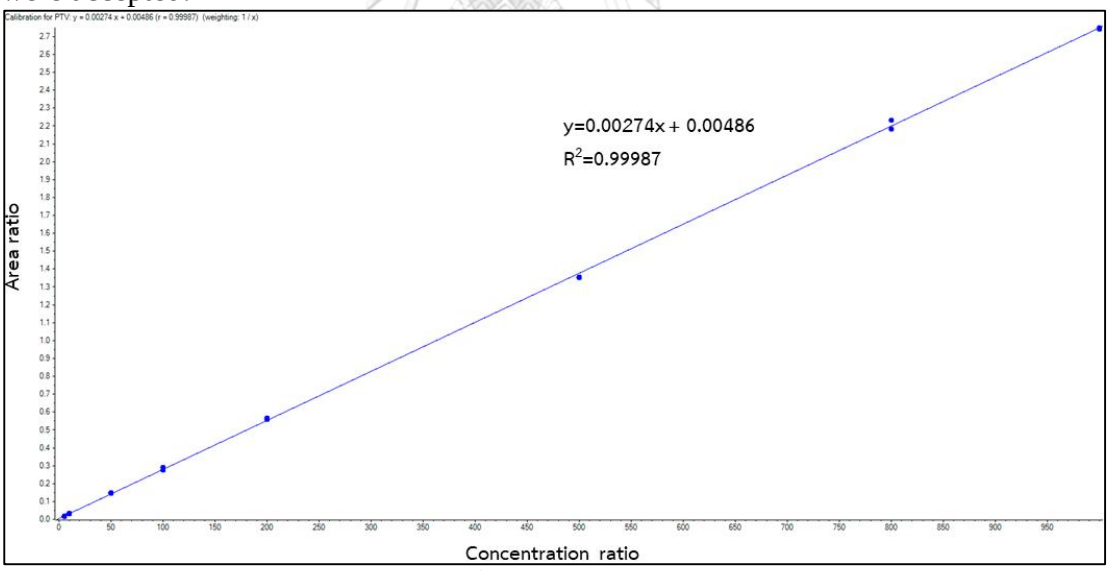


Figure 41. The standard curve and R^2 of pitavastatin

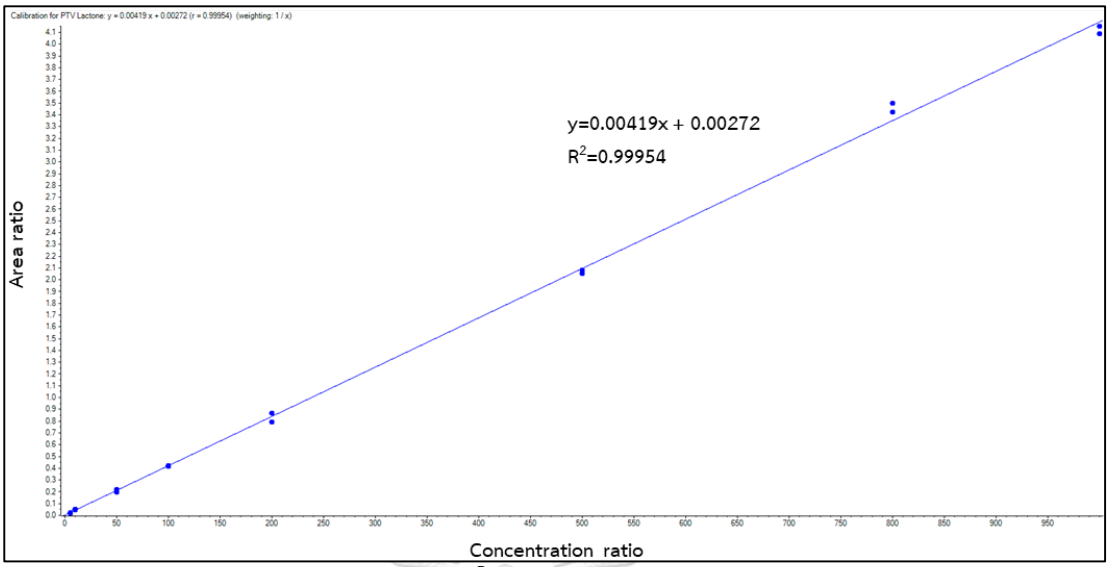


Figure 42. The standard curve and R^2 of pitavastatin lactone

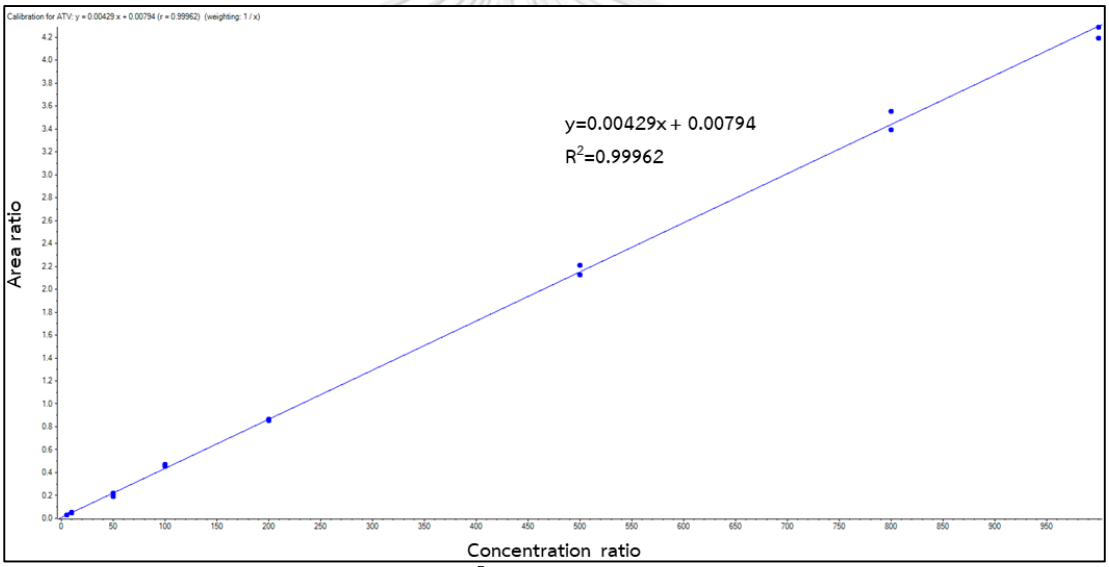


Figure 43. The standard curve and R^2 of atorvastatin

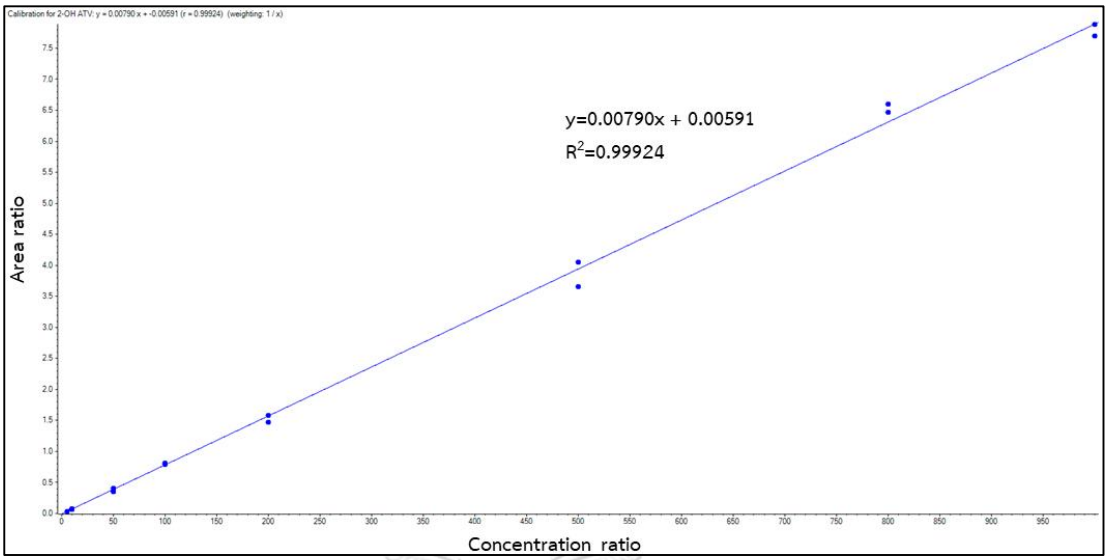


Figure 44. The standard curve and R^2 of 2-hydroxy atorvastatin

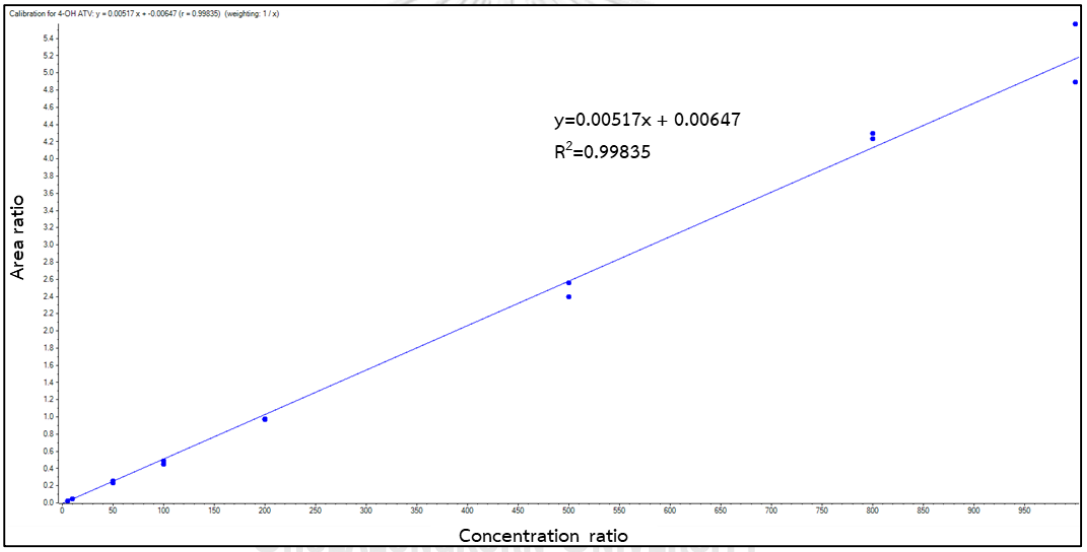


Figure 45. The standard curve and R^2 of 4-hydroxy atorvastatin

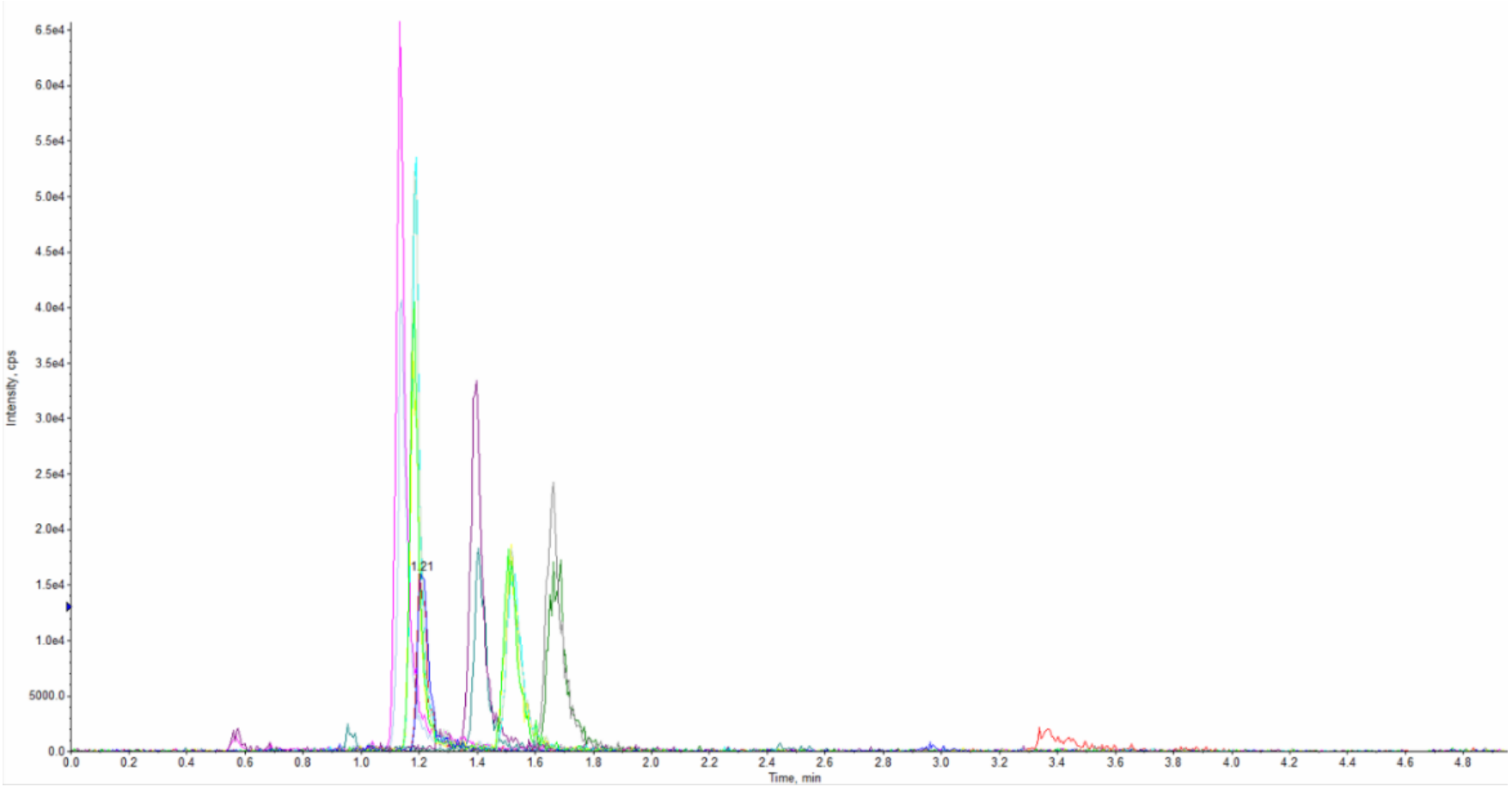


Figure 46. The chromatogram of pitavastatin, pitavastatin lactone, rosuvastatin, atorvastatin, 2-Hydroxy atovastatin, 4-Hydroxy atorvastatin, [$^2H_5$]-Pitavastatin, [$^2H_5$]-Pitavastatin lactone, [$^2H_6$]-Rosuvastatin, [$^2H_5$]-Atorvastatin, [$^2H_5$]-2-Hydroxy atovastatin, [$^2H_5$]-4-Hydroxy atorvastatin

Table 85. The retention time and partial validation results (precision and accuracy) of statins and their metabolites

Compound	Retention time (min)	Target concentration (pg/mL)	Intra-day assay (n=3)			Inter-day assay (n=6)		
			Concentration[a] (pg/mL)	Precision (% RSD)	Accuracy (%)	Concentration[a] (pg/mL)	Precision (% RSD)	Accuracy (%)
Rosuvastatin	1.74	Low (5)	6.39 (1.42)	22.20	127.87	4.67 (2.10)	45.05	93.33
		Medium (250)	260.82 (26.95)	10.33	104.33	263.29 (18.18)	6.90	105.31
		High (750)	785.71 (55.50)	7.06	104.76	772.75 (39.39)	5.10	103.03
Pitavastatin	2.27	LLOQ (1)	1.07 (0.41)	38.13	106.73	0.94 (0.25)	26.58	93.64
		Low (5)	5.20 (0.64)	12.31	103.91	5.46 (0.61)	11.23	109.22
		Medium (250)	269.83 (11.37)	4.21	107.93	269.69 (12.85)	4.76	107.88
		High (750)	778.84 (23.35)	3.00	103.85	797.02 (8.07)	1.01	106.27
Pitavastatin lactone	2.77	LLOQ (1)	0.95 (0.09)	9.14	94.81	1.00 (0.54)	54.13	100.28
		Low (5)	4.66 (0.50)	10.67	93.17	5.11 (0.28)	5.44	102.17
		Medium (250)	251.80 (7.64)	3.03	100.72	234.15 (67.01)	28.62	93.66
		High (750)	714.02 (27.88)	3.90	95.20	679.43 (185.13)	27.25	90.59
Atorvastatin	2.28	LLOQ (1)	1.49 (0.52)	34.67	148.97	1.05(0.62)	58.85	104.58
		Low (5)	4.47 (0.22)	4.99	89.39	4.56 (0.37)	8.11	91.24
		Medium (250)	233.47 (17.99)	7.71	93.39	242.57 (14.91)	6.15	97.03
		High (750)	734.59 (43.26)	5.89	97.94	725.95 (39.86)	5.49	96.79
2-OH atorvastatin	1.62	LLOQ (1)	1.03 (0.32)	31.21	102.76	1.10 (0.47)	42.30	110.14
		Low (5)	5.17 (0.56)	10.87	103.48	5.45 (0.40)	7.41	108.95
		Medium (250)	276.81 (24.12)	8.71	110.73	260.42 (10.74)	4.12	104.17
		High (750)	728.59 (21.65)	2.97	97.14	750.52 (31.01)	4.13	100.07
4-OH atorvastatin	2.14	LLOQ (1)	1.39 (0.20)	14.18	138.79	1.26 (0.43)	34.38	125.73
		Low (5)	5.31 (0.84)	15.80	106.18	5.13 (0.70)	13.62	102.59
		Medium (250)	242.04 (29.32)	12.11	96.82	278.01 (16.25)	5.85	111.20
		High (750)	716.50 (47.01)	6.56	95.53	781.49 (62.47)	7.99	104.20

[a]Data presented in mean (standard deviation, SD)

2-OH atorvastatin, 2-hydroxy atorvastatin; 4-OH atorvastatin, 4-hydroxy atorvastatin; LLOQ, lower of quantification.

Rifampicin

Reagent: Rifampicin, Rifabutin (Internal standard)
Sample matrix: Plasma
Standard calibration range: 0.1 - 20 µg/mL
Retention time: 1.89 min

The standard curves of rifampicin, rifabutin with R^2 are shown in Figure 47. The chromatogram is presented in Figure 48. Inter-day (five replications of QC in two days, n=10) and intra-day (five replications of QC, n=5) assays were performed to assess partial validation as shown in Table 86. The results of partial validation including linearity, precision, and accuracy were accepted.

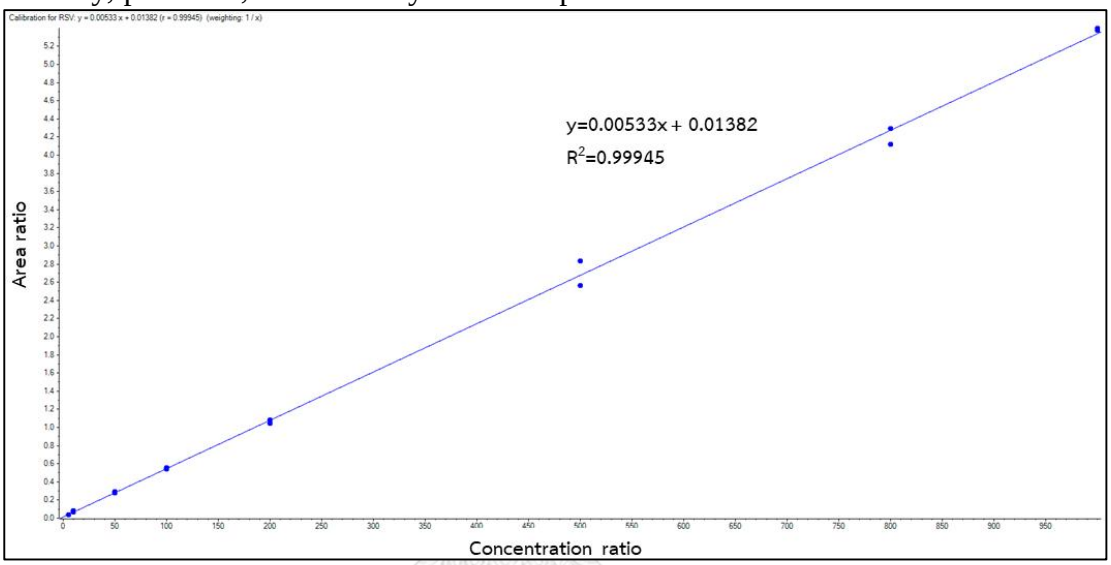


Figure 47. The standard curve and R^2 of 4-hydroxy atorvastatin

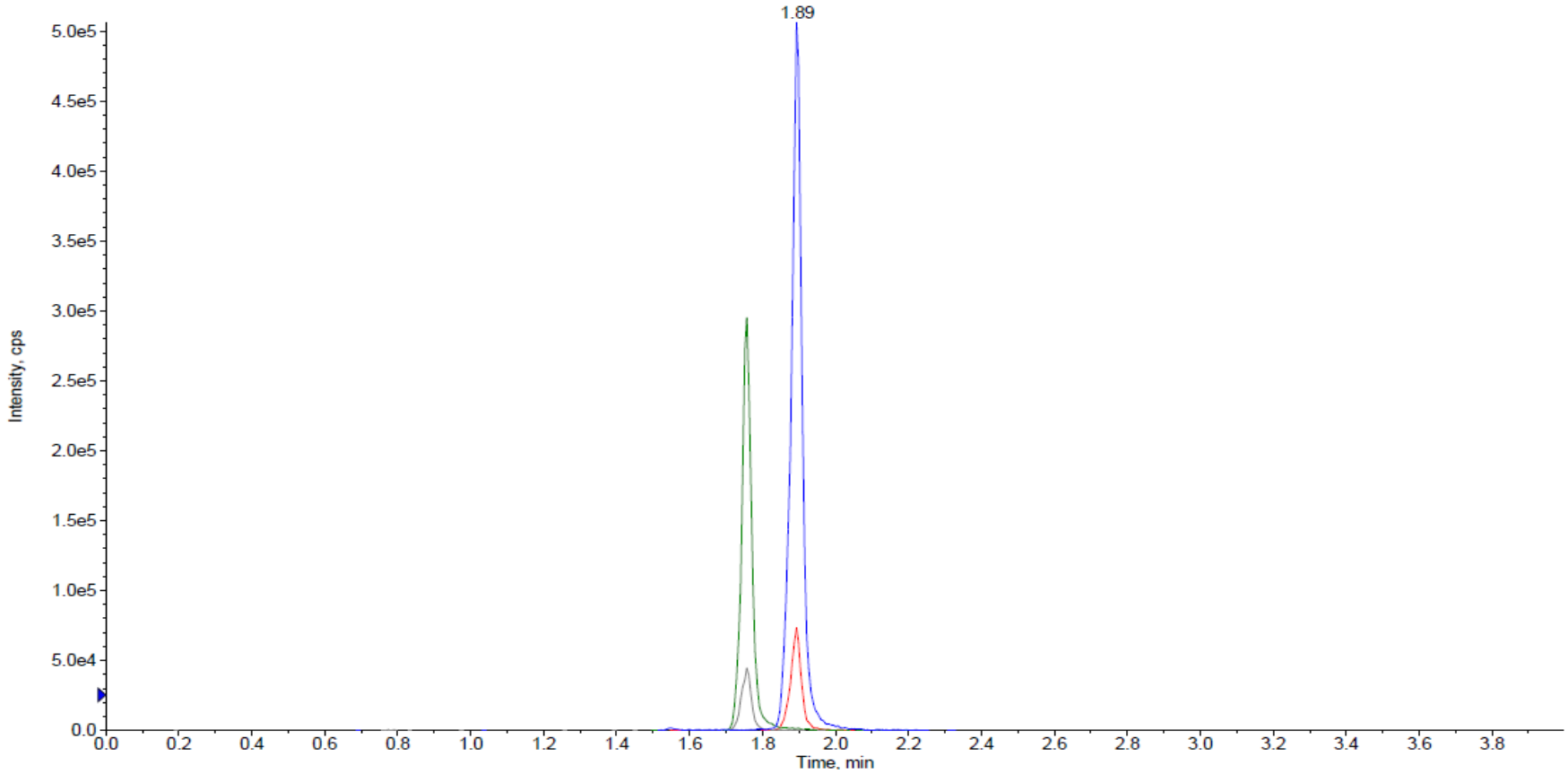


Figure 48. The chromatogram of rifampicin

Table 86. The partial validation results of rifampicin including precision and accuracy were analyzed from quality control

Target concentration (µg/mL)	**Intra-day assay (n=5)**			**Inter-day assay (n=10)**		
	Mean concentration (SD) (µg/mL)	**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (µg/mL)**	**Precision (% RSD)**	**Accuracy (%)**
LLOQ (0.1)	0.11 (0.01)	5.14	112.18	0.10 (0.01)	5.05	104.93
Low (0.4)	0.37 (0.03)	7.07	92.70	0.41 (0.01)	3.48	101.33
Medium (4)	3.85 (0.17)	4.46	96.34	4.11 (0.16)	3.97	102.73
High (15)	15.62 (0.34)	2.16	104.14	15.45 (0.51)	3.30	102.97

SD, standard deviation; LLOQ, lower of quantification

Dabigatran in urine sample

Reagent: Dabigatran, [$^{13}C_6$]-Dabigatran (Internal standard)
Sample matrix: Urine
Standard calibration range: 0.5 - 50 ng/mL
Retention time: 2.58 min
The partial validation results of dabigatran in urine corresponded to acceptance of linearity which shown as standard curve with R^2 in Figure 49, precision and accuracy as presented in Table 87. The precision and accuracy were obtained from Inter-day (five replications of QC in two days, n=10) and intra-day (five replications of QC, n=5) (Table 87). The chromatogram is shown in Figure 50.

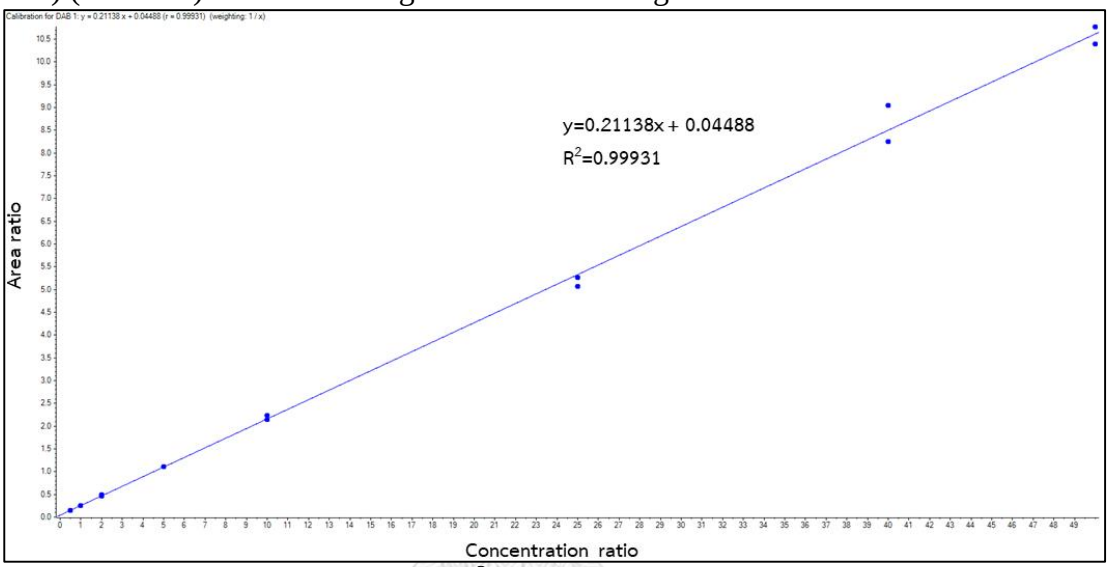


Figure 49. The standard curve and R^2 of dabigatran in urine

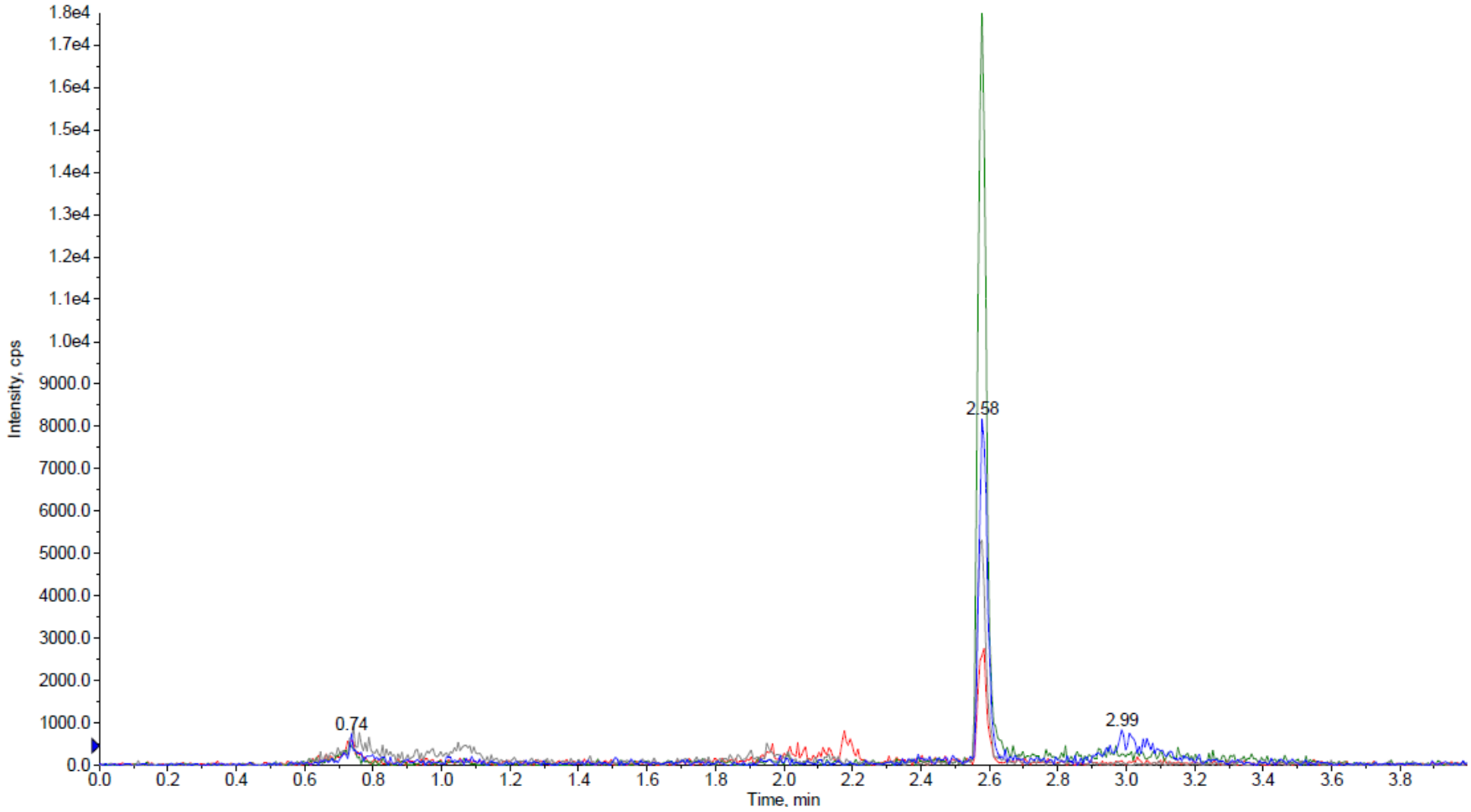


Figure 50. The chromatogram of dabigatran.

Table 87. The partial validation results of dabigatran in urine including precision and accuracy were analyzed from quality control

Target concentration (µg/mL)	**Intra-day assay (n=5)**			**Inter-day assay (n=10)**		
	Mean concentration (SD) (µg/mL)	**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (µg/mL)**	**Precision (% RSD)**	**Accuracy (%)**
LLOQ (0.5)	0.44 (0.08)	18.34	88.81	0.52 (0.08)	14.79	104.71
Low (1.5)	1.58 (0.10)	6.36	105.23	1.75 (0.14)	8.16	116.52
Medium (20)	21.70 (0.85)	3.90	108.48	21.98 (0.63)	2.87	109.90
High (40)	43.65 (1.15)	2.63	109.13	43.64 (1.50)	3.43	109.10

SD, standard deviation; LLOQ, lower of quantification

Rosuvastatin in urine sample

Reagent: Rosuvastatin, [$^{2}H_{6}$]-Rosuvastatin (internal standard)
Sample matrix: Urine
Standard calibration range: 0.5 - 50 ng/mL
Retention time: 2.50 min
The partial validation results of rosuvastatin in urine corresponded to acceptance of linearity which shown as standard curve with R^2 in Figure 51, precision and accuracy as presented in Table 88. The precision and accuracy were obtained from Inter-day (five replications of QC in two days, n=10) and intra-day (five replications of QC, n=5) (Table 88). The chromatogram is shown in Figure 52.

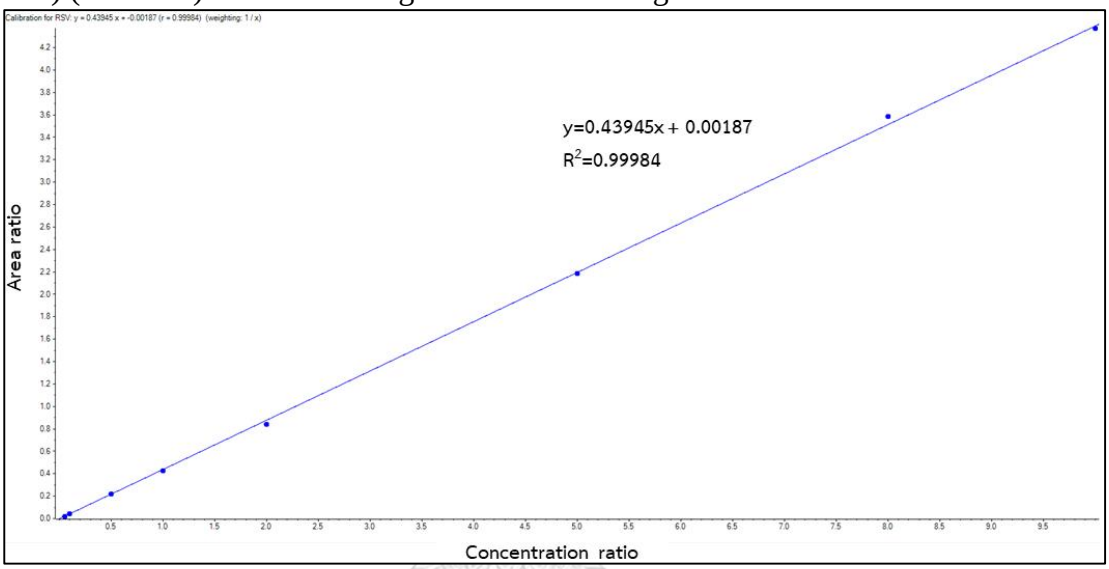


Figure 51. The standard curve and R^2 of rosuvastatin in urine

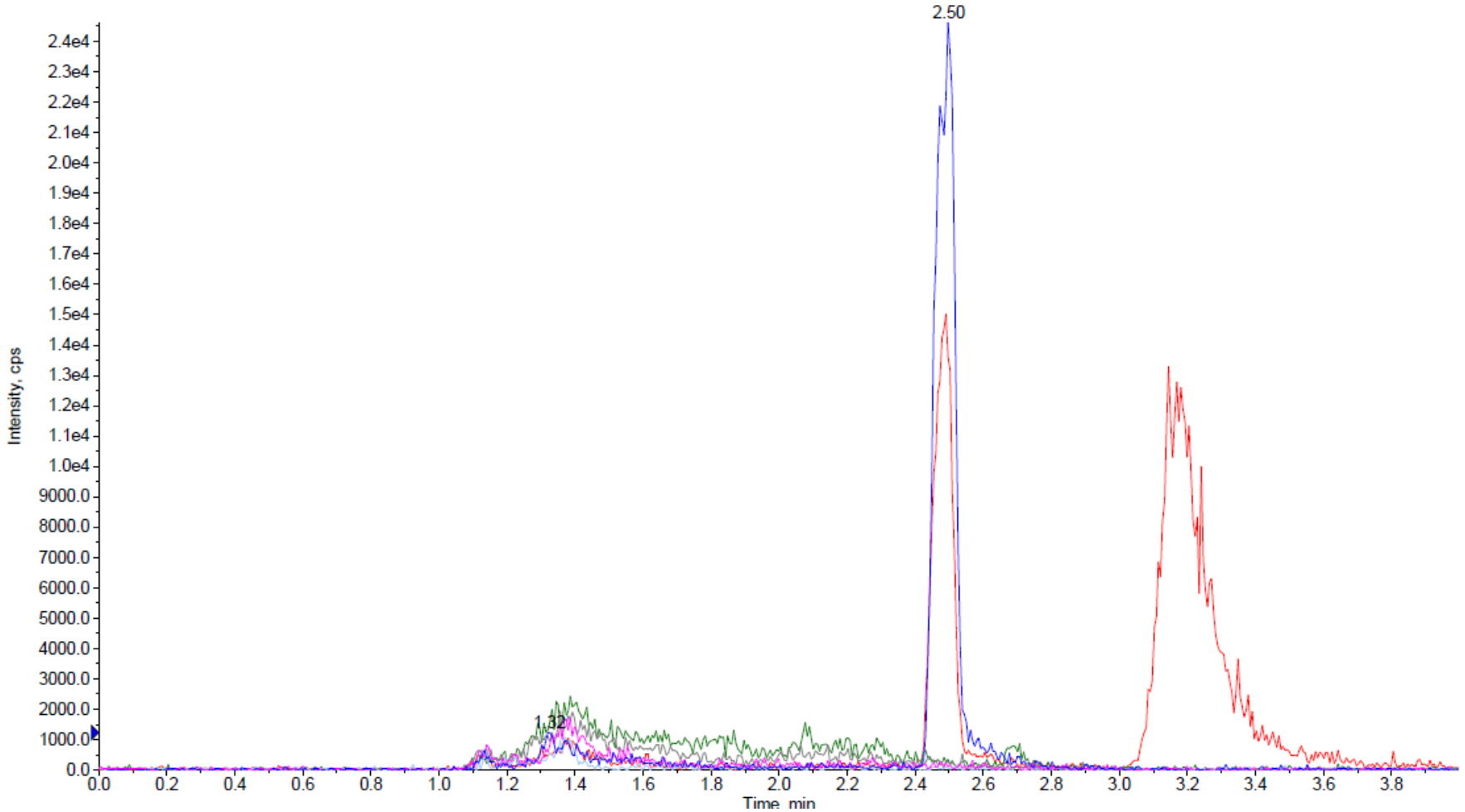


Figure 52. The chromatogram of rosuvastatin

Table 88. The partial validation results of rosuvastatin in urine including precision and accuracy were analyzed from quality control

Target concentration (µg/mL)	**Intra-day assay (n=5)**			**Inter-day assay (n=10)**		
	Mean concentration (SD) (µg/mL)	**Precision (% RSD)**	**Accuracy (%)**	**Mean concentration (SD) (µg/mL)**	**Precision (% RSD)**	**Accuracy (%)**
LLOQ (0.08)	0.08 (0.01)	9.60	97.80	0.07 (0.01)	11.72	85.57
Low (0.3)	0.30 (0.01)	2.07	99.85	0.28 (0.02)	7.91	94.41
Medium (4)	3.91 (0.06)	1.60	97.68	3.70 (0.62)	16.62	92.62
High (9)	8.97 (0.28)	3.15	99.69	8.54 (0.11)	1.33	94.94

SD, standard deviation; LLOQ, lower of quantification

C. Concomitant drugs

Table 89. Concomitant drugs of each subject

Subject number	Group	Concomitant drug	Strength	Total dose (dose per day)[a]
22	3	Sodamint	300 mg	900 mg
		Glipizide	5 mg	10 mg
		Propranolol	25 mg	25 mg
		AmLodipine	10 mg	10 mg
		Aspirin	81 mg	81 mg
		Calcium carbonate	1000 mg	1000 mg
		Folic acid	5 mg	5 mg
		Hemax injection	4000 IU	4000 IU twice a week
24	3	Alfuzosin	10 mg	10 mg
		Sennoside b	7.5 mg	15 mg
		AmLodipine	10 mg	10 mg
		Calciferol	20,000 units	40,000 units per week
25	3	Enalapril	5 mg	5 mg
		Calciferol	20,000 units	20,000 units per week
		Colchicine	0.6 mg	0.6 mg
		Furosemide	40 mg	80 mg
		Manidipine	20 mg	20 mg
		Metoprolol	100 mg	150 mg
		Sodamint	300 mg	1800 mg
		Allopurinol	300 mg	300 mg
		Benzbromarone	100 mg	50 mg
30	2	Silodosin	4 mg	8 mg
31	3	Losartan	50 mg	100 mg
		Manidipine	20 mg	30 mg
		Calciferol	20,000 units	20,000 units per week

		Pocitrin (K, Na)	231.5 mg, 195 mg	463 mg, 390 mg
		Colchicine	0.6 mg	0.6 mg
		Allopurinol	300 mg	300 mg
Subject number	**Group**	**Concomitant drug**	**Strength**	**Total dose (dose per day)[a]**
		Aspirin	81 mg	81 mg
		Simvastatin	10 mg	10 mg
		Atenolol	25 mg	25 mg
		Linagliptin	5 mg	5 mg
32	3	Calciferol	20,000 units	20,000 units per week
		Folic acid	5 mg	5 mg
		Sodamint	300 mg	300 mg
		Enalapril	5 mg	5 mg
		Perindopril	5 mg	1 mg
		Lorazepam	1 mg	300 mg
		Allopurinol	300 mg	2 mg
		Melatonin	2 mg	6 mg
		Betahistine	6 mg	18 mg
33	3	AmLodipine	10 mg	5 mg
		Enalapril	5 mg	20 mg
		Simvastatin	20 mg	1 mg
		Pioglitazone	15 mg	7.5 mg
		Insulin mixtard HM30	100 IU/mL	48 IU
34	3	Alfuzosin	10 mg	10 mg
		AmLodipine	5 mg	5 mg
		Aspirin	81 mg	81 mg
		Simvastatin	20 mg	20 mg
		Insulin novo mix penfill injection	100 IU/mL	50 IU
		Sodium chloride	300 mg	2700 mg
		Cardesartan	16 mg	8 mg

Subject number	Group	Concomitant drug	Strength	Total dose (dose per day)[a]
		Linagliptin	5 mg	5 mg
		Ceterizine	10 mg	10 mg
		Gabapentin	100 mg	100 mg
35	3	Clopidogrel	75 mg	75 mg
		Omeprazole	20 mg	20 mg
		Ferrous sulfate	200 mg	400 mg
		Folic acid	5 mg	5 mg
		Manidipine	10 mg	10 mg
36	3	Losartan	50 mg	50 mg
37	3	AmLodipine	10 mg	10 mg
		Ferrous sulfate	200 mg	600 mg
		Folic acid	5 mg	5 mg
		Hemax injection	4000 IU	4000 IU per week
		Losartan	50 mg	25 mg
		Allopurinol	100 mg	100 mg
		Sodamint	300 mg	600 mg
		Doxazosin	4 mg	4 mg
38	3	Carvedilol	25 mg	25 mg
		Hydralazine	25 mg	225 mg
		Folic acid	5 mg	5 mg
		Lercanidipine	20 mg	20 mg
		Glipizide	5 mg	5 mg
		Pioglitazone	30 mg	30 mg
39	2	Calciferol	20,000 units	20,000 units per week
		Calcium carbonate	1250 mg	1250 mg
42	2	Glucosamine sulphate	500 mg	1500 mg
		Calcium carbonate	1000 mg	1000 mg
45	3	Calciferol	20,000 units	20,000 units per week

Subject number	Group	Concomitant drug	Strength	Total dose (dose per day)[a]
		Calcium carbonate	1000 mg	1000 mg
		Aspirin	81 mg	81 mg
		Simvastatin	10 mg	10 mg
		Ferrous sulfate	200 mg	200 mg
		Folic acid	5 mg	5 mg
		Irbesartan	150 mg	50 mg
		Atenolol	25 mg	2000 mg
		Sodamint	300 mg	40 mg
		Furosemide	40 mg	25 mg
		Doxazosin	4 mg	4 mg
		Eprex	4,000 IU	4,000 IU per week
46	3	Calciferol	20,000 units	2,000 units per week
		Calcium carbonate	1000 mg	1000 mg
		Allopurinol	100 mg	50 mg
		Irbesartan	150 mg	75 mg
		Simvastatin	10 mg	10 mg
		Ezetimibe	10 mg	10 mg
		Manidipine	20 mg	20 mg
47	3	Vitamin B6	100 mg	100 mg
		Pregabalin	150 mg	150 mg
		Diacerin	50 mg	50 mg
		Enalapril	5 mg	5 mg
		Manidipine	20 mg	20 mg
		Omeprazole	20 mg	20 mg
		Nicergoline	30 mg	60 mg
		Simethicone	80 mg	180 mg
		Doxazosin	4 mg	4 mg
		Linagliptin	5 mg	5 mg

Subject number	Group	Concomitant drug	Strength	Total dose (dose per day)[a]
		Glipizide	5 mg	5 mg
48	3	Dutasteride	0.5 mg	0.5 mg
		Silodosin	4 mg	4 mg
		Aspirin	81 mg	81 mg
		Simvastatin	40 mg	40 mg
		Sodamint	300 mg	300 mg
		Allopurinol	100 mg	100 mg
		Colchicine	0.6 mg	0.6 mg
49	3	Ferrous sulfate	200 mg	400 mg
		Folic acid	5 mg	5 mg
		Furosemide	40 mg	40 mg
		Losartan	50 mg	10 mg
		Manidipine	20 mg	20 mg
		Sodamint	300 mg	400 mg
53	3	Enalapril	20 mg	40 mg
		Aspirin	81 mg	81 mg
		Carvedilol	6.25 mg	12.5 mg
		Furosemide	40 mg	20 mg
		Allopurinol	100 mg	200 mg

Table 90. Number of subjects in each concomitant drugs

Concomitant drugs	**Number of subjects**
Alfuzosin	2
Allopurinol	7
AmLodipine	5
Aspirin	5
Atenolol	2
Benzbromarone	1
Betahistine	1
Calciferol	7
Calcium carbonate	5
Cardesartan	1
Carvedilol	2
Ceterizine	1
Clopidogrel	1
Colchicine	3
Diacerin	1
Doxazosin	3
Dutasteride	1
Enalapril	5
Eprex	1
Ezetimibe	1
Ferrous sulfate	4
Folic acid	7
Furosemide	4
Gabapentin	1
Glipizide	3
Glucosamine sulphate	1
Hemax injection	2
Hydralazine	1
Insulin mixtard HM30	1
Insulin novo mix penfill injection	1
Irbesartan	2
Lercanidipine	1
Linagliptin	3
Lorazepam	1
Losartan	4
Manidipine	6
Melatonin	1
Metoprolol	1
Nicergoline	1
Omeprazole	2
Perindopril	1
Pioglitazone	2
Pocitrin (K, Na)	1
Pregabalin	1

Concomitant drugs	Number of subjects
Propranolol	1
Sennoside b	1
Silodosin	2
Simethicone	1
Simvastatin	6
Sodamint	7
Sodium chloride	1
Vitamin B6	1

D. Plasma concentration at each timepoint of microdose cocktail containing drugs in each subject

Table 91. Plasma concentration of midazolam (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	47	92	86	63	47	27	15	7	5	3	2	0	0	0
2	1	0	55	71	47	37	25	14	10	6	3	2	1	0	0	0
3	1	0	63	67	57	50	36	22	14	7	4	2	1	0	0	0
4	1	0	33	73	63	45	31	17	11	5	3	2	1	0	0	0
5	1	0	27	71	63	41	31	16	9	5	3	2	1	0	0	0
6	1	0	31	68	63	47	38	18	14	6	4	2	2	0	0	0
7	1	0	74	141	131	108	81	45	32	15	8	5	3	0	0	0
8	1	0	83	118	83	60	40	26	22	11	6	5	3	0	0	0
9	1	0	56	88	70	41	29	18	11	7	4	2	1	0	0	0
10	1	0	28	78	71	62	42	23	15	7	3	3	2	0	0	0
11	1	0	20	81	95	94	62	35	23	10	7	5	4	0	0	0
12	1	0	68	111	81	49	29	14	10	5	2	1	0	0	0	0
13	1	0	27	64	63	51	43	21	14	5	4	2	0	0	0	0
14	1	0	8	28	24	17	12	6	3	0	0	0	0	0	0	0
15	1	0	14	40	41	30	21	13	8	3	0	0	0	0	0	0
16	1	0	43	71	54	41	28	20	12	4	3	0	0	0	0	0
17	1	0	46	109	84	62	46	26	17	7	3	1	0	0	0	0
18	1	0	17	78	61	53	39	22	17	7	5	3	2	0	0	0
19	1	0	96	153	121	84	59	34	28	13	9	5	3	0	0	0
20	1	0	61	98	80	55	38	20	12	5	2	1	0	0	0	0
21	2	0	26	182	159	105	79	50	36	15	11	13	9	3	2	1
23	2	0	41	87	97	80	73	47	31	16	10	8	6	2	0	0
26	2	0	95	92	63	43	32	20	10	5	3	2	1	0	0	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
27	2	0	89	214	192	153	104	68	45	27	19	17	10	4	1	0
28	2	0	46	71	58	51	37	20	15	7	4	4	3	0	0	0
29	2	0	20	71	75	54	42	28	19	9	6	4	3	0	0	0
30	2	0	29	129	92	68	53	35	25	13	7	5	4	0	0	0
39	2	0	178	228	181	123	95	56	41	22	15	13	10	2	0	0
40	2	0	401	287	208	161	117	71	57	33	22	17	15	3	0	0
41	2	0	199	217	156	118	79	61	49	29	22	18	13	5	1	0
42	2	0	29	148	141	87	52	33	22	12	8	6	5	1	0	0
43	2	0	158	179	148	110	86	57	39	24	18	14	9	0	0	0
44	2	0	39	134	153	134	96	56	37	17	10	7	5	0	0	0
50	2	0	84	134	137	90	57	31	26	14	9	8	6	2	0	0
51	2	0	168	145	94	58	44	26	19	11	7	4	3	0	0	0
52	2	0	89	135	155	150	94	57	40	22	17	13	10	3	1	0
22	3	0	19	147	139	114	76	52	39	23	18	12	8	4	2	1
24	3	0	139	181	151	95	69	55	40	22	13	11	8	3	0	0
25	3	0	41	87	91	87	79	79	56	32	25	18	14	6	2	1
31	3	0	12	127	153	95	75	47	33	17	11	7	5	2	0	0
32	3	0	105	145	107	62	49	27	23	13	9	8	5	2	0	0
33	3	0	45	138	101	82	69	54	42	28	22	20	15	6	3	1
34	3	0	55	110	89	58	36	23	16	8	6	5	3	0	0	0
35	3	0	101	182	155	124	75	53	38	22	17	12	9	4	0	0
36	3	0	106	154	149	117	89	61	49	27	19	13	9	1	0	0
37	3	0	242	223	170	109	75	55	40	21	11	9	4	0	0	0
38	3	0	53	115	112	70	53	38	31	21	15	9	7	2	0	0
45	3	0	48	157	138	104	80	47	35	21	19	14	11	3	0	0
46	3	0	235	206	149	99	79	52	47	28	28	22	19	9	3	1

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
47	3	0	97	204	210	214	160	99	76	47	36	30	21	6	0	0
48	3	0	196	243	220	159	112	74	55	33	23	18	13	5	1	0
49	3	0	66	37	82	60	46	28	22	11	6	4	2	0	0	0
53	3	0	85	171	201	128	123	75	65	31	21	15	10	2	0	0

Table 92. Plasma concentration of midazolam (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	38	121	114	99	41	39	25	14	8	5	3	0	0	0
2	1	0	13	43	69	51	68	26	15	8	4	3	1	0	0	0
3	1	0	65	94	90	50	46	23	16	8	5	3	2	0	0	0
4	1	0	41	131	124	69	65	36	23	13	8	4	2	0	0	0
5	1	0	24	95	80	52	39	20	13	8	6	3	2	0	0	0
6	1	0	22	57	86	80	63	34	22	11	6	4	2	1	0	0
7	1	0	51	136	178	144	118	61	45	23	15	9	5	0	0	0
8	1	0	66	91	88	65	47	27	20	9	9	4	2	0	0	0
9	1	0	37	112	87	64	38	21	19	11	5	4	2	0	0	0
10	1	0	34	127	95	69	49	24	19	8	6	4	1	0	0	0
11	1	0	25	111	113	116	92	51	29	16	11	5	4	3	1	0
12	1	0	89	124	85	47	35	17	14	5	2	1	0	0	0	0
13	1	0	25	82	66	56	42	25	15	7	4	1	0	0	0	0
14	1	0	11	27	22	19	17	8	5	0	0	0	0	0	0	0
15	1	0	9	48	49	41	28	23	13	5	2	0	0	0	0	0
16	1	0	10	56	53	50	36	23	14	7	3	2	0	0	0	0
17	1	0	69	142	116	73	64	42	30	15	8	3	2	0	0	0
18	1	0	42	97	87	56	38	19	12	7	4	2	2	0	0	0
19	1	0	70	155	125	92	71	49	32	16	12	7	4	0	0	0
20	1	0	96	122	105	71	45	25	17	9	5	4	0	0	0	0

Table 93. Plasma concentration of dabigatran (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	158	448	592	535	564	478	356	239	192	126	100	35	0	0
2	1	0	233	648	527	790	633	568	486	355	266	175	138	39	0	0
3	1	0	311	424	577	538	479	398	277	196	133	99	85	29	0	0
4	1	0	60	218	298	304	320	284	269	168	131	84	64	0	0	0
5	1	0	133	350	349	387	322	225	207	141	94	73	53	0	0	0
6	1	0	148	322	471	459	405	395	256	170	122	100	60	0	0	0
7	1	0	106	375	440	488	438	398	331	216	167	124	94	30	0	0
8	1	0	112	492	574	527	450	398	296	169	119	83	64	25	0	0
9	1	0	108	307	397	372	329	295	309	158	124	98	66	0	0	0
10	1	0	38	167	235	293	260	239	173	120	80	60	46	0	0	0
11	1	0	71	283	353	402	365	269	197	126	51	44	67	0	0	0
12	1	0	74	207	288	297	267	213	183	134	88	74	56	25	0	0
13	1	0	77	145	289	269	253	196	171	124	94	75	55	0	0	0
14	1	0	38	126	120	126	105	97	71	61	41	30	25	0	0	0
15	1	0	66	196	215	226	220	182	143	97	82	48	39	0	0	0
16	1	0	47	199	222	201	219	167	151	128	88	68	49	19	0	0
17	1	0	242	543	564	572	507	478	382	274	210	164	113	45	0	0
18	1	0	30	134	178	150	193	123	135	89	72	67	60	0	0	0
19	1	0	153	347	349	370	350	289	276	214	147	125	94	37	0	0
20	1	0	132	453	668	526	457	474	356	234	178	143	106	33	0	0
21	2	0	76	613	789	744	736	632	624	414	292	219	203	54	0	0
23	2	0	110	239	333	443	464	457	375	256	200	131	114	38	0	0
26	2	0	240	462	531	620	553	461	412	278	201	162	137	39	0	0
27	2	0	140	420	640	785	868	775	644	485	380	295	237	90	34	0
28	2	0	36	137	219	284	265	231	206	153	104	83	65	0	0	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	68	144	242	231	241	181	156	92	71	53	40	0	0	0
30	2	0	70	250	265	281	266	224	196	133	112	85	59	0	0	0
39	2	0	298	560	688	620	630	514	478	336	240	187	136	35	0	0
40	2	0	215	226	259	280	259	256	208	165	145	122	100	44	0	0
41	2	0	184	447	499	461	384	347	351	290	222	173	148	48	0	0
42	2	0	150	497	625	599	483	404	318	233	190	140	109	29	0	0
43	2	0	132	267	274	302	275	239	208	126	91	83	63	31	0	0
44	2	0	27	187	381	477	498	453	380	292	234	199	144	63	31	0
50	2	0	195	370	616	604	579	342	323	242	166	130	104	32	0	0
51	2	0	196	373	398	361	374	300	239	197	158	120	90	22	0	0
52	2	0	104	275	592	846	798	820	781	609	495	398	310	99	32	0
22	3	0	32	437	622	771	838	772	701	578	580	441	419	266	159	120
24	3	0	282	644	842	970	868	810	859	720	631	553	493	342	230	159
25	3	0	34	123	187	275	249	331	375	374	325	251	234	161	98	55
31	3	0	0	205	419	474	458	428	379	294	249	200	160	87	36	0
32	3	0	147	541	583	553	573	434	398	295	233	183	169	79	39	0
33	3	0	76	265	328	319	302	310	305	275	236	200	175	105	61	27
34	3	0	81	424	674	737	682	592	538	464	377	331	320	163	105	62
35	3	0	60	342	502	698	693	600	508	442	388	363	289	196	124	83
36	3	0	179	411	541	577	622	550	502	421	352	282	266	142	73	53
37	3	0	227	430	620	779	651	635	671	515	456	448	405	307	217	164
38	3	0	81	354	532	492	488	391	365	346	311	277	293	198	136	98
45	3	0	81	229	380	383	370	360	335	301	281	254	242	169	121	85
46	3	0	456	678	862	1022	1111	1145	983	880	718	641	637	367	236	116
47	3	0	35	370	717	1207	1426	1450	1369	1236	1102	917	809	410	203	138
48	3	0	295	462	655	636	577	507	477	409	299	270	257	158	86	69

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	**0.33 hr**	**0.67 hr**	**1 hr**	**1.5 hr**	**2 hr**	**3 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**	**12 hr**	**24 hr**	**36 hr**	**48 hr**
49	3	0	163	69	227	264	299	296	293	260	255	236	196	152	120	0
53	3	0	609	978	1100	755	900	790	701	498	410	373	321	165	95	51

Table 94. Plasma concentration of dabigatran (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	135	329	653	868	869	873	738	525	368	280	237	92	59	25
2	1	0	55	251	472	680	941	998	957	760	527	370	351	154	20	0
3	1	0	345	626	789	956	1001	831	703	432	316	221	213	41	0	0
4	1	0	131	391	766	764	748	713	603	409	298	239	160	72	33	0
5	1	0	67	473	711	905	1106	870	735	487	395	243	194	54	0	0
6	1	0	114	395	513	671	815	838	733	549	409	293	317	45	0	0
7	1	0	117	342	557	766	790	982	869	657	407	330	281	83	61	0
8	1	0	120	360	573	788	789	698	489	273	279	151	113	30	0	0
9	1	0	110	264	419	721	834	709	629	486	308	283	195	0	0	0
10	1	0	133	402	482	619	755	712	594	424	351	277	198	71	24	0
11	1	0	66	326	487	778	962	680	684	400	437	169	184	46	46	0
12	1	0	106	260	319	418	434	373	344	246	166	128	109	52	0	0
13	1	0	66	238	309	355	391	347	277	197	145	115	85	35	0	0
14	1	0	96	282	395	494	531	457	383	242	199	147	105	35	0	0
15	1	0	55	241	333	446	429	381	298	224	150	107	70	38	0	0
16	1	0	28	168	262	367	446	421	350	256	170	152	111	47	0	0
17	1	0	216	468	600	812	771	848	792	577	402	302	232	86	35	25
18	1	0	67	177	259	345	362	316	298	219	156	107	77	0	0	0
19	1	0	135	382	475	654	602	585	494	349	263	209	181	51	0	0
20	1	0	241	552	706	743	695	712	581	397	281	225	160	43	0	0

Table 95. Plasma concentration of pitavastatin (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	189	242	168	84	49	25	18	14	12	7	6	3	2	0
2	1	0	176	165	97	46	29	21	17	11	9	6	7	3	0	0
3	1	0	217	220	128	80	55	25	23	12	9	6	5	3	0	0
4	1	0	97	131	93	52	34	20	16	11	10	5	4	1	0	0
5	1	0	133	211	136	78	64	34	27	13	11	7	7	4	2	2
6	1	0	71	123	109	65	40	24	22	9	7	7	5	2	0	0
7	1	0	228	216	167	78	48	30	23	16	9	6	6	6	4	0
8	1	0	195	270	231	126	65	33	26	15	12	10	7	5	2	2
9	1	0	85	205	163	166	99	57	36	19	12	9	6	6	2	3
10	1	0	144	225	157	94	50	25	18	12	8	5	4	3	2	1
11	1	0	131	186	108	55	41	21	19	9	10	8	4	2	0	0
12	1	0	110	136	99	39	30	17	11	8	7	5	4	2	0	0
13	1	0	90	102	75	53	40	29	24	14	13	8	6	4	2	0
14	1	0	61	118	101	72	45	31	26	13	12	8	7	5	2	2
15	1	0	51	75	56	35	25	18	15	9	7	5	4	2	1	0
16	1	0	111	185	132	86	53	31	26	16	17	9	7	3	1	0
17	1	0	100	149	105	70	51	36	30	21	15	9	8	6	2	3
18	1	0	36	73	57	36	28	19	14	11	9	7	6	4	2	0
19	1	0	244	226	130	73	53	35	25	18	13	9	8	6	2	0
20	1	0	85	101	76	52	35	17	17	13	11	9	6	4	1	0
21	2	0	46	113	86	49	32	16	13	9	7	7	5	4	2	0
23	2	0	44	72	67	65	46	27	16	10	7	6	5	3	1	0
26	2	0	154	146	131	83	56	23	19	13	9	7	6	3	0	0
27	2	0	46	89	82	71	51	23	17	14	9	6	5	3	0	0
28	2	0	92	131	105	50	34	21	15	10	9	7	4	1	1	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	66	139	126	85	48	36	23	14	13	8	5	3	2	0
30	2	0	293	211	139	89	60	34	27	17	15	12	10	4	2	0
39	2	0	46	113	86	49	32	16	13	9	7	7	5	4	2	0
40	2	0	199	177	146	107	86	57	39	17	19	14	11	7	3	0
41	2	0	229	290	190	113	60	39	31	20	13	10	12	7	3	2
42	2	0	120	155	95	60	37	19	16	7	8	8	6	3	2	1
43	2	0	199	216	147	111	74	36	27	18	14	15	8	7	3	1
44	2	0	32	156	141	70	40	21	15	13	9	7	5	2	2	0
50	2	0	100	130	107	61	43	20	14	7	7	5	4	1	1	2
51	2	0	219	221	164	88	49	26	18	10	7	5	5	2	0	0
52	2	0	68	104	124	119	88	46	24	14	10	7	6	6	3	6
22	3	0	61	160	128	80	40	24	15	10	10	10	6	2	1	0
24	3	0	239	257	197	131	77	44	34	21	22	13	11	5	5	0
25	3	0	87	101	86	65	49	41	26	14	11	8	8	5	3	0
31	3	0	20	187	266	184	132	54	45	22	17	11	10	5	2	2
32	3	0	105	211	155	66	63	30	23	15	10	10	7	6	2	2
33	3	0	70	115	78	58	38	28	23	10	7	6	4	2	1	1
34	3	0	63	130	108	60	39	22	13	9	6	6	6	5	2	1
35	3	0	153	236	181	95	60	38	23	16	17	13	12	7	2	5
36	3	0	395	372	226	151	103	68	53	42	22	21	19	12	5	3
37	3	0	375	364	325	127	101	68	41	20	20	13	10	7	2	3
38	3	0	199	321	213	176	87	52	36	24	17	13	9	8	5	3
45	3	0	170	300	230	136	84	55	38	22	20	17	10	7	3	1
46	3	0	156	288	220	124	81	54	42	28	19	17	14	7	3	1
47	3	0	92	274	242	186	99	44	28	16	15	13	9	6	2	1
48	3	0	414	410	264	177	120	66	49	30	23	24	19	14	8	5

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	**0.33 hr**	**0.67 hr**	**1 hr**	**1.5 hr**	**2 hr**	**3 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**	**12 hr**	**24 hr**	**36 hr**	**48 hr**
49	3	0	379	405	270	192	123	59	45	30	23	25	17	13	7	5
53	3	0	175	217	151	87	59	26	24	13	13	141	9	7	4	3

Table 96. Plasma concentration of pitavastatin (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	**0.33 hr**	**0.67 hr**	**1 hr**	**1.5 hr**	**2 hr**	**3 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**	**12 hr**	**24 hr**	**36 hr**	**48 hr**
1	1	0	261	1116	954	637	393	233	160	108	58	40	33	7	0	0
2	1	0	23	392	620	564	414	244	159	75	37	31	29	3	**3**	2
3	1	0	548	1128	775	437	312	168	99	58	34	18	17	2	0	0
4	1	0	807	1203	792	480	324	168	136	65	37	20	10	2	0	0
5	1	0	356	1110	717	449	344	189	144	63	44	26	14	4	0	0
6	1	0	62	316	643	532	400	232	159	74	42	22	14	3	0	0
7	1	0	268	896	752	624	478	272	199	98	62	43	27	5	3	0
8	1	0	950	1404	1009	644	444	274	216	75	58	31	20	4	0	0
9	1	0	48	922	854	601	400	262	179	77	46	31	15	2	2	1
10	1	0	203	1510	1127	757	566	336	221	113	59	39	20	5	1	1
11	1	0	650	1369	1169	823	592	373	219	107	63	34	23	4	1	0
12	1	0	398	729	622	399	287	211	137	63	37	25	14	2	0	0
13	1	0	633	620	481	310	216	141	90	43	30	13	9	2	**2**	1
14	1	0	126	559	410	272	184	122	84	37	22	15	9	3	1	0
15	1	0	79	511	428	259	158	74	53	29	16	11	9	1	0	0
16	1	0	76	603	594	363	274	140	94	49	32	20	12	3	0	0
17	1	0	353	948	719	538	400	269	173	103	69	36	26	5	3	2
18	1	0	202	601	431	240	160	82	55	30	18	12	6	1	0	0
19	1	0	600	1021	744	440	301	184	126	70	49	**35**	21	4	3	0
20	1	0	490	612	468	341	230	116	91	38	30	19	13	2	2	0

Table 97. Plasma concentration of pitavastatin lactone (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	14	56	81	81	79	74	58	47	40	27	22	11	9	4
2	1	0	18	45	48	44	42	39	35	23	22	14	16	7	3	3
3	1	0	22	48	61	63	66	48	45	29	20	14	10	5	3	1
4	1	0	11	52	62	77	70	54	44	35	21	17	15	6	4	3
5	1	0	11	56	72	66	69	52	47	34	26	16	15	7	5	4
6	1	0	8	31	51	44	49	40	36	25	20	16	12	6	1	0
7	1	0	14	55	58	61	66	57	49	34	26	18	14	7	5	4
8	1	0	13	51	90	85	82	74	69	43	38	35	21	14	7	5
9	1	0	4	25	35	59	62	54	56	39	25	22	15	10	4	3
10	1	0	5	31	47	61	58	49	49	30	25	13	11	7	2	0
11	1	0	13	39	40	45	67	45	32	21	19	14	11	2	0	0
12	1	0	8	41	54	35	40	36	28	18	13	9	6	2	1	0
13	1	0	8	33	62	51	48	48	39	27	23	16	12	6	4	1
14	1	0	6	24	31	39	38	31	31	17	14	9	9	6	2	1
15	1	0	5	21	25	26	28	23	18	12	8	5	4	1	0	0
16	1	0	8	38	52	61	50	44	34	28	23	13	9	5	1	0
17	1	0	12	60	74	82	75	64	52	35	27	15	10	9	3	3
18	1	0	2	21	34	35	32	29	26	18	17	12	10	6	2	2
19	1	0	24	59	65	61	53	42	35	24	18	12	9	6	0	0
20	1	0	14	43	59	60	53	41	31	22	15	12	9	7	1	0
21	2	0	5	41	55	50	55	42	41	29	22	18	15	11	6	3
23	2	0	6	19	30	39	41	33	34	24	16	16	10	5	3	2
26	2	0	13	38	50	59	56	42	36	27	24	17	13	7	2	0
27	2	0	2	14	21	30	29	27	27	16	13	10	8	5	0	0
28	2	0	4	20	36	43	40	35	32	17	14	12	8	5	2	1

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	3	23	39	38	38	36	34	25	16	13	11	5	3	2
30	2	0	34	82	94	102	85	59	56	41	34	30	24	11	5	3
39	2	0	5	41	55	50	55	42	41	29	22	18	15	11	6	3
40	2	0	32	57	76	79	80	72	70	51	45	33	29	17	5	5
41	2	0	23	66	80	83	71	51	53	35	36	28	22	12	6	6
42	2	0	7	51	62	65	56	47	41	30	26	21	15	10	5	2
43	2	0	17	58	66	78	70	62	53	38	34	30	18	13	8	3
44	2	0	3	24	50	60	53	45	36	32	22	15	14	7	0	0
50	2	0	8	22	48	52	48	32	30	20	15	13	11	5	3	2
51	2	0	10	28	42	61	55	50	40	30	24	19	15	9	3	2
52	2	0	3	12	31	48	53	56	48	41	32	25	19	10	6	4
22	3	0	2	39	60	73	78	54	57	37	30	24	15	8	4	7
24	3	0	10	41	57	71	66	60	61	47	37	28	25	13	9	5
25	3	0	3	17	31	48	47	58	58	47	38	32	31	15	11	7
31	3	0	9	17	46	65	77	55	51	40	34	20	18	9	4	2
32	3	0	5	61	85	78	81	52	50	38	29	26	18	11	4	4
33	3	0	2	17	23	30	27	24	24	18	13	9	8	4	2	2
34	3	0	3	20	38	54	48	41	34	31	21	18	14	11	3	2
35	3	0	9	42	72	103	85	81	56	47	49	43	37	21	9	6
36	3	0	14	41	51	58	66	61	63	51	35	30	28	13	10	5
37	3	0	7	27	54	49	79	113	107	74	67	52	45	19	10	6
38	3	0	7	26	44	74	47	49	47	46	40	36	27	16	11	9
45	3	0	13	56	97	72	67	54	40	34	32	26	24	7	6	2
46	3	0	17	62	79	80	66	53	49	39	33	31	21	9	5	2
47	3	0	1	63	105	116	130	81	77	49	40	34	33	16	6	4
48	3	0	29	48	92	128	128	97	96	92	69	62	51	29	15	12

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
49	3	0	27	52	88	113	131	104	93	80	65	56	50	34	17	8
53	3	0	10	36	62	29	67	58	58	38	33	30	23	17	8	7

Table 98. Plasma concentration of pitavastatin lactone (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	10	55	66	82	53	84	69	49	44	31	33	11	4	4
2	1	0	3	24	46	44	91	46	44	36	22	18	15	5	3	3
3	1	0	26	84	82	82	76	51	44	27	25	17	11	4	1	1
4	1	0	17	63	64	75	81	60	58	44	29	24	18	8	3	1
5	1	0	8	54	73	67	61	54	45	34	23	17	13	7	2	0
6	1	0	4	26	65	76	79	71	66	43	30	22	16	6	3	2
7	1	0	8	56	82	96	95	78	71	49	41	30	23	8	6	3
8	1	0	34	106	117	100	105	80	82	36	33	25	15	6	0	0
9	1	0	5	38	55	65	54	58	51	36	24	25	18	6	4	3
10	1	0	8	70	76	77	80	72	64	52	35	23	18	5	2	2
11	1	0	14	46	71	81	94	79	60	42	32	24	15	8	1	1
12	1	0	28	81	85	93	74	60	56	38	24	18	14	5	2	2
13	1	0	22	47	57	61	56	57	51	30	27	16	14	5	1	0
14	1	0	7	44	46	50	54	41	39	23	20	10	8	3	2	0
15	1	0	1	26	37	36	36	30	27	17	11	7	4	1	0	0
16	1	0	2	33	54	51	50	47	35	31	23	16	18	5	1	0
17	1	0	19	81	80	89	97	89	81	58	57	35	30	18	7	3
18	1	0	9	45	62	65	54	44	35	27	21	13	11	4	3	0
19	1	0	19	73	61	78	64	53	49	37	29	22	15	6	4	1
20	1	0	22	66	91	72	61	54	47	32	25	19	12	5	3	2

Table 99. Plasma concentration of atorvastatin (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	8	7	8	3	4	4	7	4	4	3	3	0	0	0
2	1	0	19	10	6	8	6	7	5	12	8	6	6	3	2	2
3	1	0	29	16	14	10	8	7	7	5	7	5	3	2	1	0
4	1	0	20	12	8	9	7	5	5	4	4	5	2	1	0	0
5	1	0	24	15	10	11	12	10	9	11	8	6	4	2	0	0
6	1	0	12	9	10	8	6	7	9	11	11	9	4	2	1	0
7	1	0	21	13	10	16	9	9	10	14	13	13	8	4	0	0
8	1	0	10	7	5	6	7	5	6	4	5	3	2	2	1	0
9	1	0	14	6	7	5	4	5	3	5	6	4	2	0	0	0
10	1	0	9	11	8	7	7	9	10	11	14	9	8	4	1	0
11	1	0	15	12	7	8	10	7	6	6	8	7	4	0	0	0
12	1	0	11	9	8	6	7	7	12	5	5	3	2	0	0	0
13	1	0	11	9	7	6	6	5	5	8	7	6	3	0	0	0
14	1	0	18	13	8	7	8	8	7	5	4	2	1	0	0	0
15	1	0	8	5	4	5	4	4	3	4	4	4	3	0	0	0
16	1	0	18	16	14	10	11	9	9	11	103	7	8	3	2	2
17	1	0	11	7	7	6	5	7	5	7	7	7	5	2	1	0
18	1	0	8	6	5	4	5	3	3	4	4	4	3	2	1	1
19	1	0	26	23	18	16	19	17	13	14	17	12	9	5	2	0
20	1	0	12	7	6	5	6	3	3	9	8	4	4	3	2	0
21	2	0	26	15	11	11	9	6	9	6	6	7	7	2	2	1
23	2	0	23	19	16	11	11	8	7	7	6	6	6	2	2	2
26	2	0	29	31	23	16	14	13	15	14	11	6	7	1	2	1
27	2	0	24	49	40	15	15	10	10	10	9	8	9	2	1	0
28	2	0	44	20	14	12	15	11	12	12	12	12	11	3	3	2

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	64	26	19	16	14	21	23	31	26	16	16	6	4	2
30	2	0	35	14	13	12	13	11	12	15	13	14	10	4	3	1
39	2	0	26	15	11	11	9	6	9	6	6	7	7	2	2	1
40	2	0	33	24	20	23	18	19	21	13	17	14	13	4	2	0
41	2	0	44	24	16	18	13	12	19	18	15	14	14	8	5	0
42	2	0	26	22	15	16	12	12	9	13	15	12	9	3	1	0
43	2	0	113	62	54	46	36	36	29	27	24	25	15	6	4	1
44	2	0	29	25	20	15	12	12	10	17	12	11	13	4	4	2
50	2	0	25	15	11	10	10	10	9	9	8	6	8	2	1	0
51	2	0	31	20	14	13	11	10	8	9	6	4	5	1	1	0
52	2	0	17	15	16	15	12	12	8	12	10	7	6	3	3	1
22	3	0	51	38	28	24	18	16	14	12	10	8	9	4	3	1
24	3	0	73	32	26	31	27	21	23	27	18	17	12	6	2	0
25	3	0	7	4	5	7	7	10	10	12	10	7	6	6	2	1
31	3	0	46	49	34	23	19	18	16	14	14	19	12	3	0	0
32	3	0	147	62	35	26	25	19	19	12	13	12	10	3	2	0
33	3	0	40	24	17	17	15	16	16	13	12	11	9	2	5	1
34	3	0	24	18	15	10	9	8	9	10	9	9	7	3	2	0
35	3	0	82	78	66	58	48	48	40	31	29	27	21	13	7	0
36	3	0	56	35	31	33	25	21	32	41	41	48	30	8	3	2
37	3	0	73	56	45	53	38	46	40	40	31	38	29	9	2	2
38	3	0	112	70	57	44	45	44	31	32	31	25	15	8	3	3
45	3	0	85	72	73	64	59	46	62	37	35	23	23	22	3	2
46	3	0	78	70	75	66	64	53	49	37	33	25	22	28	3	2
47	3	0	162	175	114	75	67	49	66	50	33	30	33	12	5	4
48	3	0	110	95	60	53	49	45	40	45	36	28	28	14	6	4

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	**0.33 hr**	**0.67 hr**	**1 hr**	**1.5 hr**	**2 hr**	**3 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**	**12 hr**	**24 hr**	**36 hr**	**48 hr**
49	3	0	99	99	62	56	47	44	49	45	37	31	28	13	8	4
53	3	0	58	16	14	7	14	13	12	15	13	13	12	6	5	2

Table 100. Plasma concentration of atorvastatin (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	39	450	254	213	154	108	96	65	38	15	7	0	0	0
2	1	0	17	120	211	218	178	128	91	44	25	10	5	0	0	0
3	1	0	71	225	184	120	114	69	56	35	18	15	11	0	0	0
4	1	0	221	349	223	162	102	72	81	67	26	12	3	0	0	0
5	1	0	38	290	187	135	107	85	86	45	22	14	5	0	0	0
6	1	0	9	50	152	184	164	124	85	50	27	16	7	0	0	0
7	1	0	33	296	270	357	292	163	148	113	66	45	21	0	0	0
8	1	0	364	615	402	248	211	142	114	43	31	21	6	0	0	0
9	1	0	24	294	259	177	102	85	55	32	16	12	4	.	.	1
10	1	0	18	599	389	247	232	130	82	35	16	9	4	0	0	0
11	1	0	370	654	493	275	217	183	115	59	32	17	8	0	0	0
12	1	0	58	258	291	199	171	127	94	43	23	13	5	0	0	0
13	1	0	135	147	142	105	98	63	50	31	17	8	5	0	0	0
14	1	0	12	108	95	57	37	29	26	12	5	2	0	0	0	0
15	1	0	13	126	109	74	52	32	26	27	13	10	4	1	0	0
16	1	0	13	90	130	90	89	70	49	33	32	15	6	0	0	0
17	1	0	34	312	285	208	150	99	70	56	36	21	12	0	0	0
18	1	0	22	141	113	62	51	35	28	21	14	9	3	0	0	0
19	1	0	152	445	279	197	151	113	102	73	63	43	22	2	0	0
20	1	0	50	93	110	104	80	51	51	34	22	22	9	0	0	0

Table 101. Plasma concentration of 4-hydroxy atorvastatin (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	0	.	.	.	1	3	5	4	10	8	3	3	2	0
2	1	0	0	1	2	2	5	6	7	6	7	7	7	7	2	0
3	1	0	0	2	3	3	4	3	4	7	5	5	3	6	3	0
4	1	0	0	1	1	1	2	4	4	5	4	4	4	3	0	0
5	1	0	0	.	.	.	3	3	6	6	6	5	5	4	0	0
6	1	0	0	.	.	1	1	2	2	4	2	5	2	3	0	0
7	1	0	0	.	.	.	.	.	3	5	6	6	7	4	2	2
8	1	0	0	2	3	2	2	3	2	5	3	3	5	4	4	0
9	1	0	0	.	.	.	5	4	4	2	3	1	0	0	0	0
10	1	0	0	.	.	.	4	4	10	5	7	6	5	4	0	0
11	1	0	0	2	3	3	5	6	6	6	4	10	8	3	3	0
12	1	0	0	.	.	.	2	3	3	2	3	4	4	2	2	0
13	1	0	0	2	3	3	3	4	5	6	6	6	4	2	0	0
14	1	0	0	3	7	8	9	11	12	14	10	7	7	4	1	0
15	1	0	0	1	1	1	3	3	3	3	4	5	3	3	0	0
16	1	0	0	2	5	5	5	5	6	8	8	8	6	4	3	2
17	1	0	0	2	7	7	5	4	7	6	5	7	8	4	2	2
18	1	0	0	1	3	3	3	3	3	3	2	2	3	3	2	0
19	1	0	0	3	7	9	9	11	13	11	10	12	8	7	4	2
20	1	0	0	3	4	4	4	3	3	3	5	7	4	4	3	2
21	2	0	.	.	2	1	2	2	2	3	4	3	4	2	0	0
23	2	0	.	.	2	2	2	8	6	7	6	5	4	2	0	0
26	2	0	1	2	4	5	6	5	8	8	9	8	7	2	1	0
27	2	0	.	.	2	4	4	4	8	7	9	4	6	2	0	0
28	2	0	.	.	5	5	3	4	5	10	6	5	6	0	0	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	.	.	3	2	5	10	11	13	9	10	11	4	2	1
30	2	0	1	2	2	4	5	4	7	6	8	10	11	4	2	0
39	2	0	.	.	2	1	2	2	2	3	4	3	4	2	0	0
40	2	0	1	2	3	7	8	10	13	11	23	14	16	10	5	3
41	2	0	2	3	3	6	3	6	8	8	7	9	6	3	2	0
42	2	0	.	.	3	2	2	4	2	5	6	6	6	3	1	0
43	2	0	2	4	6	6	7	10	11	8	7	12	7	2	2	0
44	2	0	1	2	2	3	3	4	4	6	5	6	4	1	0	0
50	2	0	1	2	2	3	4	5	7	5	5	6	5	2	2	0
51	2	0	1	2	4	4	6	6	8	6	6	5	6	1	0	0
52	2	0	.	.	2	4	4	4	4	7	5	4	4	2	0	0
22	3	0	.	.	2	3	3	6	5	7	3	4	4	4	4	0
24	3	0	1	2	4	4	9	7	12	11	9	12	8	5	3	1
25	3	0	2	.	.	.	2	2	.	3	5	2	3	2	1	0
31	3	0	1	2	6	5	7	7	8	7	8	9	7	3	0	0
32	3	0	2	3	4	5	6	6	8	7	6	7	6	3	1	0
33	3	0	1	1	2	4	2	7	7	8	7	8	4	4	4	2
34	3	0	.	.	2	2	2	4	4	6	4	5	6	4	2	1
35	3	0	10	12	11	23	22	25	21	16	18	17	14	7	2	0
36	3	0	1	4	3	7	6	6	13	20	21	28	16	3	2	0
37	3	0	1	2	3	15	5	7	11	11	11	16	17	3	3	0
38	3	0	5	10	17	5	17	26	21	21	23	22	17	7	6	0
45	3	0	2	3	6	10	15	15	18	9	10	7	7	4	1	0
46	3	0	1	6	5	11	11	15	15	14	6	13	7	6	5	2
47	3	0	3	5	4	8	11	11	15	15	16	15	15	5	4	2
48	3	0	1	4	8	9	12	15	15	15	15	12	10	7	2	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
49	3	0	3	3	6	9	11	13	21	21	13	15	10	4	3	1
53	3	0	1	2	3	1	3	2	5	8	5	8	7	4	2	2

Table 102. Plasma concentration of 4-hydroxy atorvastatin (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	37	73	105	120	140	129	126	101	58	26	26	0	0	0
2	1	0	3	7	60	94	131	158	165	102	74	42	28	0	0	0
3	1	0	4	53	104	85	113	71	58	71	39	25	15	3	0	0
4	1	0	7	62	67	82	82	64	90	59	32	17	6	0	0	0
5	1	0	32	64	62	72	81	131	92	46	33	36	8	0	0	0
6	1	0	2	4	32	78	88	104	85	59	50	30	11	1	0	0
7	1	0	8	16	43	71	101	95	92	93	70	58	38	4	0	0
8	1	0	19	195	230	204	181	173	170	70	58	45	17	1	0	0
9	1	0	21	43	98	92	83	89	67	34	30	18	8	0	0	0
10	1	0	52	105	188	224	263	252	176	81	52	32	10	1	0	0
11	1	0	20	95	148	155	163	165	123	89	61	38	22	1	0	0
12	1	0	26	52	104	132	114	122	100	52	42	28	14	0	0	0
13	1	0	13	55	63	68	72	64	51	35	16	11	6	1	0	0
14	1	0	35	70	114	99	83	72	58	25	15	8	4	0	0	0
15	1	0	20	41	74	68	57	39	33	34	21	11	7	0	0	0
16	1	0	13	26	59	75	77	67	57	32	32	19	13	0	0	0
17	1	0	2	66	93	107	92	80	62	57	44	32	18	1	0	0
18	1	0	13	26	41	31	34	31	25	19	13	9	5	0	0	0
19	1	0	8	88	109	83	90	80	72	69	60	45	30	3	2	0
20	1	0	4	30	33	60	63	50	52	34	26	25	12	3	0	0

Table 103. Plasma concentration of rosuvastatin (Period 1)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	6	12	15	12	13	13	16	11	0	0	0	0	0	0
2	1	0	26	67	91	117	140	117	115	68	39	14	5	7	0	0
3	1	0	8	10	46	23	20	16	21	9	8	4	2	5	0	0
4	1	0	2	6	9	20	26	27	29	17	11	6	0	0	0	0
5	1	0	9	20	23	37	32	48	49	28	17	6	7	0	0	0
6	1	0	.	.	8	7	10	13	15	11	10	0	0	0	0	0
7	1	0	13	24	31	50	49	56	62	55	34	18	10	6	0	0
8	1	0	11	29	39	38	33	34	35	23	15	9	6	8	0	0
9	1	0	4	8	13	18	15	8	6	5	0	0	0	0	0	0
10	1	0	8	21	27	46	38	31	16	9	0	0	0	0	0	0
11	1	0	12	29	41	67	62	53	54	20	17	8	0	0	0	0
12	1	0	6	18	36	36	38	28	26	8	3	3	0	0	0	0
13	1	0	3	5	7	9	11	10	11	11	6	8	0	0	0	0
14	1	0	.	.	8	7	10	13	11	7	6	0	0	0	0	0
15	1	0	4	8	6	9	6	9	37	9	7	0	0	0	0	0
16	1	0	9	13	12	19	17	17	23	16	16	0	0	0	0	0
17	1	0	6	14	19	24	31	33	44	38	33	17	12	11	0	0
18	1	0	.	.	.	.	6	6	9	10	7	0	0	0	0	0
19	1	0	30	48	62	57	70	56	48	43	30	19	8	9	0	0
20	1	0	8	10	10	26	27	35	26	26	20	6	9	0	0	0
21	2	0	3	8	13	13	15	20	22	34	19	12	6	0	0	0
23	2	0	3	6	7	14	25	30	41	34	16	11	5	0	0	0
26	2	0	6	27	15	25	30	32	45	33	17	9	8	0	0	0
27	2	0	4	8	8	12	11	14	20	13	10	9	0	0	0	0
28	2	0	9	10	14	13	19	36	23	17	11	8	0	0	0	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
29	2	0	9	13	14	17	19	27	31	21	11	10	6	0	0	0
30	2	0	15	25	33	54	52	50	63	43	23	24	13	0	0	0
39	2	0	3	8	13	13	15	20	22	34	19	12	6	0	0	0
40	2	0	12	17	20	38	35	45	54	39	35	23	13	12	0	0
41	2	0	15	23	26	26	33	43	57	39	27	16	11	0	0	0
42	2	0	11	12	16	13	14	33	41	21	17	15	7	0	0	0
43	2	0	23	40	33	34	31	28	19	13	0	0	0	0	0	0
44	2	0	6	11	21	25	31	32	32	27	11	17	10	1	0	0
50	2	0	8	17	13	15	16	17	19	17	14	10	10	2	0	0
51	2	0	11	11	33	20	19	21	26	18	13	1	5	10	0	0
52	2	0	7	23	17	27	33	38	32	32	17	13	7	11	0	0
22	3	0	7	19	25	36	34	47	31	19	18	8	10	8	0	0
24	3	0	37	54	54	82	68	76	84	65	33	21	19	19	2	3
25	3	0	8	9	12	12	12	23	16	15	22	15	9	8	3	0
31	3	0	9	18	23	27	30	38	34	19	7	0	0	0	0	0
32	3	0	13	22	18	28	34	32	43	24	16	9	0	0	0	0
33	3	0	16	34	29	32	30	31	34	23	15	11	14	6	0	0
34	3	0	4	8	11	11	18	24	18	15	10	0	0	0	0	0
35	3	0	11	19	25	35	33	41	36	30	20	13	11	0	0	0
36	3	0	22	33	44	52	63	68	85	55	27	8	8	0	0	0
37	3	0	5	11	14	53	23	18	20	14	0	0	0	0	0	0
38	3	0	17	41	65	29	45	56	46	31	25	18	11	0	0	0
45	3	0	14	42	51	49	37	36	49	32	17	7	9	0	0	0
46	3	0	23	47	54	53	50	50	37	24	19	14	9	0	0	0
47	3	0	14	77	104	121	127	116	105	53	31	28	16	11	0	0
48	3	0	28	37	39	38	43	29	34	21	25	14	12	0	0	0

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
49	3	0	29	35	38	42	41	35	28	27	24	11	10	0	0	0
53	3	0	195	37	26	24	38	30	39	36	22	25	13	7	0	0

Table 104. Plasma concentration of rosuvastatin (Period 2)

Subject number	Group	Plasma concentration at each timepoint (pg/mL)														
		0 hr	0.33 hr	0.67 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	10 hr	12 hr	24 hr	36 hr	48 hr
1	1	0	37	73	105	120	140	129	126	101	58	26	26	0	0	0
2	1	0	3	7	60	94	131	158	165	102	74	42	28	0	0	0
3	1	0	4	53	104	85	113	71	58	71	39	25	15	3	0	0
4	1	0	7	62	67	82	82	64	90	59	32	17	6	0	0	0
5	1	0	32	64	62	72	81	131	92	46	33	36	8	0	0	0
6	1	0	2	4	32	78	88	104	85	59	50	30	11	1	0	0
7	1	0	8	16	43	71	101	95	92	93	70	58	38	4	0	0
8	1	0	19	195	230	204	181	173	170	70	58	45	17	1	0	0
9	1	0	21	43	98	92	83	89	67	34	30	18	8	0	0	0
10	1	0	52	105	188	224	263	252	176	81	52	32	10	1	0	0
11	1	0	20	95	148	155	163	165	123	89	61	38	22	1	0	0
12	1	0	26	52	104	132	114	122	100	52	42	28	14	0	0	0
13	1	0	13	55	63	68	72	64	51	35	16	11	6	1	0	0
14	1	0	35	70	114	99	83	72	58	25	15	8	4	0	0	0
15	1	0	20	41	74	68	57	39	33	34	21	11	7	0	0	0
16	1	0	13	26	59	75	77	67	57	32	32	19	13	0	0	0
17	1	0	2	66	93	107	92	80	62	57	44	32	18	1	0	0
18	1	0	13	26	41	31	34	31	25	19	13	9	5	0	0	0
19	1	0	8	88	109	83	90	80	72	69	60	45	30	3	2	0
20	1	0	4	30	33	60	63	50	52	34	26	25	12	3	0	0

E. Urine concentration at each urine interval of dabigatran and rosuvastatin in each subject

Table 105. Urine concentration of dabigatran (Period 1)

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	**0-4 hr**	**4-8 hr**	**8-12 hr**	**12-24 hr**	**24-36 hr**	**36-48 hr**
1	1	0	9.95	2.76	1.90	2.70	1.55	0.39
2	1	0	10.23	4.87	3.39	2.60	1.07	0.54
3	1	0	11.58	5.58	2.10	1.79	0.46	0.51
4	1	0	3.54	2.69	0.68	1.26	0.36	0.17
5	1	0	5.14	3.20	1.29	0.51	0.79	0.41
6	1	0	8.34	3.06	1.56	2.95	1.11	0.06
7	1	0	5.37	3.67	1.80	1.33	0.83	0.28
8	1	0	6.00	1.39	1.81	1.28	1.26	0.94
9	1	0	3.62	5.26	1.60	2.66	2.21	0.45
10	1	0	2.89	1.91	1.10	1.51	0.02	0.07
11	1	0	4.17	1.74	1.86	1.17	0.68	0.11
12	1	0	2.34	2.75	1.23	0.27	0.71	0.44
13	1	0	4.30	2.43	1.05	0.19	0.79	0.13
14	1	0	1.62	1.31	0.79	1.00	0.43	0.31
15	1	0	3.37	3.58	1.62	1.95	1.23	0.51
16	1	0	1.91	3.52	1.00	1.62	1.17	0.69
17	1	0	4.07	7.66	2.66	3.12	0.41	0.73
18	1	0	2.15	4.27	1.35	2.05	1.09	1.16
19	1	0	4.24	4.00	1.33	2.68	1.18	0.41
20	1	0	5.74	5.06	2.86	3.11	2.25	1.00
21	2	0	0.85	10.98	2.40	3.47	1.14	0.36
23	2	0	4.63	5.18	4.00	3.31	1.57	0.57
26	2	0	3.75	3.11	3.32	1.66	0.95	0.44
27	2	0	4.77	2.87	3.24	2.87	1.32	0.63

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	0-4 hr	4-8 hr	8-12 hr	12-24 hr	24-36 hr	36-48 hr
28	2	0	2.82	1.16	1.59	1.10	0.91	0.15
29	2	0	2.37	2.98	0.96	1.49	0.89	0.07
30	2	0	3.74	2.25	1.45	2.37	0.87	0.35
39	2	0	7.08	2.73	1.97	1.99	1.52	1.19
40	2	0	1.15	1.13	0.65	1.18	0.58	0.30
41	2	0	4.11	4.03	1.49	2.73	1.80	0.92
42	2	0	3.86	1.82	1.29	2.60	2.14	1.27
43	2	0	1.19	1.43	0.46	0.87	0.45	0.57
44	2	0	1.40	4.35	3.78	2.04	0.72	0.82
50	2	0	4.17	4.95	1.48	2.99	1.39	0.00
51	2	0	3.18	2.64	1.70	1.59	1.08	0.00
52	2	0	3.93	2.32	4.48	3.77	1.90	0.86
22	3	0	0.78	2.58	1.37	1.81	0.83	1.13
24	3	0	0.09	2.44	1.31	5.00	1.41	1.66
25	3	0	1.03	2.53	0.44	2.70	2.36	1.36
31	3	0	2.28	1.31	1.23	2.01	0.67	0.41
32	3	0	2.73	1.81	1.02	0.86	1.97	0.48
33	3	0	1.65	1.39	3.01	3.62	2.62	1.37
34	3	0	1.35	3.62	1.77	3.61	2.05	1.19
35	3	0	1.63	2.79	1.28	2.80	2.15	1.18
36	3	0	3.22	1.09	2.51	2.85	1.92	0.00
37	3	0	0.75	2.33	0.76	3.13	2.10	0.87
38	3	0	0.39	1.91	0.41	1.86	1.44	1.21
45	3	0	0.53	0.44	0.62	0.90	1.00	0.55
46	3	0	2.81	1.71	3.39	0.90	3.18	2.33
47	3	0	4.87	5.33	3.30	1.78	3.83	2.01
48	3	0	1.47	1.40	1.23	2.68	1.41	1.07

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	0-4 hr	4-8 hr	8-12 hr	12-24 hr	24-36 hr	36-48 hr
49	3	0	0.23	0.57	0.08	0.85	0.78	0.83
53	3	0	1.03	1.01	1.83	2.73	2.08	0.78

Table 106. Urine concentration of dabigatran (Period 2)

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	**0-4 hr**	**4-8 hr**	**8-12 hr**	**12-24 hr**	**24-36 hr**	**36-48 hr**
1	1	0	8.31	7.05	2.64	3.05	0.50	0.75
2	1	0	9.97	7.69	11.77	8.10	2.17	0.55
3	1	0	7.52	8.01	2.80	2.89	0.81	0.59
4	1	0	9.39	5.13	1.95	3.65	1.13	0.41
5	1	0	10.78	5.18	3.17	7.99	1.44	0.83
6	1	0	7.93	8.49	7.20	1.84	1.35	1.28
7	1	0	9.02	16.64	5.06	5.40	0.49	0.63
8	1	0	9.79	3.66	1.58	2.51	0.37	0.51
9	1	0	11.39	10.35	3.57	5.39	1.77	1.08
10	1	0	6.48	5.88	2.71	3.66	1.53	0.41
11	1	0	10.58	3.98	4.47	2.97	0.87	0.34
12	1	0	5.10	3.03	1.87	2.05	0.94	0.33
13	1	0	5.59	1.49	4.76	1.87	0.43	0.36
14	1	0	3.68	3.58	4.93	1.50	1.39	0.97
15	1	0	2.11	5.03	4.62	2.77	0.99	0.45
16	1	0	4.90	4.31	3.12	2.08	0.37	0.65
17	1	0	1.43	14.89	4.52	4.33	1.95	0.92
18	1	0	2.81	3.59	5.02	4.06	1.48	0.70
19	1	0	5.21	5.34	3.83	4.84	1.88	0.50
20	1	0	7.19	2.09	7.46	4.54	1.72	1.22

Table 107. Urine concentration of rosuvastatin (Period 1)

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	0-4 hr	4-8 hr	8-12 hr	12-24 hr	24-36 hr	36-48 hr
1	1	0	0.92	0.46	0.14	0.18	0.23	0.07
2	1	0	4.16	2.91	1.42	0.62	0.46	0.14
3	1	0	1.08	0.88	0.33	0.23	0.10	0.09
4	1	0	0.50	1.23	0.22	0.31	0.22	0.07
5	1	0	1.16	1.25	0.27	0.05	0.18	0.07
6	1	0	0.34	0.49	0.22	0.21	0.16	0.00
7	1	0	0.95	1.25	1.53	0.71	0.51	0.13
8	1	0	1.07	0.50	0.98	0.51	0.97	0.29
9	1	0	0.27	0.60	0.17	0.18	0.25	0.06
10	1	0	1.07	0.70	0.25	0.21	0.12	0.03
11	1	0	1.34	0.98	0.95	0.22	0.20	0.04
12	1	0	0.94	0.94	0.27	0.07	0.14	0.08
13	1	0	0.37	0.46	0.16	0.04	0.10	0.00
14	1	0	0.20	0.21	0.17	0.22	0.09	0.04
15	1	0	0.30	0.70	0.35	0.20	0.15	0.04
16	1	0	0.29	0.80	0.30	0.17	0.26	0.09
17	1	0	0.21	2.78	1.36	0.61	0.15	0.19
18	1	0	0.08	0.44	0.21	0.39	0.18	0.12
19	1	0	1.45	1.98	0.60	0.58	0.37	0.04
20	1	0	0.77	1.58	0.55	0.41	0.50	0.10
21	2	0	0.04	1.40	0.59	0.42	0.25	0.07
23	2	0	0.40	1.43	1.21	0.57	0.30	0.08
26	2	0	0.32	0.63	0.61	0.10	0.16	0.05
27	2	0	1.02	0.60	0.54	0.36	0.16	0.08
28	2	0	0.25	0.20	0.28	0.12	0.17	0.00
29	2	0	0.30	0.93	0.24	0.21	0.17	0.00

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	0-4 hr	4-8 hr	8-12 hr	12-24 hr	24-36 hr	36-48 hr
30	2	0	0.43	0.48	0.27	0.24	0.18	0.08
39	2	0	1.42	1.09	0.91	0.29	0.30	0.11
40	2	0	0.51	3.18	0.28	0.27	0.10	0.03
41	2	0	2.27	2.03	0.54	0.42	0.32	0.18
42	2	0	0.78	0.93	0.38	0.34	0.14	0.13
43	2	0	0.43	0.47	0.31	0.44	0.39	0.13
44	2	0	0.44	0.92	1.05	0.33	0.08	0.08
50	2	0	0.31	2.55	1.18	0.36	0.57	0.04
51	2	0	0.32	1.96	0.28	0.15	0.33	0.03
52	2	0	0.27	0.31	0.56	0.20	0.36	0.19
22	3	0	0.07	0.29	0.13	0.06	0.03	0.01
24	3	0	0.01	0.56	0.08	0.64	0.06	0.03
25	3	0	0.09	0.26	0.04	0.21	0.20	0.05
31	3	0	0.38	0.23	0.15	0.13	0.01	0.00
32	3	0	0.35	0.38	0.14	0.09	0.20	0.02
33	3	0	0.19	0.17	0.38	0.26	0.10	0.02
34	3	0	0.07	0.32	0.13	0.09	0.03	0.00
35	3	0	0.15	0.38	0.13	0.14	0.06	0.02
36	3	0	0.99	0.37	0.72	0.32	0.24	0.07
37	3	0	0.08	0.27	0.05	0.10	0.06	0.00
38	3	0	0.07	0.48	0.05	0.11	0.05	0.02
45	3	0	0.06	0.06	0.04	0.01	0.00	0.00
46	3	0	0.72	0.35	0.47	0.07	0.17	0.10
47	3	0	1.01	0.83	0.35	0.11	0.12	0.05
48	3	0	0.31	0.41	0.27	0.52	0.19	0.08
49	3	0	0.10	0.24	0.02	0.10	0.06	0.03
53	3	0	0.03	0.14	0.26	0.26	0.20	0.05

Table 108. Urine concentration of rosuvastatin (Period 2)

Subject number	Group	Urine concentration at each urine interval (ng/mL)						
		Predose	0-4 hr	4-8 hr	8-12 hr	12-24 hr	24-36 hr	36-48 hr
1	1	0	4.31	1.26	0.27	0.40	0.10	0.08
2	1	0	10.09	3.67	2.71	0.82	0.19	0.04
3	1	0	3.14	2.12	0.37	0.19	0.11	0.04
4	1	0	5.35	1.64	0.30	0.17	0.17	0.02
5	1	0	7.11	1.65	0.55	0.54	0.19	0.04
6	1	0	3.30	1.71	0.73	0.07	0.16	0.04
7	1	0	11.19	3.42	0.46	0.36	0.07	0.03
8	1	0	8.43	1.61	0.60	0.69	0.09	0.03
9	1	0	4.47	1.86	0.27	0.15	0.10	0.04
10	1	0	4.24	0.86	0.32	0.15	0.08	0.03
11	1	0	8.17	1.24	1.05	0.23	0.20	0.05
12	1	0	4.45	1.07	0.52	0.33	0.09	0.02
13	1	0	2.74	0.53	1.13	0.11	0.06	0.01
14	1	0	1.44	1.49	1.50	0.10	0.10	0.03
15	1	0	1.05	1.79	1.12	0.16	0.18	0.03
16	1	0	2.69	1.44	0.52	0.18	0.01	0.02
17	1	0	1.46	9.94	1.20	0.50	0.22	0.06
18	1	0	1.59	1.32	1.62	0.18	0.15	0.07
19	1	0	4.97	2.93	1.44	0.75	0.37	0.05
20	1	0	3.28	0.65	1.74	0.46	0.22	0.06

F. Genotype frequency of CYP3A5*3 (rs776746, 6986A > G) in three groups of subjects and pharmacokinetic parameters of midazolam including in wild type subjects and differential genotypes.

Table 109. Genotype frequency of CYP3A5*3 in three groups of subjects

Genotype frequency (n)	**Group 1 (n=20)**	**Group 2 (n=16)**	**Group 3 (n=17)**	**Total (n=53)**
rs776746 (6986A > G)				
6986AA (wild type)	3	0	5	8
6986AG	9	8	5	22
6986GG	8	8	7	23

Table 110. Pharmacokinetic parameters of midazolam in wild-type CYP3A5 subjects

PK parameters	**Group 1 (n=3)**	**Group 1 with rifampicin (n=3)**		**Group 2 (n=0)**		**Group 3 (n=5)**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**
$AUC_{0\text{-last}}$ (pg/mL.hr)	244 (140-424)	344 (183-646)	1.41 (0.82-2.42)	-	-	619 (411-931)	2.53 (1.49-4.31)*
$AUC_{0\text{-inf}}$ (pg/mL.hr)	254 (136-473)	360 (195-665)	1.42 (0.81-2.49)	-	-	653 (435-980)	2.57 (1.49-4.42)*
C_{max} (pg/mL)	95 (89-102)	119 (111-128)	1.26* (1.18-1.34)	-	-	174 (144-210)	1.83 (1.46-2.28)*
T_{max} (hr)[a]	0.7 (0.7-1.0)	0.7 (0.7-1.5)	-	-	-	0.7 (0.7-1.0)	-
$T_{1/2}$ (hr)	2.9 (0.9-8.7)	4.7 (0.5-42.2)	1.65 (0.34-8.06)	-	-	8.4 (5.2-13.7)	2.96 (1.42-6.13)*
Kel (hr^{-1})	0.242 (0.080-0.739)	0.147 (0.016-1.316)	0.61 (0.12-2.96)	-	-	0.082 (0.051-0.133)	0.34 (0.16-0.70)*

[a]Data presented in median IQR (interquartile range)

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease.

*p-value<0.05, Group 1 as a reference group

PK, pharmacokinetic; GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-last}}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-inf}}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

Table 111. Pharmacokinetic parameters of midazolam in Group 1 subjects (n=20) categorized by CYP3A5 genotypes.

PK parameters	**6986AA (wild type, n=3)**	**6986AG (n=9)**		**6986GG (n=8)**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**
$AUC_{0\text{-}last}$ (pg/mL.hr)	244 (140-424)	177 (111-280)	0.72 (0.38-1.40)	192 (147-252)	0.78 (0.40-1.53)
$AUC_{0\text{-}inf}$ (pg/mL.hr)	254 (136-473)	184 (117-289)	0.72 (0.38-1.38)	200 (153-261)	0.79 (0.41-1.52)
C_{max} (pg/mL)	95 (89-102)	77 (51-118)	0.82 (0.46-1.45)	78 (63-97)	0.82 (0.46-1.47_
T_{max} (hr)[a]	0.7 (0.7-1.0)	0.7 (0.7-0.7)	-	0.7 (0.7-0.7)	-
$T_{1/2}$ (hr)	2.9 (0.9-8.7)	2.3 (1.5-3.4)	0.79 (0.43-1.44)	2.9 (2.4-3.6)	1.02 (0.55-1.88)
Kel (hr^{-1})	0.242 (0.080-0.739)	0.308 (0.204-0.465)	1.27 (0.70-2.32)	0.238 (0.195-0.299)	0.98 (0.53-1.81)

[a]Data presented in median IQR (interquartile range).

Group 1, healthy young subjects; Group 2, healthy elderly subjects; Group 3, elderly subjects with chronic kidney disease

*p-value<0.05, Group 1 as a reference group

PK, pharmacokinetic; GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-}last}$, area under the concentration-time curve of time zero to the last time point; $AUC_{0\text{-}inf}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; $T_{1/2}$, half-life; Kel, elimination rate constant CL_R, renal clearance

G. Pharmacokinetic parameters of dabigatran in moderate and severe renal impairment

Table 112. Pharmacokinetic parameters of dabigatran in moderate and severe renal impairment

PK parameters	**Normal renal function (n=35)**	**Moderate renal function (n=9)**		**Severe renal function (n=8)**	
	GM (95% CI)	**GM (95% CI)**	**GMR (95% CI)**	**GM (95% CI)**	**GMR (95% CI)**
$AUC_{0\text{-}inf}$ (pg/mL.hr)	3687 (3147-4318)	11373 (7504-17236)	3.08 (2.19-4.35)*	15715 (12490-19773)	4.26 (2.96-6.11)*
C_{max} (pg/mL)	417 (357-488)	664 (447-987)	1.59 (1.12-2.25)*	613 (435-863)	1.47 (1.02-2.11)*

Normal renal function, eGFR >60 ml/min/1.73m^2; Moderate renal function, eGFR 30-60 ml/min/1.73m^2; Severe renal function, eGFR 15-29 ml/min/1.73m^2

*Significant level, p-value<0.05

PK, pharmacokinetic; GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-}inf}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; eGFR, estimated glomerular filtration rate

H. Pharmacokinetic parameters of pitavastatin in moderate and severe renal impairment

Table 113. Pharmacokinetic parameters of pitavastatin in moderate and severe renal impairment

PK parameters	Normal renal function (n=35)	Moderate renal function (n=9)		Severe renal function (n=8)	
	GM (95% CI)	GM (95% CI)	GMR (95% CI)	GM (95% CI)	GMR (95% CI)
$AUC_{0\text{-}inf}$ (pg/mL.hr)	479 (431-531)	772 (559-1068)	1.61 (1.24-2.09)*	776 (536-1123)	1.62 (1.23-2.13)*
C_{max} (pg/mL)	156 (137-179)	230 (158-335)	1.47 (1.08-2.00)*	256 (183-358)	1.64 (1.18-2.26)*

Normal renal function, eGFR >60 ml/min/1.73m^2; Moderate renal function, eGFR 30-60 ml/min/1.73m^2; Severe renal function, eGFR 15-29 ml/min/1.73m^2

*Significant level, p-value<0.05

PK, pharmacokinetic; GM, geometric mean; CI, confidence interval; GMR, geometric mean ratio; $AUC_{0\text{-}inf}$, area under the concentration-time curve of time zero to infinity; C_{max}, maximum plasma concentration; eGFR, estimated glomerular filtration rate

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