Short Communication

Recommendation to Exclude Bile-Duct-Cannulated Rats with Hyperbilirubinemia for Proper Conduct of Biliary Drug Excretion Studies[S]

ABSTRACT

Hyperbilirubinemia (HB) is sometimes encountered following bile-duct cannulation in rats. It possibly originates from the reduced functioning of multidrug resistance-associated protein 2 (Mrp2) and subsequent adaptive alterations in the expression of Mrp3 and the organic anion transporting polypeptides (Oatps). Our aim was to clarify the importance of excluding bile-duct-cannulated (BDC) rats with HB for proper conduct of drug excretion studies. We detected HB [serum total bilirubin concentration (TBIL) ≥0.20 mg/dl] in 16% of all BDC rats prepared. The serum activities of aspartate aminotransferase, alanine aminotransferase, leucine aminopeptidase, and alkaline phosphatase were within the respective normal ranges in the BDC rats with mild HB (TBIL, 0.20–0.79 mg/dl), indicating the absence of hepatic failure. In the pharmacokinetics of pravastatin, an Oatps/Mrp2 probe drug in the BDC rats, the apparent volume of distribution and the clearance were smaller in the mild HB group as compared with the normal group, suggesting the reduction of apparent hepatic uptake and hepatobiliary elimination. The biliary excretion (percentage of dose) was significantly reduced by 54%, suggesting that the biliary efflux activity via Mrp2 was reduced to a greater extent relative to metabolic activity in hepatocytes. The serum γ-glutamyltransferase (GGT) activity correlated with TBIL and inversely correlated with biliary excretion of pravastatin, a finding which could serve as a clue to uncover the regulatory system involving cooperation between GGT and Mrp2. In conclusion, BDC rats with HB, however mild, should be excluded from drug excretion studies to avoid the risk of underestimation of the biliary excretion of drugs.

Introduction

Characterization of the elimination pathways of candidate drugs is one of the most important studies in drug development. Rats with bile-duct cannulation (BDC) have been used to obtain valuable information about the biliary excretion of drugs. An extracorporeal BDC bypass loop is implanted into the rats by surgery (Lipsky and Berkley, 1977) to collect bile specimens from the animals under unrestrained freely moving conditions (Balabaud et al., 1981). However, the BDC surgery sometimes causes hyperbilirubinemia (HB) (Faure et al., 2006). Decreased hepatobiliary transport of bilirubin and its glucuronides is a possible cause of the HB in such rats. Multidrug resistance-associated protein 2 (Mrp2) plays a crucial role in the efflux of endogenous and exogenous anionic compounds from the hepatocytes into bile (van der Schoor et al., 2015). The Eisai hyperbilirubinemic rat, an Mrp2-deficient mutant rat, has been reported to show reduced biliary elimination of bilirubin glucuronides (Kato et al., 2012). It would be reasonable to consider that Mrp2 function is reduced by BDC, considering the common knowledge that Mrp2 expression is downregulated in intrahepatic and obstructive cholestasis (Trauner et al., 1997), and that cholestasis can be induced even by partial obstruction of the bile duct (Rodriguez-Garay et al., 2004). Under such conditions of Mrp2 deficiency and cholestasis, the expression of Mrp3, a basolateral efflux pump to transport bilirubin glucuronides back into the bloodstream, is strongly upregulated (Soroka et al., 2001; Kuroda et al., 2004) and is responsible for the conjugated HB (Keppler, 2014). As an additional adaptive change against Mrp2 function impairment, the expression of the organic anion transporting polypeptide 1 (Oatp1) and Oatp2 is downregulated (Dumont et al., 1997; Kuroda et al., 2004). The rat model with partial obstruction of the bile duct has been reported to have the impairment of Oatp1 function along with that of Mrp2 function (Rodriguez-Garay et al., 2004). Since the Oatps play a role in the uptake of both unconjugated and conjugated bilirubin across the sinusoidal membrane from the bloodstream into the hepatocytes, reduced functioning of these proteins leads to both unconjugated and conjugated HB (van de Steeg et al., 2010; Chu et al., 2015; Watanabe et al., 2015). Therefore, there is a risk that the use of BDC rats with HB can lead to underestimation of the biliary elimination of Oatp and/or Mrp2 substrates in drug excretion studies.

In this study, we investigated the frequency distribution of HB, the serum enzyme activities for evaluating hepatobiliary function in BDC rats with HB, and the pharmacokinetics and biliary excretion of pravastatin, an Oatps/Mrp2 probe drug. Our goal is to verify the reduced hepatobiliary elimination of the anionic drugs via the Oatps and/or Mrp2 in the BDC rats with HB and to propose the cutoff value of the serum concentration of total bilirubin (TBIL) to exclude an animal from a drug excretion study. Our findings would serve as useful information for proper conduct of biliary excretion studies during drug development.

ABBREVIATIONS: AUC, area under the plasma concentration–time curve; AUC_h, AUC in the liver; BDC, bile-duct cannulation; CL_{bile}, biliary clearance; $CL_{int,bile}$, intrinsic clearance for biliary efflux; $CL_{int,h}$, intrinsic hepatic clearance; $CL_{int,met}$, intrinsic clearance for metabolism; $CL_{non-bile}$, nonbiliary clearance; CL_{total}, total body clearance with regard to the plasma concentration; DBIL, direct bilirubin; GGT, γ-glutamyltransferase activity; HB, hyperbilirubinemia; IDBIL, indirect bilirubin; Mrp, multidrug resistance-associated protein; Oatp, organic anion transporting polypeptide; PS_{eff}, intrinsic clearance for cellular efflux; PS_{inf}, intrinsic clearance for cellular uptake (influx); TBIL, total bilirubin concentration.

TABLE 1

Serum biochemistry parameters in the bile-duct-cannulated rats

Data represent the mean ± S.E.M.

Parameter	Normal BDC Rats (TBIL < 0.2)	BDC Rats with Hyperbilirubinemia		
		Mild (TBIL = 0.2–0.79)	Moderate (TBIL = 0.8–1.99)	Severe (TBIL ≥ 2.0)
n	79	11	3	1
TBIL (mg/dl)	0.05 ± 0.01	0.39 ± 0.03**	1.19 ± 0.18**	4.96
DBIL (mg/dl)	0.04 ± 0.005	0.35 ± 0.03**	1.11 ± 0.17**	4.39
IDBIL (mg/dl)	0.005 ± 0.001	0.05 ± 0.01**	0.08 ± 0.02**	0.57
AST (IU/l)	207 ± 12	236 ± 27	430 ± 50**	549
ALT (IU/l)	62 ± 3	78 ± 11	135 ± 24**	211
LAP (IU/l)	85.2 ± 1.4	86.5 ± 4.4	97.4 ± 6.6	95.6
ALP (IU/l)	1196 ± 35	1850 ± 176**	1460 ± 308	1697
GGT (IU/l)	2.39 ± 0.23	6.06 ± 0.89**	22.13 ± 11.01**	50.79

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LAP, leucine aminopeptidase.
**$P < 0.01$ versus normal BDC rats.

Materials and Methods

Animals and Reagents. Eight-week-old male Sprague-Dawley rats with an extracorporeal BDC bypass loop were purchased from Charles River Laboratories (Yokohama, Japan) and Japan SLC (Hamamatsu, Japan). All of the experimental procedures involving animal handling were approved by the Institutional Animal Care and Use Committee of Taisho Pharmaceutical Co., Ltd., and were in accordance with the Guidelines for the Proper Conduct of Animal Experiments (http://www.scj.go.jp/en/report/index.html). Pravastatin and pravastatin-d_3 were obtained from Toronto Research Chemicals (North York, Canada). All other chemicals used were of high-performance liquid chromatography grade or of the highest purity grade available commercially.

Biochemical Assays. Blood specimens were collected from the tail vein of the BDC rats at 4–8 days postsurgery. The serum levels of parameters reflecting hepatobiliary function were assayed using the Hitachi 7180 Auto Analyzer (Hitachi High-Technologies, Tokyo, Japan).

Pharmacokinetics and Biliary Excretion Study. Pravastatin (5 mg/kg) in saline was injected via the internal jugular vein into the BDC rats. Bile specimens were collected for 1.5 hours with the animals under freely moving conditions (Balabaud et al., 1981). Blood specimens were collected through the tail vein at 0.083, 0.25, 0.5, 0.75, 1, and 1.5 hours after the administration of pravastatin, transferred into EDTA tubes, and then centrifuged to prepare plasma samples. Then, after the animals were sacrificed, their livers were removed, weighed, and homogenized in 4 volumes of ice-cold water. The concentrations of pravastatin in the biologic samples were measured by liquid chromatography–tandem mass spectrometry (see Supplemental Methods for details). The pharmacokinetics of pravastatin was analyzed using the software Phoenix WinNonlin 6.2 (Pharsight, Mountain View, CA). The total body clearance (CL_{total}) and biliary clearance (CL_{bile}) were calculated by dividing the dose and cumulative biliary excretion amount, respectively, by the area under the plasma concentration–time curve (AUC). The nonbiliary clearance ($CL_{non\text{-}bile}$) was calculated by subtracting CL_{bile} from CL_{total}.

Statistical Analysis. Data are presented as the mean ± S.E.M. P values <0.05 were considered to indicate statistical significance. All statistical analyses were performed using the SAS 9 software (SAS Institute, Cary, NC) (see Supplemental Methods for details).

Results and Discussion

The frequency distribution of the serum concentrations of TBIL was assessed based on the background data ($n = 94$) of the BDC rats prepared for biliary excretion studies of candidate drugs. The median and lower (25th) and upper (75th) quartiles of TBIL were 0.05, 0.01, and 0.11, respectively. Animals with TBIL < 0.20, 0.20–0.79, 0.80–1.99, and ≥2.0 mg/dl were defined as having normal TBIL levels, mild HB, moderate HB, and severe HB, respectively, in this study; the percentages of animals in these four categories were 84, 12, 3, and 1%, respectively (Table 1). As might be expected, the frequency distribution probably changes with dependence on surgical technique. The serum activities of aspartate aminotransferase and alanine aminotransferase were slightly increased in the BDC rats with mild HB as compared with those in the normal BDC rats, and were increased by approximately 2-fold in the BDC rats with moderate HB. There were no significant differences in the leucine aminopeptidase activity. The alkaline phosphatase was found to be lower in the normal BDC rats, considering the level of 1543 ± 46 IU/l found in the untreated rats (see Supplemental Table 1). The serum levels of these four enzymes were within 2-fold as compared with the untreated rats (see Supplemental Table 1) in the BDC rats with mild HB as well as in the normal BDC rats, indicating that hepatobiliary failure scarcely developed in these rats.

The levels of both direct bilirubin (DBIL) and indirect bilirubin (IDBIL) increased with increasing TBIL, although IDBIL to a lesser degree than
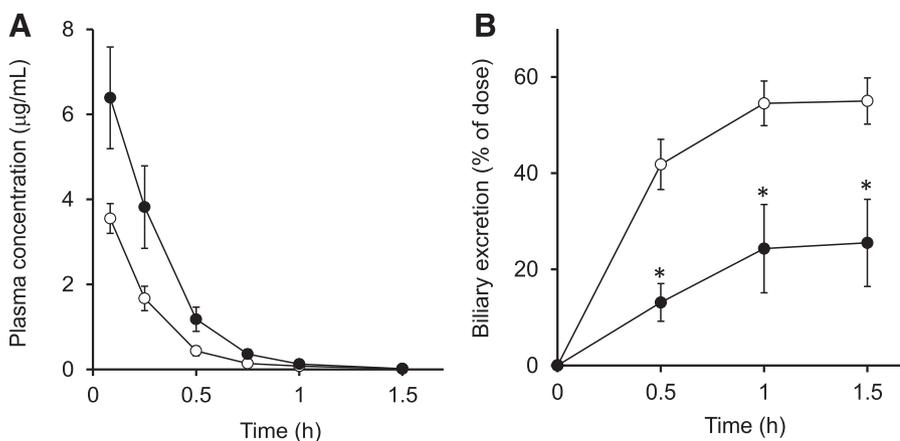
Fig. 1. Plasma concentration–time (A) and cumulative biliary excretion–time (B) profiles in BDC rats after intravenous administration of pravastatin (5 mg/kg). The concentrations in the normal group (open circles) and mild HB group (closed circles) were measured by liquid chromatography–tandem mass spectrometry. The TBIL in the normal and mild HB groups ranged from 0.00 to 0.19 and 0.33 to 0.75 mg/dl, respectively. Each point and vertical bars represent the mean ± S.E.M. of three animals. *$P < 0.05$ versus normal BDC rats.

TABLE 2

Pharmacokinetic parameters after intravenous administration of pravastatin
(5 mg/kg) to BDC rats and the serum biochemistry parameters predose

The individual TBIL values in the normal and mild HB groups ranged from 0.00 to 0.19 and 0.33 to 0.75 mg/dl, respectively. The $C_{plasma,1.5h}$ and $C_{liver,1.5h}$ represent the concentrations of plasma and liver, respectively, collected at 1.5 hours postdose. Data represent the mean ± S.E.M. of three animals.

Parameter	Normal Group	Mild HB Group
AUC (ng·h/ml)	1180 ± 159	2380 ± 505
CL_{total} (ml/h/kg)	4400 ± 699	2310 ± 521
V_d (ml/kg)	840 ± 69	497 ± 77*
CL_{bile} (ml/h/kg)	2390 ± 247	661 ± 367*
$CL_{non-bile}$ (ml/h/kg)	2020 ± 505	1650 ± 241
Biliary excretion (% of dose)	55.0 ± 4.8	25.5 ± 9.1*
$C_{plasma,1.5h}$ (ng/ml)	12.9 ± 7.6	17.9 ± 9.5
$C_{liver,1.5h}$ (ng/g)	<5 (3.39 ± 0.93)[a]	14.3 ± 4.5
C_{liver}-to-C_{plasma} ratio	<0.80 ± 0.42 (0.40 ± 0.12)[a]	1.2 ± 0.4
Biliary flow rate (ml/h/kg)	5.78 ± 0.46	6.89 ± 0.74
TBIL (mg/dl)	0.09 ± 0.06	0.55 ± 0.12*
GGT (IU/l)	2.20 ± 0.98	7.14 ± 1.35*

[a]The values in parentheses were calculated by extrapolation of standard curves below the lower limit of quantification (5 ng/g).
*$P < 0.05$ versus normal group.

DBIL (Table 1). The accumulation of IDBIL can be caused by the decreased functioning of the Oatps, and that of DBIL can be caused by the decreased functioning of Mrp2 and/or Oatps, and/or the increased functioning of Mrp3 (van de Steeg et al., 2010; Keppler, 2014). A number of anionic drugs and metabolites are excreted into bile by the OATP- and/or MRP2-mediated transport (Ieiri et al., 2009). To verify whether these transport activities are reduced in the BDC rats with HB, we conducted a pharmacokinetic and biliary excretion study of pravastatin, an Oatp/Mrp2 probe drug (Watanabe et al., 2009). The mean plasma concentration–time and cumulative biliary excretion–time profiles of pravastatin are shown in Fig. 1. The pharmacokinetic parameters are listed with the biliary flow rate, TBIL, and γ-glutamyltransferase activity (GGT) in Table 2. Scarce difference was observed between the normal (TBIL = 0.00, 0.07, and 0.19 mg/dl) and mild (TBIL = 0.33, 0.57, and 0.75 mg/ml) HB groups with regard to the biliary flow rate. The plasma concentrations and AUC were higher, whereas the CL_{total} was lower, in the mild HB group as compared with the values in the normal group, although the differences were not statistically significant. The apparent volume of distribution (V_d) and CL_{bile} were significantly reduced by 41 and 72%, respectively, in the mild HB group. The reduced V_d suggests the reduction of apparent hepatic uptake, which catalyzes the marked liver-specific distribution of pravastatin in rats (Komai et al., 1992). The reduction of apparent hepatic uptake can be led by the decreased functioning of the Oatps (Hsiang et al., 1999; Tokui et al., 1999) and/or the increased functioning of Mrp3, although the involvement

of Mrp3 has been little reported in the disposition of pravastatin. The $CL_{non-bile}$ is considered as the metabolic clearance in the liver, since the renal clearance of pravastatin is negligible in rats (Komai et al., 1992). Thus, the CL_{bile} and the $CL_{non-bile}$ account for hepatic clearance. The intrinsic hepatic clearance ($CL_{int,h}$) is expressed by the following equation (Kusuhara and Sugiyama, 2009):

$$CL_{int,h} = \frac{PS_{inf}}{PS_{eff} + CL_{int}} \times CL_{int}$$
$$= \frac{PS_{inf}}{PS_{eff} + (CL_{int,bile} + CL_{int,met})} \times (CL_{int,bile} + CL_{int,met}) \quad (1)$$

where PS_{inf} and PS_{eff} represent the intrinsic clearance for cellular uptake (influx) and efflux into the systemic circulation, respectively. CL_{int} represents the intrinsic clearance based on the unbound concentration in the hepatocytes and consists of two intrinsic clearance values for biliary efflux from hepatocytes ($CL_{int,bile}$) and for metabolism in hepatocytes ($CL_{int,met}$). When the CL_{int} is much greater than the PS_{eff}, the $CL_{int,h}$ can be approximated to the PS_{inf}. Indeed, the uptake is rate limiting for the hepatic elimination of pravastatin in normal rats (Kusuhara and Sugiyama, 2009). Therefore, the reduced uptake likely caused the reduction of the CL_{bile} and $CL_{non-bile}$ in this study. Furthermore, in BDC rats with HB, the uptake may no longer be the only rate limiting process for the $CL_{int,h}$, as decreased $CL_{int,bile}$ and increased PS_{eff}, which can be caused by altered functioning of Mrp2 and Mrp3, respectively, progressed. The reduction of CL_{bile} was much greater than that of $CL_{non-bile}$, supporting that the CL_{bile} was affected by the $CL_{int,bile}$, namely Mrp2 activity, in addition to the hepatic uptake.

The liver concentration and liver-to-plasma concentration ratio at 1.5 hours postdose were greater in the HB group as compared with that in the normal group. Since renal clearance of pravastatin is negligible in rats, the AUC in the liver (AUC_h) is expressed by the following equation (Kusuhara and Sugiyama, 2009):

$$AUC_h = \frac{Dose}{f_{u,h} \cdot CL_{int}} \quad (2)$$

where $f_{u,h}$ represents the unbound fractions of pravastatin in the hepatocytes. The AUC_h is not influenced by change in PS_{inf} and PS_{eff}, for which Oatps and Mrp3, respectively, are responsible. Thus, the elevation of liver concentration suggests reduced CL_{int}. When renal clearance is negligible, the biliary excretion ($f_{e,bile}$, percentage of dose) is determined by the following equation:

$$f_{e,bile} = \frac{CL_{bile}}{CL_{bile} + CL_{non-bile}} \times 100 = \frac{CL_{int,bile}}{CL_{int,bile} + CL_{int,met}} \times 100 \quad (3)$$

The $f_{e,bile}$ (percentage of dose) was significantly reduced by 54% in the mild HB group (Table 2), suggesting that the biliary efflux activity via
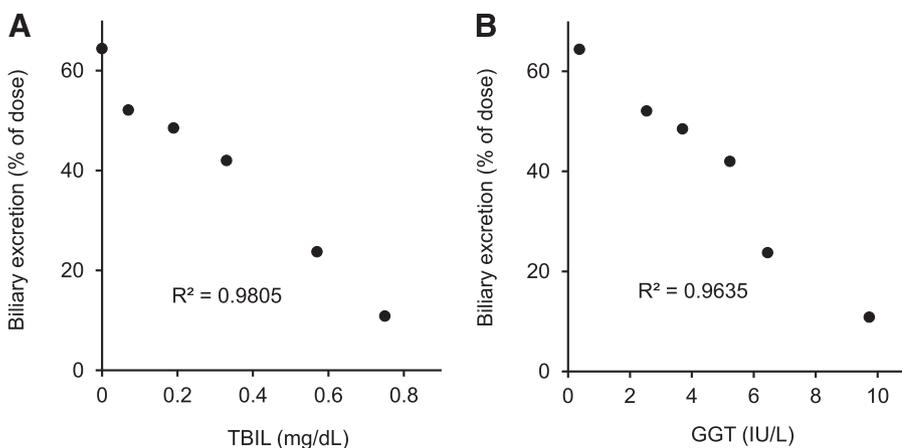
Fig. 2. Relationships between TBIL (A) or GGT (B) and the biliary excretion of the drug (percentage of dose) in six BDC rats after intravenous administration of pravastatin (5 mg/kg). R, correlation coefficient.

Mrp2 was reduced relative to the metabolic activity in hepatocytes. Given the $CL_{int,met}$ is little affected by BDC, the $f_{e,bile}$ is dependent on the $CL_{int,bile}$, which is controlled by Mrp2 activity.

The downregulation of mRNA, reduced protein expression, and internalization of Mrp2 (Sekine et al., 2006) are assumed as candidates of mechanism responsible for the reduced Mrp2 activity. Then, the expression of Oatps and Mrp3 seems to be adaptively altered, as observed in rats with impairment of Mrp2 (Dumont et al., 1997; Soroka et al., 2001; Kuroda et al., 2004), leading to the reduced hepatic uptake. Furthermore, endogenous and food-derived compounds, such as bilirubin glucuronides and phase II metabolites of isoflavonoids, which are accumulated by the impairment of Mrp2 (Kato et al., 2012), might contribute to the reduced hepatic uptake.

The individual values of TBIL were inversely correlated with the $f_{e,bile}$ value of pravastatin, with high correlation coefficients (Fig. 2A). Marked differences in the $f_{e,bile}$ values were observed between the individuals with the minimum and maximum TBIL values of 0.00 and 0.75 mg/dl, respectively (64.4 vs. 10.8%). Considering these data, we recommend that the cutoff value of the TBIL is set below 0.20 mg/dl. The purpose of the experiment should also be considered for determining the cutoff value. A stricter exclusion criterion is likely adequate in the Good Laboratory Practice-like studies for the new drug application. On the other hand, in the early drug discovery stage, the experiment including BDC rats with HB may be useful to explore the involvement of transporters in the biliary elimination of anionic candidate drugs.

As an unexpected result, the GGT activity increased with increasing TBIL in the BDC rats (Table 1). Then, the GGT was inversely correlated with the $f_{e,bile}$ of pravastatin, which likely reflects the Mrp2 activity (Fig. 2B). We can propose two hypotheses about the relationship between GGT and Mrp2 as follows. First, since GGT is present in many tissues and plays an important role in initiating the metabolism of glutathione conjugates to mercapturic acids (Zhang et al., 2005), it may be induced as an adaptive response in the presence of Mrp2 function impairment to prevent the accumulation of glutathione conjugates, which are Mrp2 substrates, by changing the elimination pathway from bile to urine. Second, since both GGT and Mrp2 are responsible for glutathione homeostasis (Lu et al., 1996; Ballatori and Rebbeor, 1998; Zhang et al., 2005), the alterations might be regulatory responses to the negative influence on glutathione homeostasis exerted by the BDC. These hypotheses may serve as clues to find the unrecognized relationship between GGT and Mrp2.

In conclusion, our findings suggest that the use of BDC rats with HB can lead to underestimation of the hepatic uptake and biliary efflux of the anionic drugs via Oatps and Mrp2, respectively. Therefore, such animals with HB, however mild, should be generally excluded by TBIL measurement for proper conduct of drug excretion studies.

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Wrote or contributed to the writing of the manuscript: Kato, Hasegawa, Iwata, Yamaguchi.

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Drug metabolism and Disposition

Recommendation to Exclude Bile-Duct-Cannulated Rats with Hyperbilirubinemia for Proper Conduct of Biliary Drug Excretion Studies

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Data Supplement

Supplemental TABLE 1

Serum biochemistry parameters in untreated SD rats (n=30).

Parameter	Mean ± S.E.M.	Minimum	Maximum
TBIL (mg/dL)	0.010 ± 0.002	0.00	0.04
DBIL (mg/dL)	0.003 ± 0.001	0.00	0.02
IDBIL (mg/dL)	0.007 ± 0.002	0.00	0.03
AST (IU/L)	142 ± 8	75	248
ALT (IU/L)	42 ± 1	28	66
LAP (IU/L)	92 ± 1	79.1	109.2
ALP (IU/L)	1543 ± 46	1140	2112
GGT (IU/L)	0.28 ± 0.06	0.00	1.01

Supplementary Methods

Pharmacokinetics and Biliary Excretion Study

Quantification of Pravastatin in Biological Samples. A quadruple volume of acetonitrile/methanol (9:1, v/v) containing pravastatin-d_3, an internal standard (IS), was added to a biological sample (50 µL). The mixture was vortexed and then centrifuged at 3639 ×g for 10 min at 4°C. The supernatant was then subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The LC system used was an LC-30AD (Shimadzu, Kyoto, Japan) equipped with a Shim-pack XR-ODS column (2.2 µm, 30 mm × 3.0 mm; Shimadzu). The mobile phase was prepared by combining solvents A (0.1% formic acid) and B (acetonitrile) and delivered at 1.3 mL/min with 2% solvent B, followed by a linear gradient to 80% solvent B at 1.00 min and 98% solvent B at 1.01 min, and then an isocratic elution to 1.20 min with 98% solvent B. MS data were acquired using a Triple Quad 5500 tandem mass spectrometer (AB Sciex, Foster City, CA) equipped with an electrospray ionization source. The analytes and IS were detected in positive ionization mode by exploiting the transitions m/z 423.1/321.0 for pravastatin and m/z 426.1/321.0 for pravastatin-d_3.

Statistical Analysis

Statistical Methods. For comparison between two groups, an unpaired Student t-test for equal variances or the Welch test for unequal variances was used. For comparison among three groups, Dunnett's multiple comparison for equal variances ($P > 0.05$ using the Bartlett's test) or the Steel test for unequal variances was used.