

DMD #58354

Gut microbiota-mediated drug interactions between lovastatin and antibiotics

Dae-Hyeong Yoo, In Sook Kim, Thi Kim Van Le, Il-Hoon Jung, Hye Hyun Yoo, and Dong-Hyun Kim

Department of Pharmacy, College of Pharmacy, Kyung Hee University, Dongdaemun-gu, Seoul 130-701, Korea: DHY, TKVL, IHJ, DHK

Institute of Pharmaceutical Science and Technology and College of Pharmacy, Hanyang University, Ansan, Gyeonggi-do 426-791, Korea: ISK, HHY

DMD #58354

Running title: Gut microbiota-mediated drug interactions with lovastatin

Correspondence:

Prof. Dong-Hyun Kim

Department of Pharmacy, College of Pharmacy, Kyung Hee University, Dongdaemun-gu,
Seoul 130-701, Republic of Korea

Phone: +82-2-961-0374; Fax: + 82-2-966-2297; E-mail: dhkim@khu.ac.kr

Prof. Hye Hyun Yoo, Ph.D.

Institute of Pharmaceutical Science and Technology and College of Pharmacy, Hanyang
University, Gyeonggi-do 426-791, Republic of Korea

Phone: +82-31-400-5804; Fax: + 82-31-400-4783; E-mail: yoohh@hanyang.ac.kr

Number of text pages: 18

Number of Tables: 2

Number of Figures: 5

Number of References: 21

Number of words in the *Abstract*: 192

Number of words in the *Introduction*: 511

Number of words in the *Discussion*: Results and Discussion: 1760

Abbreviations: ESI, electrospray ionization; LC-MS/MS, liquid chromatography- tandem
mass spectroscopy.

DMD #58354

Abstract

Orally-administered drugs may be metabolized by intestinal microbial enzymes before absorption into the blood. Accordingly, co-administration of drugs affecting the metabolic activities of gut microbes (e.g. antibiotics) may lead to drug-drug interactions (DDI). In this study, gut microbiota-mediated DDI were investigated by studying the pharmacokinetics of lovastatin in antibiotic-treated rats. Incubation of lovastatin with human and rat fecalase preparations produced four metabolites, **M1** (demethylbutyryl metabolite), **M4** (hydroxylated metabolite), **M8** (the active hydroxy acid metabolite), and **M9** (hydroxylated M8) indicating involvement of the gut microbiota in lovastatin metabolism. The plasma concentration-time profiles of **M8** were compared after oral administration of lovastatin to control rats or those treated with either ampicillin (100 mg/kg), or an antibiotic mixture consisting of cefadroxil (150 mg/kg), oxytetracycline (300 mg/kg), and erythromycin (300 mg/kg). Pharmacokinetic analyses indicated that systemic exposure to **M8** was significantly lower in antibiotic-treated rats, as compared with controls. In addition, fecal **M8** formation decreased by 58.3% and 59.9% in the ampicillin- and antibiotic mixture-treated rats, respectively. These results suggested that antibiotic intake may reduce the biotransformation of orally-administered drugs by gut microbiota and that the subsequent impact on microbiota metabolism could result in altered systemic concentrations of either the intact drug and/or its metabolite(s).

DMD #58354

Introduction

The term “xenobiotic metabolism” refers to the biochemical modification of drugs or food constituents by living organisms, including humans (Croom, 2012). This process generally converts hydrophobic xenobiotic compounds into more hydrophilic products, in order to facilitate their excretion. The liver is known to be a major site of xenobiotic metabolism. However, orally-administered xenobiotics may also be metabolized by gut microbial enzymes before being absorbed from the gastrointestinal tract into the blood (Scheline, 1973; Sousa et al., 2008; Haiser and Turnbaugh, 2013). The role of gut microbiota in the metabolism of orally-administered compounds or phytochemicals has been investigated extensively. Representative examples of compounds metabolized by gut microbiota include ginsenosides, cycasin, rutin, baicalin, hesperidin, and genistin (Saad et al., 2012; Choi et al., 2011). Some drugs, such as acetaminophen, chloramphenicol, digoxin, and sulfasalazine have also been shown to be affected by gut microbial metabolism (Saad et al., 2012). Once these compounds are orally administered, they are transformed to bioactive, bio-inactive, or toxic metabolites by intestinal microbiota prior to their absorption into the blood (Li and Jia, 2013).

Drug-drug interactions (DDIs) occur when a drug affects the activity of another drug. DDI may result from various processes, including pharmacokinetic and pharmacodynamic interactions. Alteration of drug pharmacokinetics (absorption, distribution, metabolism, and excretion) generally occurs due to inhibition or induction of drug metabolizing enzymes, such as cytochrome P450 enzymes (CYP) or transporters involved in absorption or excretion processes (König et al., 2013; Isoherranen et al., 2012). However, modulation of gut microbial enzyme activities represents another possible cause of DDI. Thus, drugs (generally antibiotics) affecting gut microbe metabolic activities may alter the pharmacokinetics of co-

DMD #58354

administered drugs that are metabolized by gut microbiota. As the impact of gut microbiota on drug metabolism has gradually been gaining recognition, the potential DDIs occurring via the interactions with microbiota metabolism studied, and the impact of gut microbes on DDIs has been discussed (Lindenbaum et al., 1981; Saha et al., 1983; Wilson and Nicholson, 2009). Furthermore, the role and significance of microbial β -glucuronidase in connection with enterohepatic recycling of xenobiotics have been emphasized (Roberts et al., 2013). The impact of gut microbiota on drug metabolism was evidenced by experimental studies involving animal models, in particular, with germ-free animals and animals in which human microflora had been introduced (Bowey et al., 2003).

Lovastatin is a statin, a class of drugs used as cholesterol-lowering agents to reduce cardiovascular disease risk. Lovastatin is a lactone prodrug which is readily hydrolyzed *in vivo* to yield the active β -hydroxy acid metabolite, a strong inhibitor of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase (Krukemyer and Talbert, 1987; Vyas et al., 1990). In our preliminary study, lovastatin was found to disappear by incubation with human and rat fecalase preparations (unpublished data). Accordingly, we wished to investigate the involvement of gut microbiota in the metabolism of lovastatin, in particular its biotransformation to the β -hydroxy acid metabolite, and the potential for this to associate with antibiotic DDI. In this study, the metabolism of lovastatin by gut microbial enzymes was characterized and the potential for pharmacokinetic DDI between lovastatin and antibiotics was investigated in rats.

Materials and methods

Materials. Lovastatin, lovastatin β -hydroxyl acid, 4-nitrophenyl- β -D-glucuronide, 4-

DMD #58354

nitrophenyl- β -D-xylopyranoside, 4-nitrophenyl- α -L-rhamnopyranoside, and 4-nitrophenyl- β -D-glucopyranoside were purchased from Sigma (St. Louis, MO, USA). Pooled human and rat liver microsomes were obtained from BD Gentest (Woburn, MA, USA).

Subjects. The study subjects were 10 healthy Koreans with an average age of 40.00 ± 9.58 years (range, 23-51 years). These subjects (5 male and 5 female) were non-smokers who were not taking any medication, and who were not regular or current users of antibiotics. The protocols for recruitment of subjects and collection of their stools were approved by the Committee for the Care and Use of Clinical Study in the Medical School, Kyung Hee University (IRB No KHP-2013-03-04-R1).

Animals. Male Sprague-Dawley rats (210-240 g) were supplied by the Koatech Experimental Animal Breeding Center (Gyeonggi-do, Korea). All animals were housed in wire cages (two rats per cage) at 20-22°C and $50 \pm 10\%$ humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea) and allowed water *ad libitum*. The rats were segregated into three groups: control group and two antibiotic-treated groups ($n = 10$ for each). All experiments were performed in accordance with the NIH and Kyung Hee University Guides for Laboratory Animal Care and Use, and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University (KHP-2013-03-04-R1).

Fecalase preparation. Human and rat fecal specimens were prepared for fecalase preparation according to a method previously published by Lee et al. (2002).

Assay of lovastatin-metabolizing activity. The reaction mixture (total volume of 2.5 mL) consisted of 1 mL of 1 mM lovastatin, 1 mL of 0.1 M phosphate buffer, pH 7.0, and 0.5 mL of fecalase suspension. The reaction mixture was incubated at 37°C for 12 h. The reaction

DMD #58354

was stopped by the addition of 2.5 mL of MeOH, and centrifuged at $3000 \times g$ for 10 min. The amount of lovastatin in the resulting supernatant was assayed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a Hewlett Packard series 1050 with an Eclipse Plus C18 column (4.6×100 mm i.d., 5.0 μ m, Agilent Technologies, Santa Clara, CA, USA) and a UV detector at 238 nm (Ramsey, MN, USA). The elution solvent (23% water, 77% acetonitrile) flow rate was 0.6 mL/min for 20 min and the injection volume was 8 μ L.

Assay of fecal β -D-glucuronidase, β -D-glucosidase, and α -L-rhamnosidase activities.

Fecal β -D-glucuronidase, β -D-glucosidase, and α -L-rhamnosidase activities of rat fecalase samples were tested a method previously published by Lee et al. (2002).

Isolation of lovastatin metabolites produced by intestinal microbiota. A fresh human stool sample (5 g) was suspended in 45 mL anaerobic dilution broth, and centrifuged at $500 \times g$ for 10 min. The resulting supernatant was centrifuged at $10000 \times g$ for 10 min. The pellet was suspended in 1 L anaerobic dilution broth; lovastatin (0.75 g) was added and anaerobically incubated for 5 days. Then the reaction mixture was extracted 3 times (3×3 L) with ethylacetate (EtOAc), and evaporated by a rotary evaporator under vacuum to produce 1.25 g of EtOAc extract. The EtOAc extract was fractionated through a silica gel column (3 cm \times 30 cm) using hexane : EtOAc (1 : 0 to 1 : 1); then CHCl_3 : EtOAc (7 : 3 to 8 : 2); and finally CHCl_3 : MeOH (7 : 3 to 0 : 1). Seven fractions (L-1 to L-7) were obtained. The fraction L-3 was loaded onto a silica gel column (1.5 cm \times 20 cm) and eluted using CHCl_3 : EtOAc (1 : 0 to 6 : 4); then CHCl_3 : acetone (7 : 3 to 0 : 1), to produce **M8** (2.9 mg). The fraction L-4 was also applied to a silica gel column (1.5 \times 20 cm) and eluted using CHCl_3 : acetone (1 : 0 to 6 : 4) and CHCl_3 : methanol (7 : 3 to 0 : 1) to isolate **M4** (2.0 mg).

DMD #58354

The fraction L-6 was loaded onto a silica gel column (1.5 cm × 20 cm) and eluted using CHCl₃:MeOH (1: 0 to 0 : 1) to obtain **M1** (2.2 mg). The chemical structures of the isolated compounds were identified by nuclear magnetic resonance (NMR) analysis and comparison of the NMR data (Supplemental data 1) with published information [13-16].

Fecal sample preparation. Two hundred microliters of rat fecalase samples were percolated through a solid phase extraction (SPE) cartridge (Oasis hydrophilic-lipophilic-balanced 96-well plate). The SPE method involved the following steps. First, the SPE cartridge was preconditioned with methanol (1 mL) and equilibrated with 0.1% acetic acid (1 mL). The fecalase sample was loaded to the cartridge, which was washed with 0.1% acetic acid (2 × 1 mL). The sample was eluted with 1 mL methanol, and the eluent was evaporated to dryness under anitrogen stream at 40°C. The residue was dissolved in 100 μL of 0.1% formic acid in acetonitrile and 0.1% formic acid in distilled water (30:70, v/v), and a 5-μL aliquot was injected into the HPLC column for LC-QTOF MS analyses.

Liver microsomal incubation and sample preparation. Lovastatin (20 μM, final concentration) was incubated with human or rat liver microsomes (1 mg/mL) in 0.1 M potassium phosphate buffer, pH 7.4, at 37°C for 1 h in the presence of an NADPH-generating system (0.8 mM NADP⁺, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase). The reaction was stopped by the addition of acetonitrile with 0.1% acetic acid. The reaction solutions were then prepared using SPE, as described above.

Animal experiments. The control rats were administered water and the antibiotic-treated groups were administered ampicillin (AP; 100 mg/kg) or an antibiotic mixture (COE) consisting of cefadroxil (150 mg/kg), oxytetracycline (300 mg/kg), and erythromycin (300 mg/kg) 3 days before oral administration of either lovastatin (20 mg/kg) or the vehicle. Blood

DMD #58354

was drawn (0.2 mL) from the tail vein at 0.17, 0.5, 1, 2, 4, 6, 12, and 24 h after lovastatin or vehicle administration. All samples were stored at -20°C until analysis.

Blood sample preparation and calibration curves. Two hundred microliters of rat blood samples were centrifuged for 5 min at 13000× g to obtain plasma supernatants. The plasma samples (100 μL) were deproteinized with acetonitrile (200 μL) containing 50 ng/mL digoxin (internal standard, IS). The sample was vigorously vortex-mixed, and then centrifuged at 13000 × g for 5 min. The resulting supernatant was transferred to LC vials and a 5-μL aliquot was injected into the LC-MS/MS analysis system.

Calibration standards were prepared by spiking 10 μL of working standard solutions (lovastatin β-hydroxyl acid) into 90 μL of blank rat plasma at final concentrations of 1-250 ng/mL. The calibration standards were treated as described above prior to analysis. Calibration curves were generated by plotting the peak area ratio of the analyte to IS versus the concentration of the analyte, using least-square linear regression. Each standard was prepared in triplicate. The correlation coefficients of the calibration curves were greater than 0.99. The calibration curve equation was $y = 0.0390x + 0.0669$.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses. The LC-MS/MS conditions used for metabolite identification and pharmacokinetic study are provided as a supplementary material (Supplemental data 2).

Pharmacokinetic analysis. The maximum plasma concentration (C_{max}) and the time taken to reach C_{max} (T_{max}) for lovastatin and lovastatin β-hydroxyl acid were estimated directly from the plasma concentration-time profiles. A non-compartmental model provided by WinNonlin Professional 3.1 software was used to calculate the area under the plasma drug concentration-time curve (AUC).

DMD #58354

Statistics. All the data were expressed as the mean \pm standard deviation, and statistical significance was analyzed by one way ANOVA, followed by Student's t-test.

Results and discussion

Lovastatin was incubated with human and rat fecalase and the remaining amount of lovastatin was measured by HPLC analysis. The levels of lovastatin were reduced by 8-19% in the human and rat fecalase samples after a 12-h incubation. Based on this data, the lovastatin-metabolizing activities of human and rat fecalase were calculated to be 0.083 ± 0.020 nmol/h/mg and 0.053 ± 0.017 nmol/h/mg, respectively (Fig. 1).

To investigate the effects of antibiotics on this metabolic activity, antibiotics were orally-administered to rats prior to analysis of fecalase activity. The gut microflora consists of microorganisms that mainly belong to *Enterobacteriaceae* and the genera *Bacteroides*, *Clostridium*, *Fusobacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, and *Bifidobacterium*; thus, the microflora has both gram-positive and gram-negative organisms (Guarner and Malagelada, 2003). Therefore, broad-spectrum antibiotics that can effectively suppress all types of gut bacteria were chosen. Subsequently, we examined the effects of several selected antibiotics and their combinations on the gut microbiota on the basis of their glycosidase activities and optimized the antibiotic systems (AP and COE) while considering antibiotic-induced adverse physiological alterations (e.g., diarrhea) and possible pharmacokinetic interference via CYP enzymes or drug efflux pumps. AP is a broad-spectrum antibacterial agent that is effective against gram-positive organisms and some gram-negative organisms (Wilkowske, 1991). In the case of COE, cefadroxil and oxytetracycline are effective against both gram-positive and gram-negative organisms and erythromycin has activities predominantly against gram-positive bacteria (Wilkowske, 1991).

DMD #58354

Our data showed that oral administration of AP and COE significantly reduced the lovastatin-metabolizing enzyme activity by 58.3 % and 59.9 %, respectively, compared to that of control rats. Antibiotic treatment also significantly suppressed other metabolic enzyme activities such as α -D-glucosidase, β -D-glucosidase, and α -L-rhamnosidase activities (Fig. 2).

To investigate the biotransformation of lovastatin by the intestinal microbiota, lovastatin was incubated with human and rat fecalase prior to analysis using LC-QTOF/MS. Representative data from the human and rat fecalase samples are shown in Fig. 3. The accurate mass data for lovastatin and its postulated metabolites were tabulated in Table 1 and the relevant MS/MS spectra were provided as supplementary data (Supplemental Figure 2). Four metabolites were detected in the human fecalase samples (Fig. 4), that is, **M1**, **M4**, **M8**, and **M9** at m/z 343, m/z 443, m/z 445, and m/z 461, respectively, were detected as protonated ($[M+H]^+$) or sodium adduct ions ($[M+Na]^+$). **M1** was postulated as a demethylbutyryl metabolite, based on its accurate mass data (m/z 321.2048, $C_{19}H_{28}O_4H^+$); **M4** was postulated as an hydroxylated metabolite, based on a molecular weight increase of 16 Da and its MS/MS fragmentation pattern; **M8** was identified as an hydroxy acid metabolite, by comparison with the accurate mass data of the authentic reference compound and its MS/MS fragmentation pattern; **M9** was postulated as an hydroxylated hydroxy acid metabolite, based on a molecular weight increase of 16 Da, as compared with **M8**. The rat fecalase samples also exhibited these 4 metabolites with comparable MS/MS data to those observed in the human samples. **M1**, **M4**, and **M8** were isolated from the fecalase samples and their chemical structures were confirmed by 1H and ^{13}C -NMR analyses (Supplemental data 1). To investigate the actual contribution of microbiota-mediated metabolism to lovastatin pharmacokinetics, the intestinal microbiota-mediated metabolism pattern was compared with

DMD #58354

those in liver microsomal samples and rat plasma. In human and rat liver microsomes, 8~9 metabolites were detected (Supplemental Figure 1A and 1B); the human and rat metabolism profiles were comparable, although a slight difference was observed. The chemical structures of the metabolites were determined and/or postulated on the basis of their accurate mass data and MS/MS fragmentation patterns referring to the data available in the literature (Vyas KP et al., 1990). The **M4** and **M8** metabolites identified in human or rat fecal samples were also observed in liver microsomal samples. In rat plasma, a total of 4 metabolites was detected: **M1**, **M6**, **M8**, and **M11** (Supplemental Figure 1C). **M1**, **M8**, and **M11** were the observed main circulating metabolites, and our findings indicated that gut microbiota-mediated metabolism could have contributed to their plasma level. The accurate mass data for lovastatin and its postulated metabolites were tabulated in Table 1 and their relevant MS/MS spectra were provided as supplementary data (Supplemental Figure 2).

To investigate the effects of antibiotics on lovastatin pharmacokinetics, plasma lovastatin levels were analyzed following its oral administration to antibiotic-treated rats (AP or COE). Because oral lovastatin is readily metabolized to the hydroxy acid (the active metabolite; **M8**), the plasma concentrations of **M8** were determined. The mean plasma concentration-time profiles of **M8** after oral lovastatin administration to control rats, and those treated with AP or COE, are shown in Fig. 5. The resultant pharmacokinetic parameters are summarized in Table 2. As shown in Table 2, The C_{\max} values for **M8** in control, AP, and COE groups were 188.1 ± 67.8 , 114.7 ± 26.0 , and 93.1 ± 78.0 ng/mL, and the AUC values were 907.9 ± 309.7 , 589.3 ± 117.6 , and 448.4 ± 143.7 ng-h/mL, respectively. Thus, the C_{\max} and systemic exposure (AUC) of the active lovastatin metabolite, **M8**, were significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) in both groups of antibiotic-treated rats, compared with

DMD #58354

control rats.

The present study investigated the metabolism of lovastatin by gut microbiota, and the consequent pharmacokinetic interactions between lovastatin and antibiotics. Incubation of lovastatin with fecalase revealed that gut microbial enzymes may be involved in the metabolism of this drug and may have contributed to the formation of its active metabolite (**M8**). The *in vitro* metabolism profile with liver microsomes indicated a considerable overlap between gut microbial and liver microsomal enzymes in terms of their contributions to lovastatin metabolism. Furthermore, the *in vivo* metabolism data suggested that a considerable portion of circulating lovastatin metabolites, including the active **M8** metabolite, could be generated by metabolic processes mediated by intestinal microbiota. These findings indicated a strong probability of DDI between lovastatin and antibiotics. The pharmacokinetic results of the present study showed that oral administration of antibiotics prior to oral lovastatin reduced plasma **M8** levels. To rule out any other possible pharmacokinetic interference by antibiotics, such as effects on CYP enzymes or drug efflux pumps, lovastatin was administered 3 days after antibiotic treatment, when the antibiotics should have been eliminated from the body. Although erythromycin, which was included in COE, is considered as a time-dependent inactivator of CYP3A, it has been found that a single dose of erythromycin does not form a metabolic intermediate complex, i.e., it does not inactivate CYP3A4 (Zhang et al., 2010). Thus, the potential inhibition of the hepatic metabolism of lovastatin by erythromycin would be negligible. Furthermore, AP, which does not have notable DDI issues, also showed comparable effects in **M8** pharmacokinetics in this study. This finding shows that the pharmacokinetic alteration following antibiotic treatment may be mainly due to an antibacterial effect rather than inhibition of hepatic pathways. Thus,

DMD #58354

the decreased plasma level of **M8** in rats treated with antibiotics is likely to have resulted from decreased biotransformation of lovastatin to its hydroxy acid metabolite by gut microbiota. This suggested mechanism was supported by the finding of reduced metabolic activity in antibiotic-treated rat fecalase. Further experiments with germ-free animals and animals in which human flora has been introduced could aid in understanding the contribution of hepatic and gut microbes to **M8** formation.

Following oral administration, lovastatin is metabolized to the active open-ring lovastatin acid and further metabolized to numerous active or inactive compounds (Krukemyer and Talbert, 1987; Vyas et al., 1990). The systemic bioavailability of the lovastatin acid is only 5% and the cholesterol-lowering effects of lovastatin are dependent on the production of this acid metabolite (Neuvonen and Jalava, 1996). As lovastatin is mainly metabolized in the liver by CYP3A4, there are well-established DDI between lovastatin and CYP3A4 inhibitors (Neuvonen, 2010). Itraconazole, erythromycin, and grapefruit juice have been shown to significantly elevate serum levels of lovastatin and its hydroxy acid metabolite, potentially leading to serious side effects, such as rhabdomyolysis. The findings of the current study suggest that there is the additional potential for DDI between lovastatin and antibiotics. Whilst CYP3A4-mediated DDI result in increased systemic exposure to lovastatin and subsequent side effects, antibiotic-induced gut microbiota-mediated DDI reduce bioavailability of the active metabolite, which may attenuate lovastatin's therapeutic effects. The present study showed a 35-50% decrease in the **M8** AUC in antibiotic-treated rats (Table 2). Lovastatin is generally used as a long-term maintenance therapy. Our human fecalase lovastatin metabolism activity data (Fig. 1A) showed considerable inter-individual variability. In cases where patients taking lovastatin are treated with antibiotics for a long time,

DMD #58354

appropriate maintenance of serum cholesterol levels may not be achieved in some individuals. Thus, for some patients, co-administration of antibiotics might lead to serious outcomes due to a failure to control serum cholesterol levels.

Antibiotics also suppressed a range of glycosidase activities (Fig. 2). The suppression of intestinal microbial enzyme activity lasted for at least 3 days after treatment with antibiotics. This indicated that the effects of antibiotics on xenobiotic metabolism might be more extensive and potent than previously recognized. We have previously reported that antibiotic treatment altered the pharmacokinetics of hesperidin, a bioactive citrus flavonoid glycoside (Jin et al., 2010). In addition, there have been several reports demonstrating that antibiotics altered the pharmacokinetics of dietary compounds or phytochemicals (Saad et al., 2012). However, studies of this issue using synthetic drugs are still limited. For synthetic drugs, the liver is the primary focus of drug metabolism. Hepatic metabolism generally includes oxidation and conjugation reactions, producing polar high molecular weight metabolites. In contrast, bacterial metabolism primarily involves reductive and hydrolytic reactions, generating non-polar low molecular weight products (Croom, 2012). However, our finding suggested that there could be a considerable overlap between intestinal bacterial and hepatic metabolisms in some drugs. Thus, the gut microbiota could act as an organ with a drug-metabolic potential at least equal to that of the liver, depending on the type of drug involved. For this reason, more attention should be paid to gut microbiota-mediated drug metabolism as another possible determinant of DDI.

In conclusion, this study demonstrated the involvement of gut microbiota in the metabolism of lovastatin to its bioactive metabolite. Furthermore, we found that DDI between lovastatin and antibiotics were likely, due to antibiotic-mediated inhibition of intestinal

DMD #58354

bacteria. To our knowledge, this is the first report of gut microbe-mediated lovastatin metabolism and the consequent pharmacokinetic interactions. These findings indicated that administration of antibiotics to patients taking lovastatin may lead to decreased systemic exposure of the lovastatin active metabolite, reducing its pharmacological effects. Similar antibiotic-induced pharmacokinetic effects may occur with other drugs metabolized by gut microbial enzymes, and further studies on gut microbiota-mediated metabolism are therefore required, with a wider range of drugs.

Authorship contributions

Participated in research design: Kim DH and Yoo HH

Conducted experiments: Yoo DH, Le TKV, Kim IS, Jung IH

Contributed new reagents or analytic tools: Kim IS

Performed data analysis: Yoo DH and Kim IS

Wrote or contributed to the writing of the manuscript: Yoo DH, Kim DH and Yoo HH

DMD #58354

References

- Bowey E, Adlercreutz H, and Rowland I (2003) Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. *Food Chem Toxicol* 41: 631-636.
- Choi JR, Hong SW, Kim Y, Jang SE, Kim NJ, Han MJ, and Kim DH (2011) Metabolic activities of ginseng and its constituents, ginsenoside Rb1 and Rg1, by human intestinal microflora. *J Ginseng Res* 35: 301-307.
- Croom E (2012) Metabolism of xenobiotics of human environments. *Prog Mol Biol Transl Sci* 112: 31-88.
- Guarner F, Malagelada JR (2003) Gut flora in health and disease. *Lancet* 361: 512-519.
- Haiser HJ and Turnbaugh PJ (2013) Developing a metagenomic view of xenobiotic metabolism. *Pharmacol Res* 69: 21-31.
- Isoherranen N, Lutz JD, Chung SP, Hachad H, Levy RH, and Ragueneau-Majlessi I (2012) Importance of multi-p450 inhibition in drug-drug interactions: evaluation of incidence, inhibition magnitude, and prediction from in vitro data. *Chem Res Toxicol* 25: 2285-2300.
- Jin MJ, Kim U, Kim IS, Kim Y, Kim DH, Han SB, Kim DH, Kwon OS, and Yoo HH. Effects of gut microflora on pharmacokinetics of hesperidin: a study on non-antibiotic and pseudo-germ-free rats. *J Toxicol Environ Health A* 73: 1441-1450.
- König J, Müller F, and Fromm MF (2013) Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacol Rev* 65: 944-966.
- Krukemyer JJ and Talbert RL (1987) Lovastatin: a new cholesterol-lowering agent. *Pharmacotherapy* 7: 198-210.

DMD #58354

Lee DS, Kim YS, Ko CN, Cho KH, Bae HS, Lee KS, Kim JJ, Park EK, and Kim DH (2002)

Fecal metabolic activities of herbal components to bioactive compounds. *Arch Pharm Res* 25; 165–169.

Li H and Jia W (2013) Cometabolism of microbes and host: implications for drug metabolism and drug-induced toxicity. *Clin Pharmacol Ther* 94: 574-581.

Lindenbaum J, Rund DG, Butler VP Jr, Tse-Eng D, and Saha JR (1981) Inactivation of digoxin by the gut flora: reversal by antibiotic therapy. *N Engl J Med* 305: 789-794.

Neuvonen PJ (2010) Drug interactions with HMG-CoA reductase inhibitors (statins): the importance of CYP enzymes, transporters and pharmacogenetics. *Curr Opin Investig Drugs* 11: 323-332.

Neuvonen PJ and Jalava KM (1996) Itraconazole drastically increases plasma concentrations of lovastatin and lovastatin acid. *Clin Pharmacol Ther* 60: 54-61.

Roberts AB, Wallace BD, Venkatesh MK, Mani S, and Redinbo MR (2013) Molecular insights into microbial β -glucuronidase inhibition to abrogate CPT-11 toxicity. *Mol Pharmacol* 84: 208-217.

Saad R, Rizkallah MR, and Aziz RK (2012) Gut Pharmacobiomics: the tip of an iceberg of complex interactions between drugs and gut-associated microbes. *Gut Pathog* 4: 16.

Saha JR, Butler VP Jr, Neu HC, and Lindenbaum J (1983) Digoxin-inactivating bacteria: identification in human gut flora. *Science* 220: 325-327.

Scheline RR (1973) Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol Rev* 25: 451-523.

DMD #58354

Sousa T, Paterson R, Moore V, Carlsson A, Abrahamsson B, and Basit AW (2008) The gastrointestinal microbiota as a site for the biotransformation of drugs. *Int J Pharm* 363: 1-25.

Vyas KP, Kari PH, Prakash SR, and Duggan DE (1990) Biotransformation of lovastatin; II. In vitro metabolism by rat and mouse liver microsomes and involvement of cytochrome P-450 in dehydrogenation of lovastatin. *Drug Metab Dispos* 2: 218-222.

Wilkowske CJ (1991) General principles of antimicrobial therapy. *Mayo Clin Proc* 66: 931-941.

Wilson ID and Nicholson JK (2009) The role of gut microbiota in drug response. *Curr Pharm Des* 15: 1519-1523.

Zhang X, Galinsky RE, Kimura RE, Quinney SK, Jones DR, and Hall SD (2010) Inhibition of CYP3A by erythromycin: in vitro-in vivo correlation in rats. *Drug Metab Dispos* 38: 61-72.

DMD #58354

Footnotes

This research was supported by a grant from Ministry of Food and Drug Safety in 2013 [12182MFDS652].

Dae-Hyeong Yoo and In Sook Kim contributed equally to this work.

DMD #58354

Legends for figures

Figure 1. Lovastatin-metabolizing activities of human and rat fecalase. (A) Human fecalase; (B) Rat fecalase from control (NOR) or antibiotic-treated rats. AP, ampicillin; COE, antibiotic mixture [cefadroxil (150 mg/kg), oxytetracycline (300 mg/kg), and erythromycin (300 mg/kg)].

Figure 2. Effects of antibiotics on rat fecalase glycosidase activities. Antibiotics (arrow) were orally administered to rats and (A) β -D-glucuronidase, (B) α -L-rhamnosidase, and (C) β -D-glucosidase activities were measured. Closed circle, saline only; open triangle, ampicillin (AP, 250 mg/kg); open square, antibiotic mixture (COE) [cefadroxil (150 mg/kg), oxytetracycline (300 mg/kg), and erythromycin (300 mg/kg)].

Figure 3. Representative extracted ion chromatograms for lovastatin and its metabolites in (A) human and (B) rat fecalase samples.

Figure 4. Chemical structures of lovastatin and its major metabolites obtained from human and rat fecal samples.

Figure 5. Plasma concentration of lovastatin hydroxy acid in control and antibiotic-treated rats. Lovastatin (20 mg/kg) was orally administered to rats 3 days after a single dose of ampicillin (AP, 250 mg/kg) or antibiotic mixture (COE) (cefadroxil [150 mg/kg], oxytetracycline [300 mg/kg], and erythromycin [300 mg/kg]) ($n = 10$ for each).

DMD #58354

Table 1. Accurate mass data for lovastatin and its metabolites

Compound	RT	Elemental composition	Theoretical Mass(Da)	Measured mass(Da)	Error	Remark
Lovastatin	15.4	$C_{24}H_{36}O_5Na^+$	427.2482	427.2440	0.7	HF, RF, HLM, RLM
M1	13.6	$C_{19}H_{28}O_4H^+$	321.2060	321.2048	0.6	HF, RF, RP
M2	14.7	$C_{24}H_{34}O_5Na^+$	425.2326	425.2321	1.2	HLM, RLM
M3	9.0	$C_{24}H_{36}O_6Na^+$	443.2404	443.2427	5.2	HLM, RLM
M4	9.4	$C_{24}H_{36}O_6Na^+$	443.2404	443.2422	4.1	HF, RF, HLM, RLM
M5	11.5	$C_{24}H_{36}O_6Na^+$	443.2404	443.2426	5.0	RLM
M6	13.0	$C_{24}H_{36}O_6Na^+$	443.2404	443.2425	4.7	RP, HLM
M7	14.1	$C_{24}H_{36}O_6Na^+$	443.2404	443.2424	4.5	HLM
M8	13.6	$C_{24}H_{38}O_6Na^+$	445.2584	445.2584	4.9	HF, RF, HLM, RLM, RP
M9	5.4	$C_{24}H_{38}O_7Na^+$	461.2510	461.2498	2.6	HF, RF, HLM, RLM
M10	7.3	$C_{24}H_{38}O_7Na^+$	461.2510	461.2535	5.4	HLM
M11	8.2	$C_{24}H_{38}O_7Na^+$	461.2510	461.2538	6.1	HLM, RLM, RP

*HF; human feces; RF: rat feces; HLM: human liver microsomes; RLM: rat liver microsomes; RP: rat plasma

DMD #58354

Table 2. Pharmacokinetic parameters of lovastatin hydroxy acid in control and antibiotic-treated rats ($n=10$)

	Control	Ampicillin	Antibiotic mixture
C_{\max} (ng/mL)	188.1 \pm 67.8	114.7 \pm 26.0*	93.12 \pm 78.0 *
T_{\max} (hr)	2.2 \pm 2.2	1.9 \pm 2.3	1.8 \pm 1.3
AUC(ng·hr/mL)	907.9 \pm 309.7	589.3 \pm 117.6**	448.4 \pm 143.7**

* : $P < 0.05$, ** : $P < 0.01$

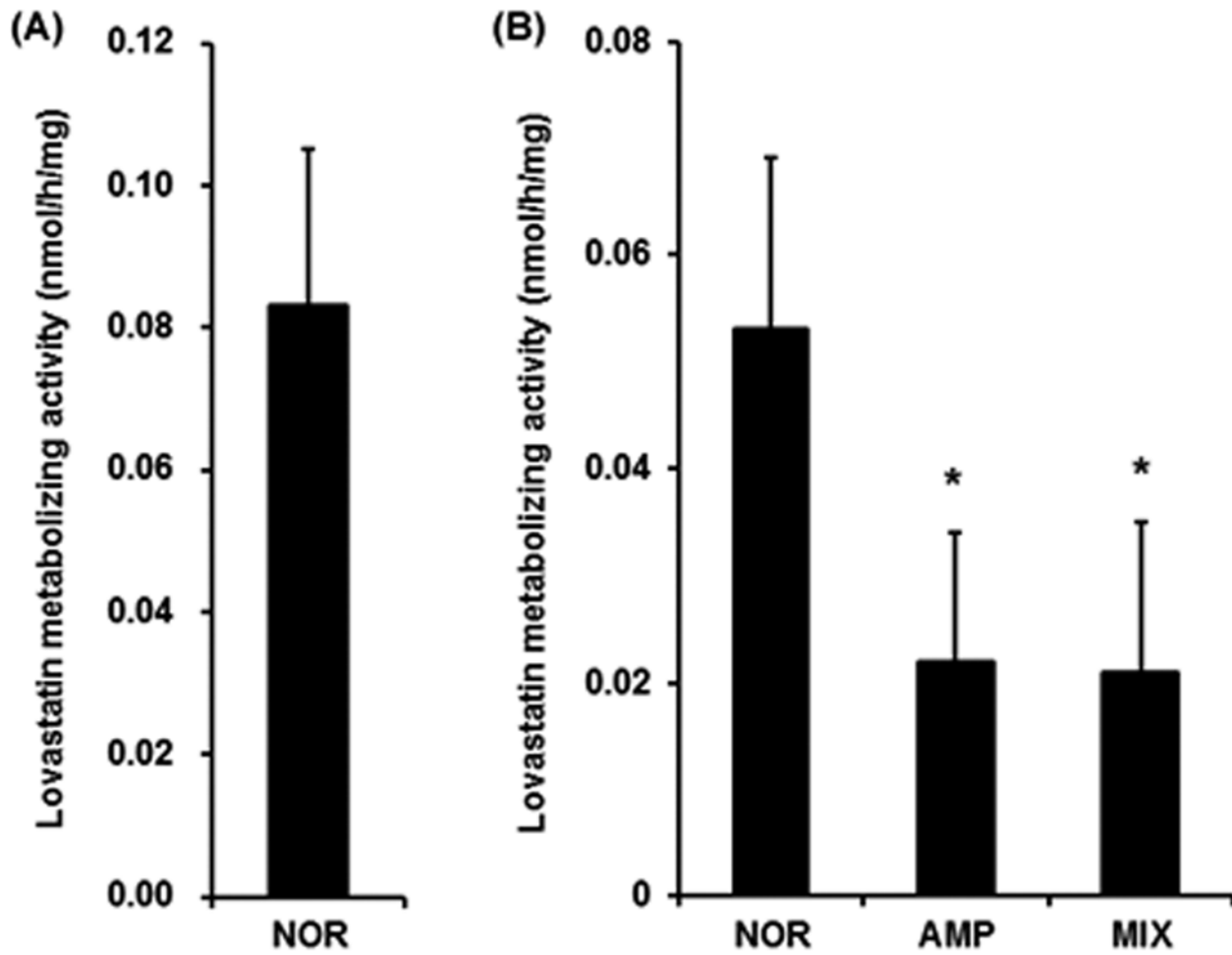


Figure 1

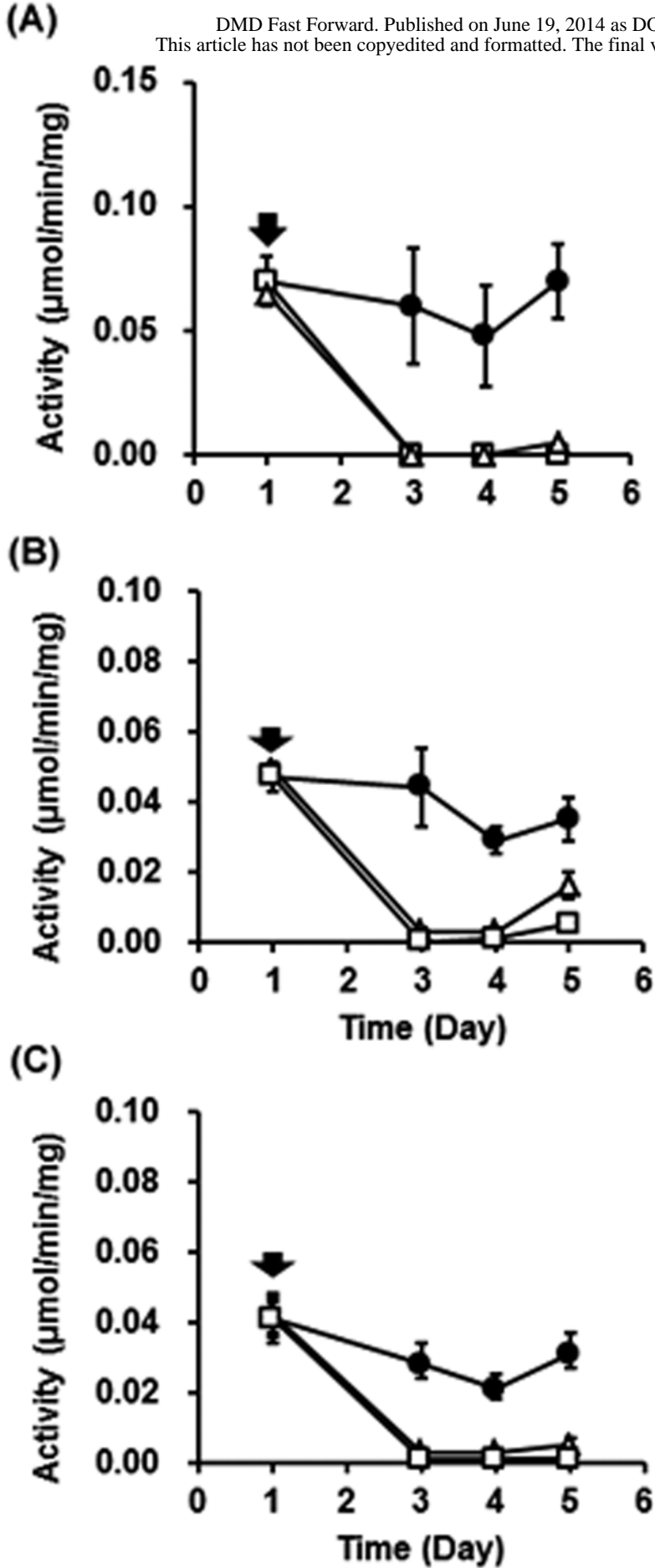


Figure 2

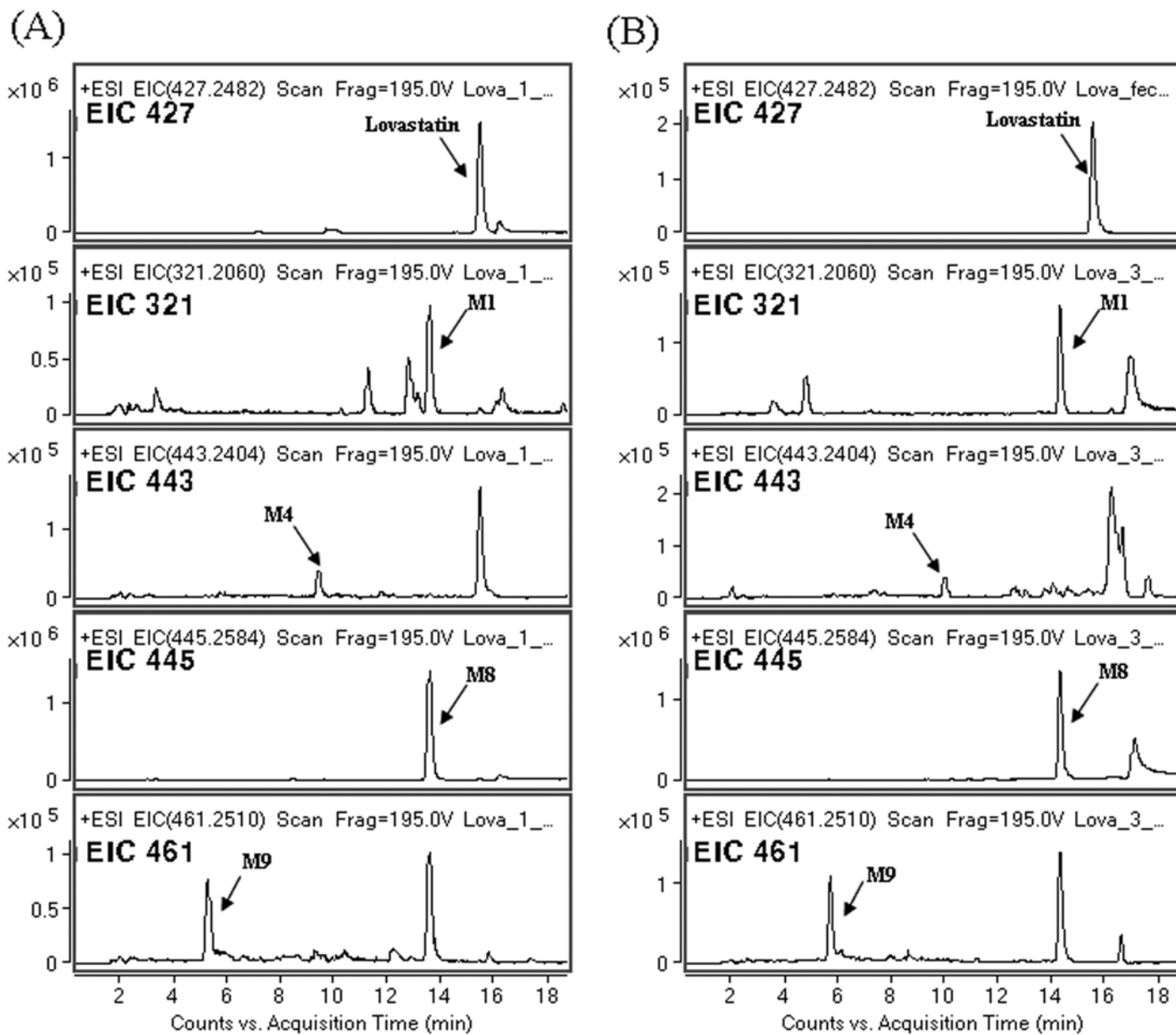


Figure 3

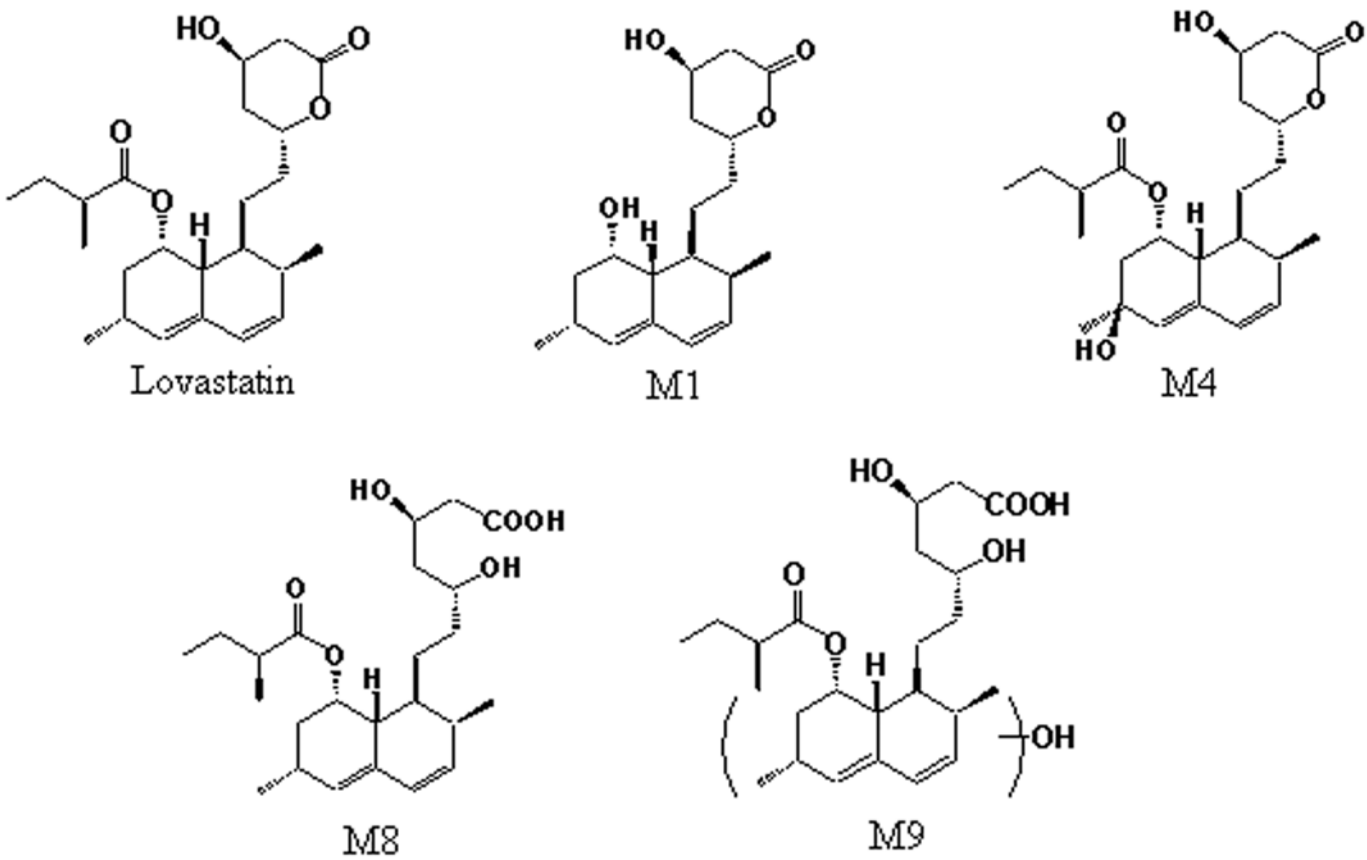


Figure 4

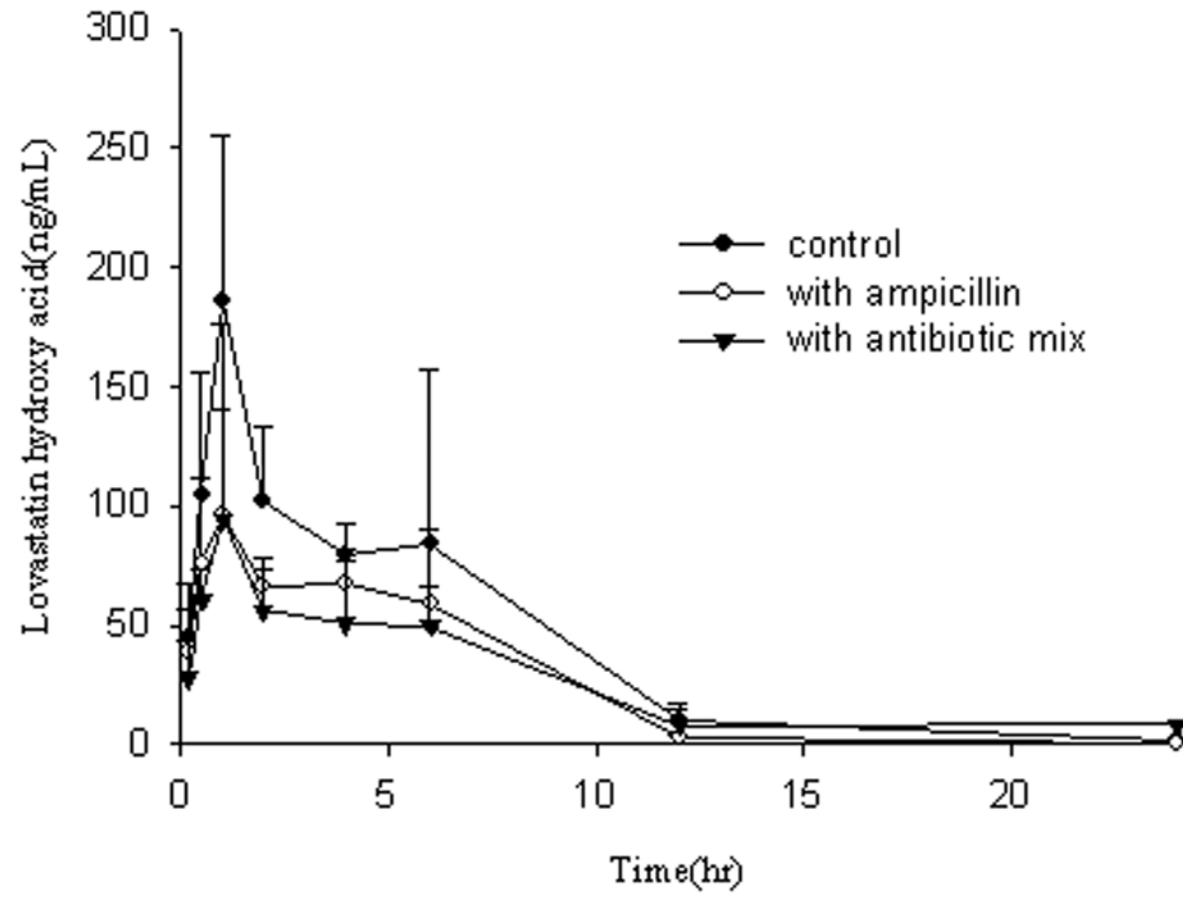


Figure 5

Supplemental data (DMD #58354)

Gut microbiota-mediated drug interactions between lovastatin and antibiotics

Dae-Hyeong Yoo, In Sook Kim, Thi Kim Van Le, Il-Hoon Jung, Hye Hyun Yoo, Dong-Hyun Kim

<Legends for Supplemental data>

Supplemental data 1. NMR data for lovastatin metabolites isolated from human fecalase samples

Supplemental data 2. LC-MS/MS conditions for metabolite identification and pharmacokinetic study

Supplemental Figure 1. Extracted ion chromatograms of lovastatin and its metabolites: (A) Human liver microsomes, (B) Rat liver microsomes, and (C) Rat plasma

Supplemental Figure 2. Product ion spectra of lovastatin and its metabolites

Supplemental data 1. NMR data for lovastatin metabolites isolated from human fecalase samples

M1

¹H-NMR (500 MHz, CDCl₃) δ (ppm) : 6.00 (1H, d, J = 12.5 Hz, H-4), 5.79 (1H, dd, J = 8.0/12.0 Hz, H-3), 5.52 (1H, brs, H-6), 4.61 (1H, m, H-13), 4.35 (1H, t, J = 4.5, H-15), 3.88 (1H, d, J = 3.5, H-9), 2.74 (1H, d, J = 3.0 Hz, H-16a), 2.70 (1H, d, J = 6.5 Hz, H-16b), 2.44 (1H, m, H-2), 2.25 (1H, m, H-14a), 2.10 (1H, m, H-14b), 1.94 (1H, m, H-10a), 1.66 (1H, m, H-8a), 1.45 (1H, m, H-14b), 1.25 (1H, m, H-8b), 1.10 (3H, d, J = 8.5Hz, H-18), 1.07 (3H, d, J = 9.5Hz, H-19).

¹³C-NMR (125 MHz, CDCl₃) δ (ppm) : 176.5 (C-17), 133.3 (C-5), 131.5 (C-3), 129.6 (C-4), 128.3 (C-6), 76.4 (C-13), 67.9 (C-9), 62.5 (C-15), 41.5 (C-10), 38.5 (C-16), 37.3 (C-8), 36.6 (C-14), 32.9 (C-1), 30.6 (C-12), 29.7 (C-2), 27.4 (C-7), 26.2 (C-11), 17.8 (C-18), 13.8 (C-19).

M4

¹H-NMR (500 MHz, CDCl₃) δ (ppm) : 6.05 (1H, d, J = 12.5 Hz, H-4), 5.79 (1H, dd, J = 8.0/12.0 Hz, H-3), 5.52 (1H, brs, H-9), 5.40 (1H, d, J = 3.5, H-6), 4.63 (1H, m, H-13), 4.33 (1H, t, J = 4.5, H-15), 2.72 (1H, d, J = 3.0 Hz, H-16a), 2.70 (1H, d, J = 6.5 Hz, H-16b), 2.44 (1H, m, H-8a), 2.36 (1H, m, H-11), 2.25 (1H, m, H-10), 1.94 (2H, m, H-12), 1.66 (2H, m, H-19), 1.45 (2H, m, H-21), 1.40 (1H, m, H-8b), 1.25 (3H, s, H-23), 1.10 (3H, d, J = 8.5Hz, H-20), 1.07 (3H, d, J = 9.5Hz, H-24), 0.88 (3H, t, J = 10.0 Hz, H-22).

¹³C-NMR (125 MHz, CDCl₃) δ (ppm) : 178.0 (C-17), 170.5 (C-18), 133.3 (C-5), 131.5 (C-3), 129.6 (C-4), 128.3 (C-6), 77.8 (C-7), 76.4 (C-13), 67.9 (C-9), 62.5 (C-15), 41.5 (C-10), 38.5 (C-16), 37.3 (C-8), 36.6 (C-14), 32.9 (C-12), 30.6 (C-2), 29.7 (C-19), 37.4 (C-1), 24.2 (C-11), 22.7 (C-20), 16.8 (C-22), 27.6 (C-23), 13.8 (C-24), 12.3 (C-21).

M8

$^1\text{H-NMR}$ (500 MHz, MeOD) δ (ppm) : 5.98 (1H, d, $J = 10.0$ Hz, H-4); 5.79 (1H, m, H-3), 5.50 (1H, brs-t, H-9), 5.37 (1H, dd, $J = 3.0/6.0$ Hz, H-6), 4.63 (1H, m, H-13), 4.24 (1H, m, H-15), 2.71 (1H, dd, $J = 4.5/17.5$ Hz, H-10), 2.51 (2H, m, H-16), 2.38 (1H, m, H-19), 2.32 (1H, m, H-2), 1.97 (2H, m, H-8), 1.91 (2H, m, H-14), 1.82 (2H, m, H-7), 1.72 (1H, m, H-1), 1.66 (1H, m, H-11a), 1.54 (1H, m, H-12a), 1.43 (1H, m, H-12b), 1.35 (1H, m, H-11b), 1.28 (3H, br, H-23), 1.11 (3H, d, $J = 8.5$ Hz, H-20), 1.07 (3H, d, $J = 9.5$ Hz, H-24), 0.86 (3H, t, $J = 10.0$ Hz, H-22).

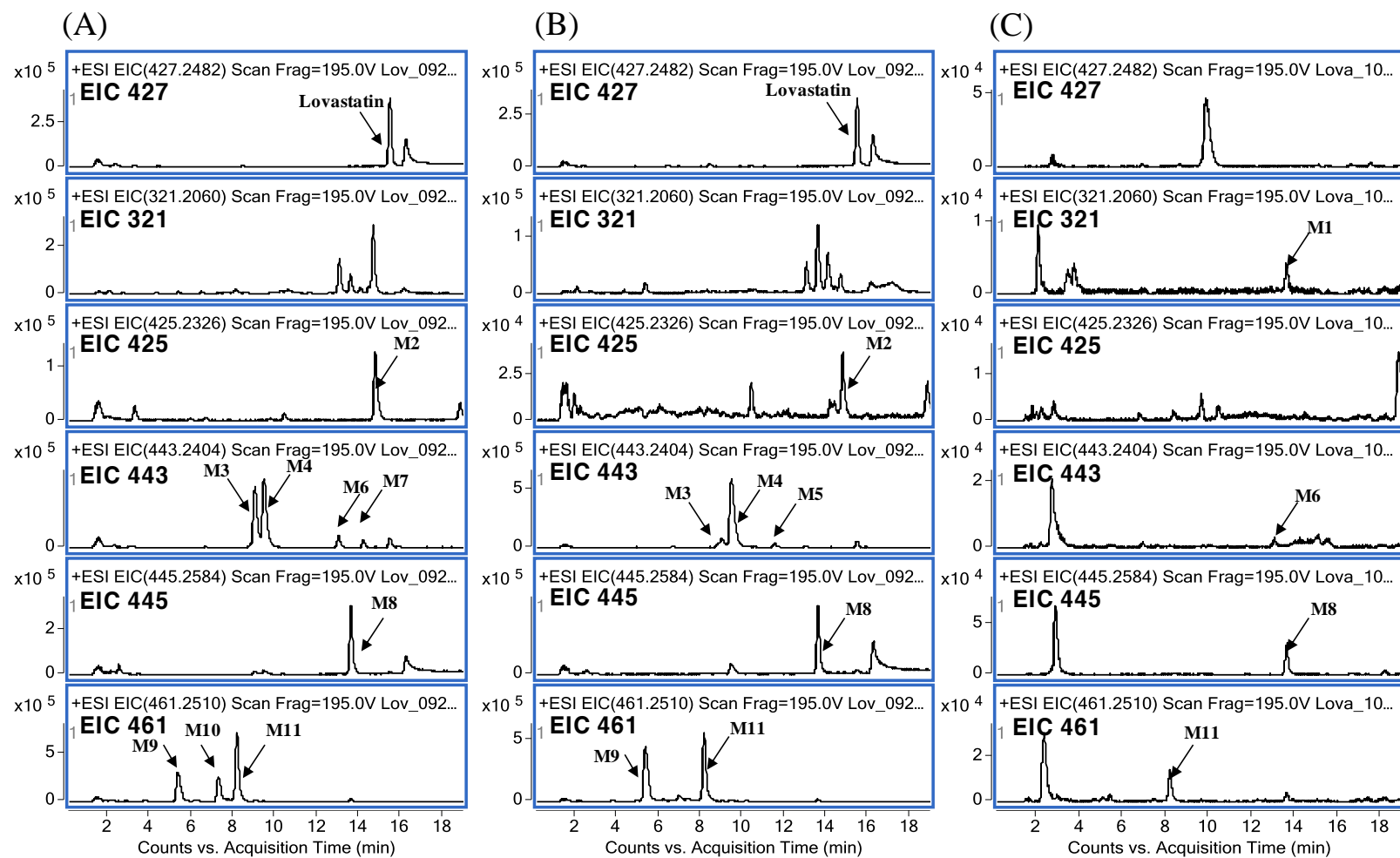
$^{13}\text{C-NMR}$ (125MHz, MeOD) δ (ppm) : 176.8 (C-17), 171.9 (C-18), 132.5 (C-6), 131.7 (C-4), 128.9 (C-3), 128.1 (C-5), 76.6 (C-9), 68.1 (C-13), 61.8 (C-15), 41.4 (C-16), 37.7 (C-10), 37.1 (C-8), 36.5 (C-1), 35.2 (C-14), 32.6 (C-19), 32.2 (C-12), 30.5 (C-2), 27.3 (C-7), 26.5 (C-11), 23.7 (C-20), 21.9 (C-21), 15.1 (C-24), 12.6 (C-23), 10.4 (C-22).

Supplemental data 2. LC-MS/MS conditions for metabolite identification and pharmacokinetic study

LC-QTOF MS analyses for lovastatin metabolites. The LC-MS system consisted of an Agilent 1260 Infinity HPLC system with an Agilent 6530 Q-TOF mass spectrometer (Agilent Technologies) equipped with a JetStream™ electrospray ionization (ESI) source. Chromatographic separation was achieved with a Hypersil gold column (150 mm × 2.0 mm i.d, 5 μm, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the oven temperature was maintained at 40°C. The mobile phase consisted of 0.1% formic acid (solvent A) and 90% acetonitrile with 0.1% formic acid (solvent B). A gradient program was used for the HPLC separation, with a flow rate of 0.25 mL/min. The initial composition of the mobile phase was 30% solvent B, which was changed to 80% solvent B over 15 min, maintained for 3 min, and followed by a 7-min re-equilibration to the initial condition. The mass spectrometer was operated in positive ion mode. The drying gas (nitrogen) temperature was 300°C, with a flow rate of 10 L/min, and the nebulizer pressure was 20 psi. Capillary and fragmentor voltages were 3500 V and 85 V, respectively. Both centroid and profile data within mass range m/z of 100-500 were acquired at a rate of 4 spectra/s using a Mass Hunter workstation (Agilent Technologies). For targeted MS/MS analysis, the product ion scan range was m/z 50–500. High-purity nitrogen was introduced into a collision cell as the fragmentation gas.

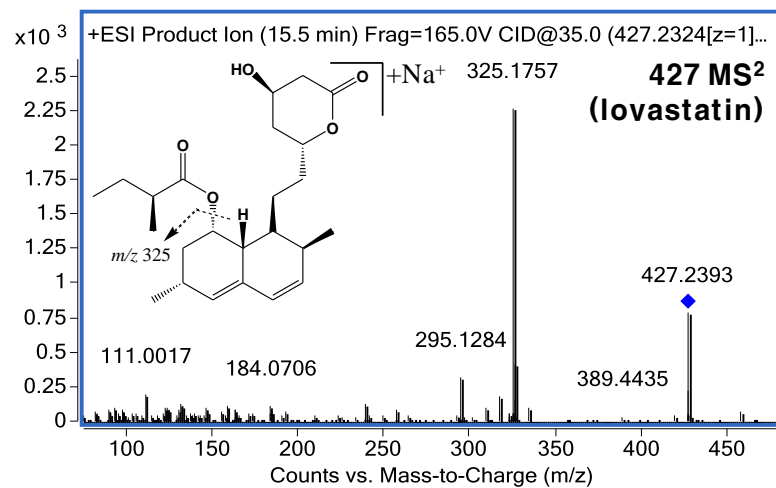
LC-MS/MS analyses for pharmacokinetic study. The LC-MS/MS system consisted of an Agilent 1260 Infinity HPLC system with an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies) equipped with a JetStream™ ESI source. Chromatographic separation was achieved with a Poroshell C18 EC120 column (50 mm × 3.0 mm i.d, 2.7μm, Agilent Technologies) and the oven temperature was maintained at 40°C. The mobile phase consisted of 0.1% formic acid (solvent A) and 90% acetonitrile with 0.1% formic acid (solvent B). A gradient program was used for the HPLC separation, with a flow-

rate of 0.3 mL/min. The initial composition of the mobile phase was 30% solvent B, which was changed to 90% solvent B over 3 min, maintained for 3.5 min, and followed by a 4.5-min re-equilibration to the initial condition for 0.1 min. The drying gas (nitrogen) temperature was 300°C, with a flow rate of 10 L/min. The nebulizer pressure was 20 psi, and the capillary voltage was 3500 V. Fragmentor voltages were set at 135 V for lovastatin β -hydroxy acid and 170 V for digoxin (IS). Multiple-reaction monitoring detection was employed using nitrogen as the collision gas; the precursor-product ion pairs monitored were 421.2 \rightarrow 100.8 for lovastatin β -hydroxy acid and 825.2 \rightarrow 779.2 for digoxin (IS). The retention times of lovastatin β -hydroxy acid and digoxin (IS) were 4.9 and 3.0 min, respectively.

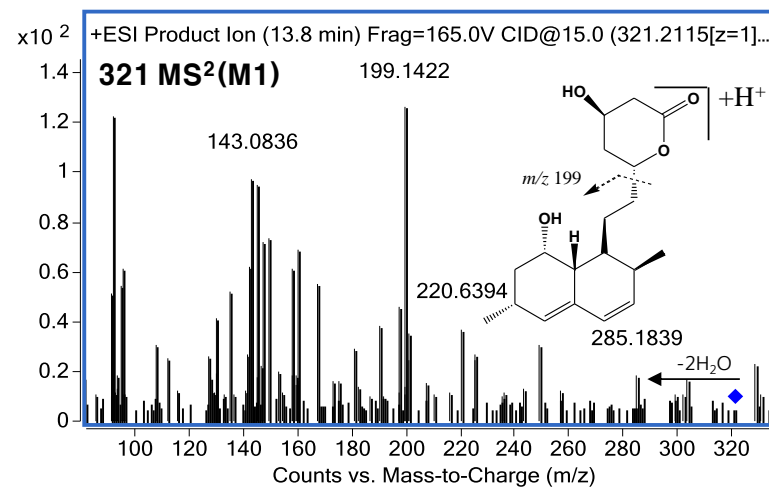


Supplemental Figure 1. Extracted ion chromatograms of lovastatin and its metabolites: (A) Human liver microsomes, (B) Rat liver microsomes, and (C) Rat plasma

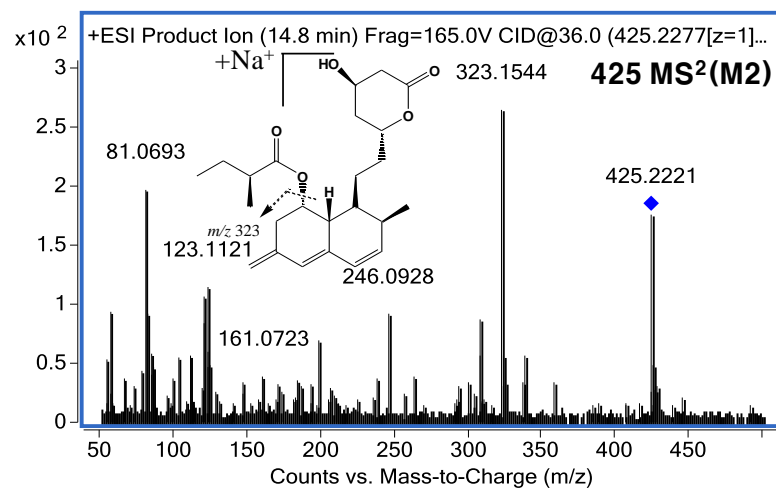
(A)



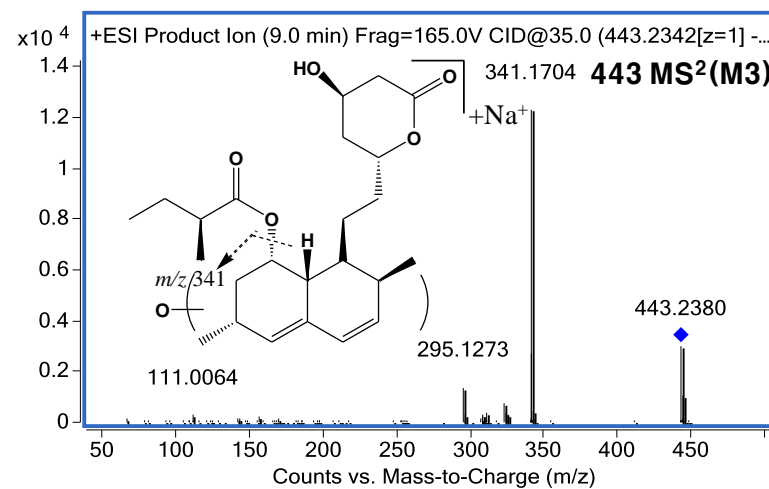
(B)



(C)

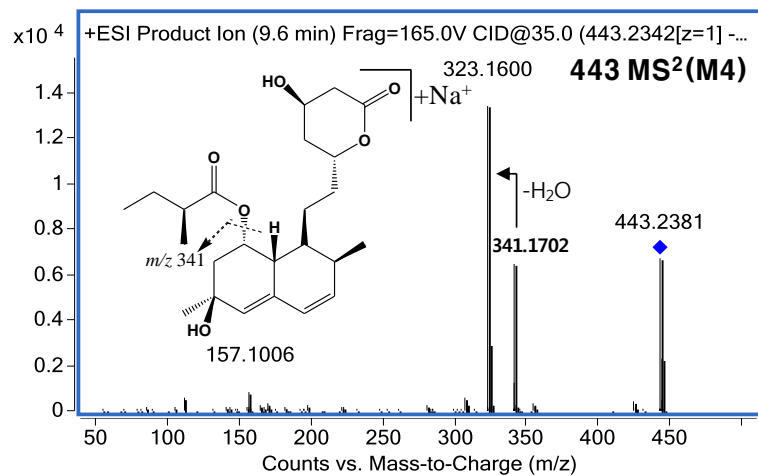


(D)

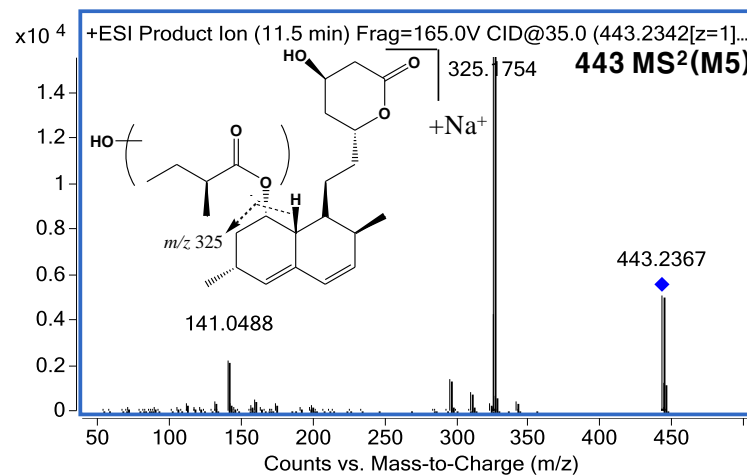


Supplemental Figure 2. Product ion spectra of lovastatin and its metabolites

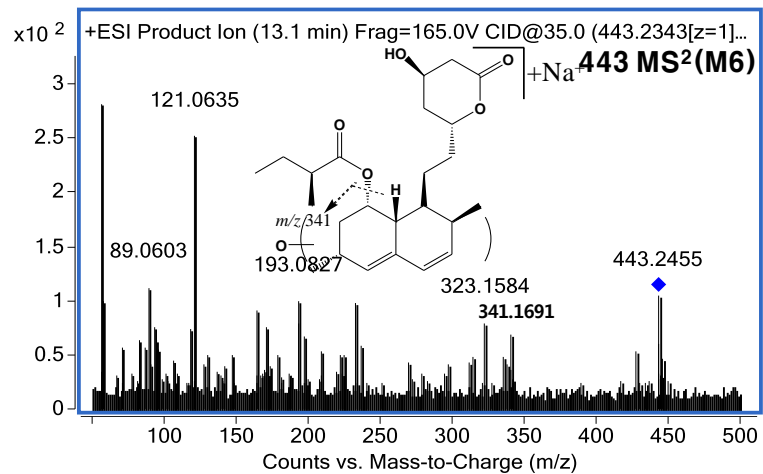
(E)



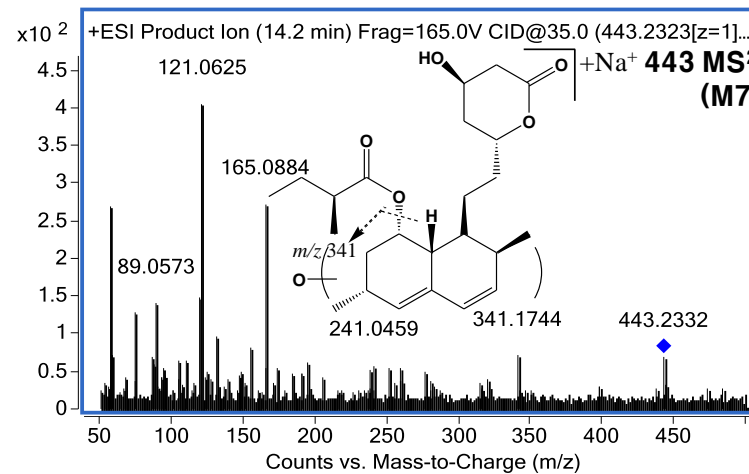
(F)



(G)

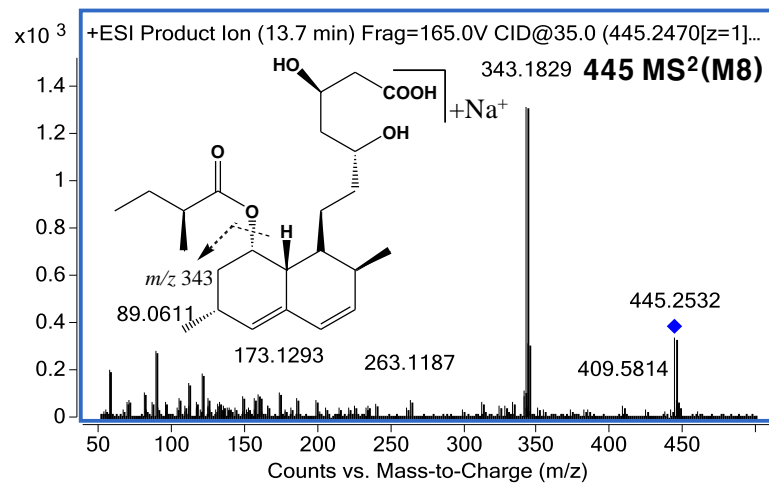


(H)

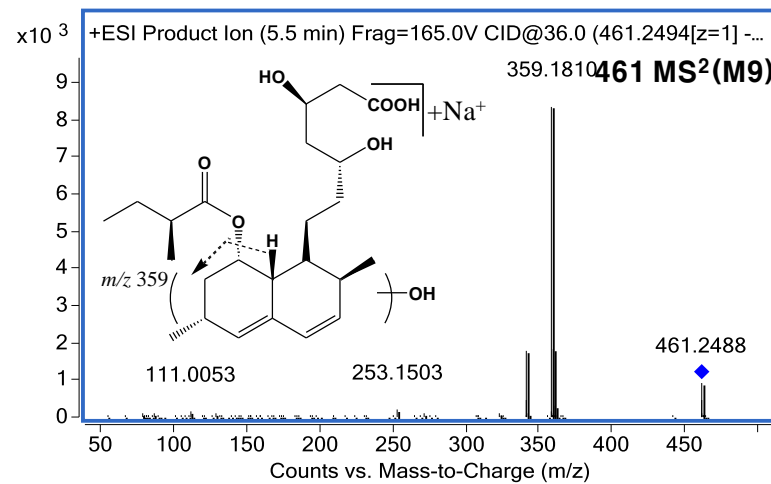


Supplemental Figure 2 continued

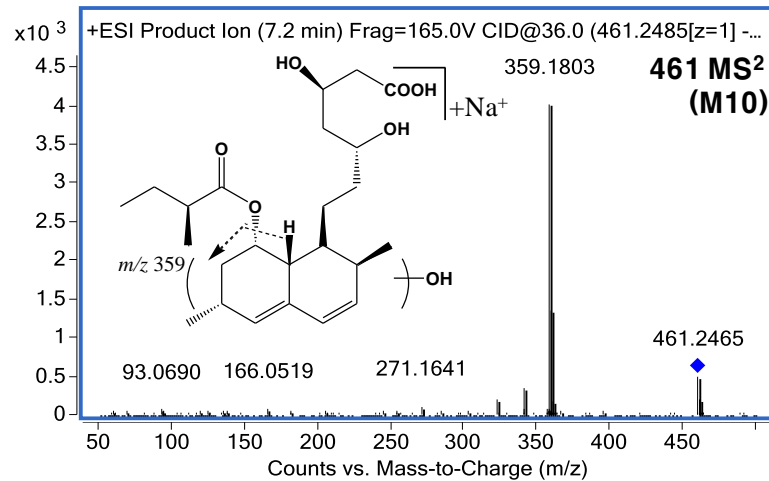
(I)



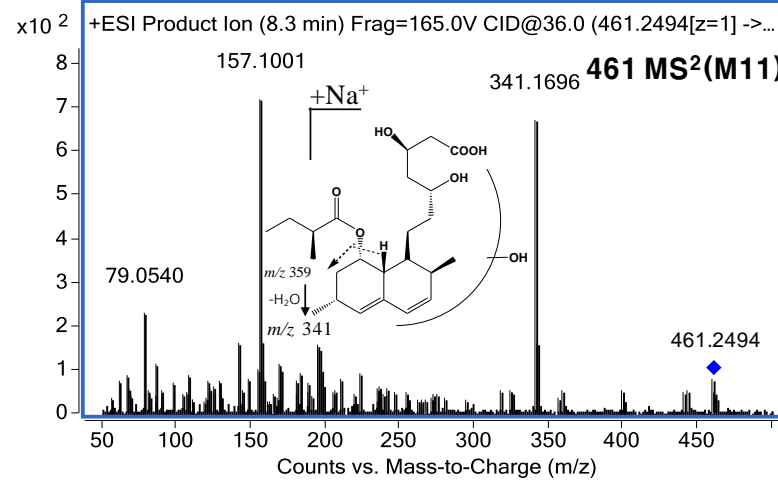
(J)



(K)



(L)



Supplemental Figure 2 continued