

IDENTIFICATION OF HUMAN UDP-GLUCURONOSYLTRANSFERASE ENZYME(S) RESPONSIBLE FOR THE GLUCURONIDATION OF EZETIMIBE (ZETIA)

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ABSTRACT:

Ezetimibe [1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidione] (Zetia; Schering-Plough, Kenilworth, NJ) is the first in a new class of cholesterol-lowering agents known as cholesterol absorption inhibitors. The objective of this study was to identify the isoform(s) of human liver and intestinal UDP-glucuronosyltransferase (UGT) enzymes responsible for the glucuronidation of ezetimibe. The main circulating metabolite of this drug in human plasma is SCH 60663, the phenolic glucuronide conjugate of ezetimibe. SCH 60663 [$m/z = 584$ Thompsons (Th)] is also the major in vitro metabolite formed by human liver microsomes supplemented with UDP glucuronic acid (UDPGA). In contrast to the liver, human jejunum microsomes supplemented with UDPGA converted ezetimibe to two glucuronides with the same mass ($m/z = 584$ Th) by liquid chromatography-mass

spectrometry. One corresponds to the phenolic glucuronide (1-O-[4-*trans*-2S,3R]-1-(4-fluorophenyl)-4-oxo-3-[3(S)-hydroxy-3-(4-fluorophenyl)propyl]-2-azetidiny]phenyl- β -D-glucopyranuronic acid; SCH 60663) and the other was identified as the benzylic glucuronide of ezetimibe (1-O-[1(S)-(4-fluorophenyl)-3-[1-(4-fluorophenyl)-2(S)-(4-hydroxyphenyl)-4-oxo-3(R)-azetidiny]propyl]- β -D-glucopyranuronic acid; SCH 488128). Recombinant human UGT1A1, UGT1A3, and UGT2B15 all exhibited catalytic activity with respect to the formation of the phenolic glucuronide. However, UGT2B7 exclusively formed SCH 488128, a trace metabolite detected in dog and human plasma samples after oral administration of ezetimibe. In conclusion, the formation of SCH 60663 is mediated via UGT1A1, UGT1A3, and UGT2B15, and the formation SCH 488128 is mediated via UGT2B7.

UDP glucuronosyltransferases (UGTs)¹ are a superfamily of membrane-bound enzymes located in the endoplasmic reticulum that catalyze the conjugation of endogenous substances (e.g., bilirubin, steroids, thyroid hormone) and xenobiotics with D-glucuronic acid. UGTs catalyze the conversion of lipophilic molecules into more polar hydrophilic glucuronides, thereby facilitating their elimination via bile, feces, and urine. The role of hepatic and extrahepatic UGT enzymes and their expression in gastrointestinal tract are described by Fisher et al. (2001). In this review, the authors reported the presence of UGT1A1, UGT1A3, UGT1A4, UGT2B7, and UGT2B15 in human liver and small intestine.

Ezetimibe is an inhibitor of intestinal absorption of cholesterol for treatment of primary hypercholesterolemia, a leading cause of mortality and morbidity in many countries (Garber et al., 1989; Hancock,

1991). It effectively blocks intestinal absorption of dietary and biliary cholesterol (van Heek et al., 2000). Ezetimibe undergoes primarily phase II metabolism in vivo to the pharmacologically active phenolic glucuronides (Patrick et al., 2002). The pharmacokinetic, excretion, and metabolite profiles of ezetimibe indicate extensive conjugation and enterohepatic recirculation after oral administration (Patrick et al., 2002).

The objective of this study was to identify the predominant in vitro UGT conjugation pathway(s) for ezetimibe. In vitro incubations with cDNA-expressed recombinant human UGT enzymes were performed to determine the isozymes responsible for the glucuronidation of ezetimibe in human liver and jejunum. Glucuronide formation was verified by LC/MS, and the susceptibility of the products to hydrolysis by β -glucuronidase. Inhibition of metabolite formation by inhibitors was used to confirm the UGT isozymes responsible for metabolism of the drug.

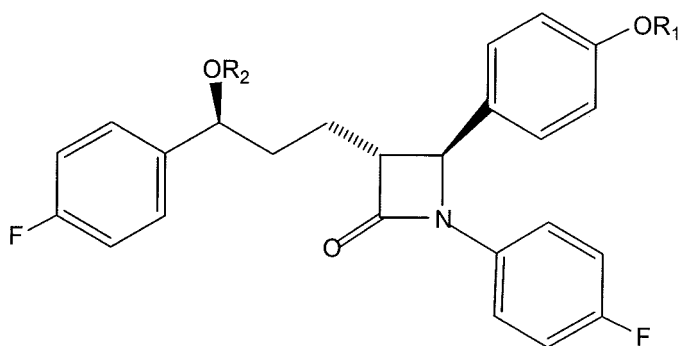
Materials and Methods

Chemicals. UDP-glucuronic acid, bovine liver β -glucuronidase in acetate buffer (Glucurase; Sigma-Aldrich), magnesium chloride, Trizma base, saccharolactone (D-saccharic acid 1,4-lactone), alamesticin, flunitrazepam, diclofenac, 4-trifluoromethyl-7-hydroxycoumarin glucuronide (4-trifluoromethylumbelliferyl glucuronide), and trifluoperazine were purchased from Sigma-Aldrich (St. Louis, MO).

Acetonitrile (HPLC grade) was obtained from Burdick and Jackson Laboratories (Muskegon, MI). Trifluoroacetic acid and ammonium acetate were obtained from Sigma-Aldrich. Dimethyl sulfoxide, isopropanol, ethyl acetate, and dichloromethane were obtained from Fisher Scientific Co. (Pittsburgh,

¹ Abbreviations used are: UGT, UDP-glucuronosyltransferase; LC/MS, liquid chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; UDPGA, uridine 5'-diphosphate-glucuronic acid; SPE, solid-phase extraction; saccharolactone, D-saccharic acid 1,4-lactone; SCH 60663, 1-O-[4-*trans*-(2S,3R)-1-(4-fluorophenyl)-4-oxo-3-[3(S)-hydroxy-3-(4-fluorophenyl)propyl]-2-azetidiny]phenyl]- β -D-glucopyranuronic acid; SCH 488128, 1-O-[1(S)-(4-fluorophenyl)-3-[1-(4-fluorophenyl)-2(S)-(4-hydroxyphenyl)-4-oxo-3(R)-azetidiny]propyl]- β -D-glucopyranuronic acid.

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SCH 58235; $R_1 = R_2 = H$

SCH 60663; $R_1 = \text{Glucuronide}, R_2 = H$

SCH 488128; $R_1 = H, R_2 = \text{Glucuronide}$

FIG. 1. Chemical structure of ezetimibe (SCH 58235).

PA). Unlabeled and radiolabeled ezetimibe (^{14}C -SCH 58235; specific activity 120.9 $\mu\text{Ci}/\text{mg}$, radiochemical purity 98.6%), and its phenolic glucuronide (SCH 60663) were prepared at Schering-Plough Research Institute (Kenilworth, NJ). Pooled human liver microsomes ($n = 10$) were purchased from XenoTech, LLC (Lenexa, KS). UGT Supersomes and 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) were purchased from BD Gentest (Woburn, MA), and baculosomes were obtained from PanVera Corp. (Madison, WI). Human jejunum microsomes (pooled, $n = 4$) and microsomes from two donors (HJ-004, white male and HJ-025, white female) were purchased from Human Cell Culture Center, Inc. (Laurel, MD).

Analysis of Ezetimibe and Metabolite(s). The analysis was performed on a Waters Alliance HPLC system (model 2690; Waters, Milford, MA), equipped with model 996 photodiode array detector (Waters), model 500TR PerkinElmer Life Sciences (Boston, MA) radioactivity detector, and a 5- μm C8 Inertsil 150 \times 4.6 mm column (MetaChem Technologies, Torrance, CA). The analytical column was maintained at room temperature for all HPLC experiments. The mobile phase, which consisted of 20 mM ammonium acetate adjusted to pH 7.0 with 1% aqueous ammonium hydroxide (A) and acetonitrile (B), was operated at a constant flow rate (1 ml/min), and the metabolite was detected at 245 nm. The gradient was 30% B for 0.1 min, followed by a rapid increase to 100% B, 5 min at 100%, and a re-equilibrium at 30% B. After LC analysis, the concentrations of each metabolite (phenolic and benzylic glucuronides) were calculated based on radiometric detection and a standard curve (linear) of 5 points. LC/MS and LC/tandem MS analyses of the metabolites were performed as described in the literature (Patrick et al., 2002).

Enzyme Assays. *Incubation of ezetimibe with pooled human liver microsomes.* Substrate concentrations of 1 to 100 μM , microsomal protein concentrations of 0.05 to 2 mg/ml, and incubation times of 5 to 120 min were used to optimize the condition of the assay. The microsomes were treated with alamethicin (a 20-amino acid α -helical pore-forming peptide) at 50 μg of alamethicin/mg of microsomal protein on ice for 15 min to diminish the latency of UGT activity (Fisher et al., 2000). All microsomal incubations contained microsomes treated with alamethicin, 10 mM magnesium chloride, 5 mM saccharolactone (an inhibitor of β -glucuronidase), and ^{14}C -ezetimibe in 0.5 ml of 0.1 M Tris-HCl buffer, pH 7.4 (Pless et al., 1999). Before the addition of UDPGA, the incubation mixtures were prewarmed for 3 min at 37°C. The reactions were initiated by the addition of 2 mM UDPGA, allowed to proceed for 120 min at 37°C, and terminated with ice-cold methanol. The incubation mixtures were vortexed and centrifuged (10,010g) at 4°C for 10 min, and the supernatants were analyzed by HPLC. Incubations without UDPGA and boiled human liver microsomes served as negative controls. For LC/MS analyses, the reactions were either terminated as described above or terminated by cooling in ice water followed by solid-phase extraction (SPE). For SPE, a tC18 Sep-Pak cartridge (Waters) was preconditioned by sequential washing with

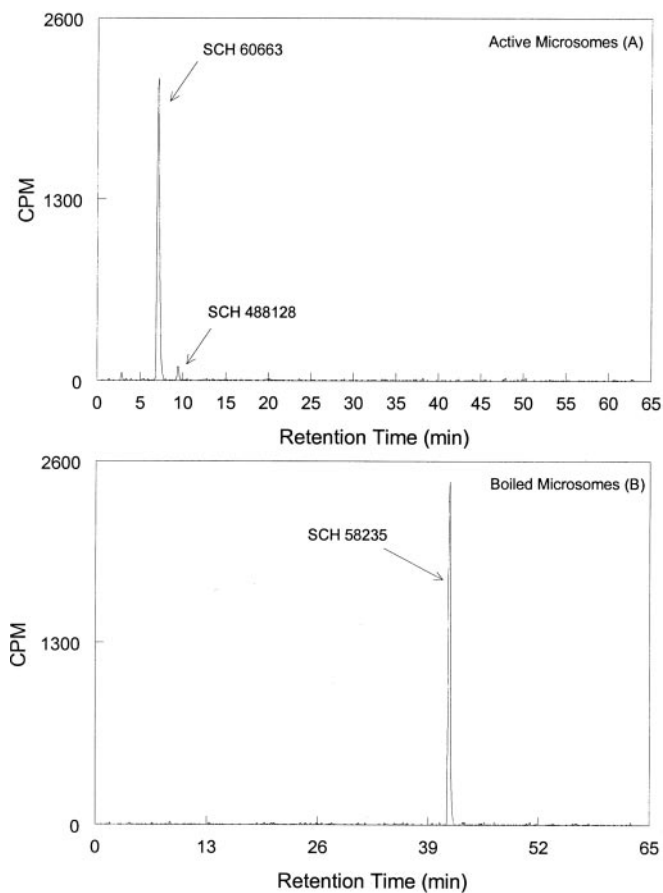


FIG. 2. Radiometric profile of drug-derived entities after 120-min incubation of ^{14}C -ezetimibe (5 μM) with active human liver microsomes supplemented with UDPGA (A) and with boiled human liver microsomes with UDPGA (B).

methanol and water. The incubation material was then loaded onto the cartridge. Each cartridge was washed with water, and the drug-derived materials were eluted with methanol.

Hydrolysis of glucuronide conjugate. The incubation mixture consisting of human liver microsomes incubated with ^{14}C -ezetimibe in the presence of saccharolactone, alamethicin, and UDPGA was divided into two parts. One part was terminated with cold methanol, centrifuged, and injected onto HPLC for metabolite profiling. The other part was terminated by cooling in ice water and subjected to SPE. The resulting eluent was dried, resuspended in 2.5 ml of Glucurase (5000 units/ml, pH 5.0), and incubated (loosely capped) for ~18 h at 37°C with gentle shaking. The sample (posthydrolysis) was subjected to SPE and injected onto HPLC for profiling of metabolites.

Incubation of ezetimibe with human jejunum microsomes. Human jejunum microsomes (1 mg/ml) from a single donor (HJ-004) were incubated (120 min) with substrate concentrations of 1 to 100 μM to determine K_m and V_{max} . The incubation was performed as described above, and boiled microsomes served as negative control. Pooled human jejunum microsomes were also incubated with 100 μM ezetimibe to compare the metabolite profile to the single donor. Ezetimibe glucuronides obtained after the incubation of ezetimibe and human jejunum microsomes (two individual donors, HJ-025 and HJ-004) supplemented with UDPGA were hydrolyzed separately with Glucurase as described above. The hydrolysis times were 5, 10, 20, 30, 180 min, and 18 h. The samples (posthydrolysis) were subjected to SPE and profiled by HPLC for metabolites.

Determination of enzyme activities of UGT Supersomes and microsomes. The activities of UGT Supersomes (UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) and human liver and jejunum microsomes were determined in assays using HPLC and 7-HFC as substrate according to Ghosal et al. (2004). UGT1A4 Supersomes do not form glucuronides with 7-HFC as a substrate. Therefore, the activity of UGT1A4 Super-

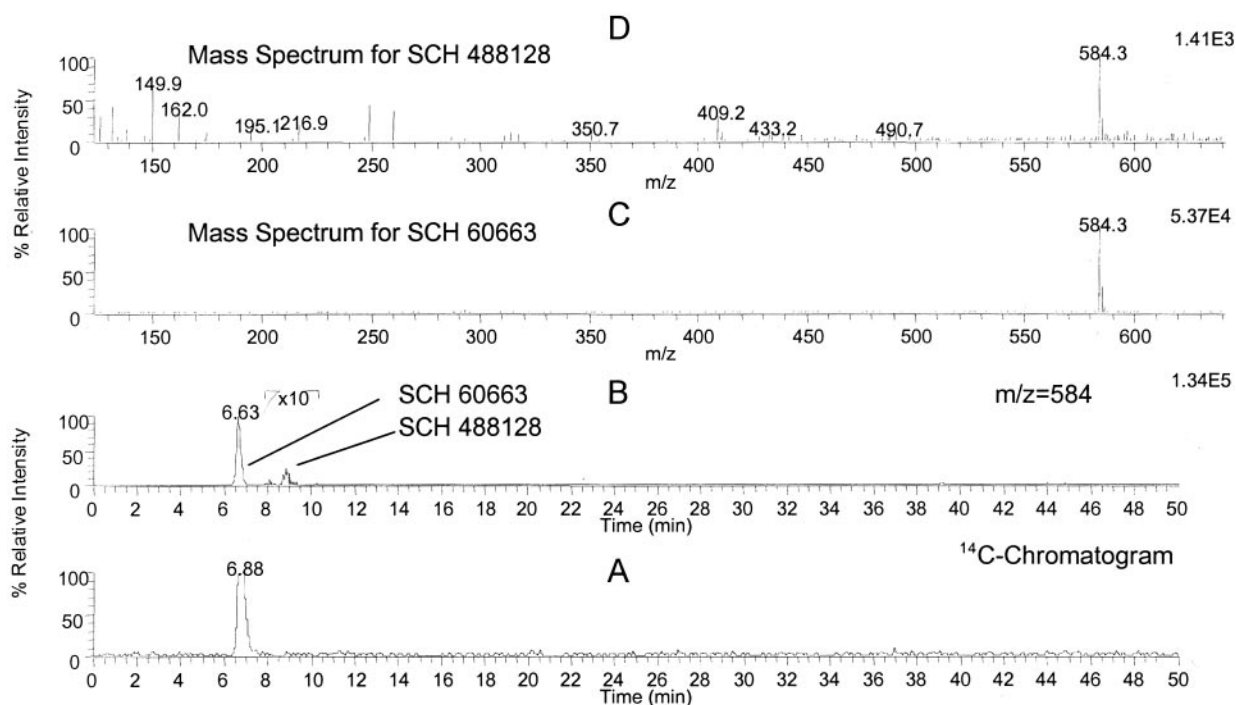


FIG. 3. Radiometric profile (A) of drug-derived entities after 120-min incubation of ^{14}C -ezetimibe ($5\ \mu\text{M}$) with active human liver microsomes supplemented with UDPGA. The selected ion chromatogram at m/z 584 (B) shows peaks for SCH 60663 and a trace amount of SCH 488128. The mass spectra are shown in panels C and D, respectively.

somes was determined in assays using HPLC and trifluoperazine substrate as described previously (Dehal et al., 2001; Ghosal et al., 2004).

Screening of nine human UGT Supersomes. In vitro screening of nine human UGT Supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) with ezetimibe was performed using a constant amount of microsomal protein (1 mg/ml) and $100\ \mu\text{M}$ ezetimibe. All incubations with Supersomes were carried out as described earlier. Insect cell microsomes without UGT cDNA served as the negative control. These samples were also analyzed by LC/MS.

Kinetic parameters (K_m and V_{max}) of ezetimibe glucuronidation by UGT1A1, UGT1A3, and UGT2B7 Supersomes were determined as described below. Substrate concentrations of 1 to $100\ \mu\text{M}$, UGT protein concentrations of 1 mg/ml, and an incubation time of 120 min were used to incubate human UGT Supersomes to determine K_m and V_{max} . The incubation was performed as described above, and insect microsomes without UGT cDNA served as the negative control.

Inhibition with chemical inhibitors of UGTs. Inhibition of ezetimibe metabolism was evaluated using known chemical inhibitors of UGTs. Human liver microsomes (0.05 mg/ml), human jejunum microsomes (1 mg/ml), or human UGT Supersomes (1 mg/ml) were preincubated with various concentrations of inhibitors for 15 min at room temperature followed by the addition of buffer, alamethicin, saccharolactone, cofactor, and substrate. Flunitrazepam and diclofenac were dissolved in methanol. The final concentration of the organic solvent in the incubation system was 2%, and all control incubations contained the same volume of appropriate vehicle. All organic solvents tested up to 2% had no effect on the formation of glucuronides. All incubations contained microsomes treated with alamethicin and were carried out as described before, except that the concentration of ^{14}C -ezetimibe was $25\ \mu\text{M}$.

Analysis of kinetic data. Untransformed enzyme kinetic data were analyzed by a nonlinear regression data analysis program (GraFit 4.04; Erithacus Software, Horley, Surrey, UK), assuming Michaelis-Menten kinetics over the substrate range studied.

Results

Optimization and Incubation with Pooled Human Liver Microsomes. After incubation of $5\ \mu\text{M}$ ^{14}C -ezetimibe (Fig. 1) with pooled human liver microsomes and UDPGA, one major metabolite (SCH

60663) was observed by radiometric detection (Fig. 2A). As expected, incubation without UDPGA and with boiled microsomes did not yield any glucuronide formation (Fig. 2B). LC/MS analysis of these incubations from human liver microsomes confirmed that the major peak in Fig. 2A was SCH 60663 and the minor peak was SCH 488128 (Fig. 3). Although both SCH 60663 and SCH 488128 exhibited identical LC/MS spectra, their NMR spectra were distinct and identified the location of the glucuronide moiety in the two compounds. Optimal protein concentration and incubation time were found to be 0.05 mg/ml and 120 min based on the linearity and percent of conversion to SCH 60663. The effect of various substrate concentrations (5 – $50\ \mu\text{M}$) on ezetimibe glucuronides demonstrated that the apparent K_m and V_{max} values for SCH 60663 formation were $21.2 \pm 3.10\ \mu\text{M}$ and $4.40 \pm 0.27\ \text{nmol/mg protein/min}$, respectively (Fig. 4). Intrinsic clearance (V_{max}/K_m) value for the glucuronide was estimated to be 0.21. A substrate concentration of $25\ \mu\text{M}$ was chosen for further experiments considering the linearity and sensitivity of detection. After hydrolysis (18 h) of SCH 60663 and SCH 488128 with β -glucuronidase (Glucurase), only the parent (ezetimibe) was observed (Table 1).

Incubation with Human Jejunum Microsomes. ^{14}C -Ezetimibe was also incubated with human jejunum microsomes to understand the role of jejunum in the glucuronidation of ezetimibe. Incubation of $100\ \mu\text{M}$ ^{14}C -ezetimibe with pooled and individual human jejunum microsomes (donor HJ-025; 1 mg/ml) supplemented with UDPGA demonstrated the formation of two separate glucuronides (Fig. 5A). LC/MS/radiometric analysis of these incubations from human jejunum microsomes confirmed the formation of approximately equal amounts of SCH 60663 and SCH 488128. Incubation of various concentrations of ezetimibe with jejunum microsomes (from a single donor, HJ-004) demonstrated that the apparent K_m and V_{max} values for SCH 60663 were $18.5 \pm 2.68\ \mu\text{M}$ and $3.02 \pm 0.24\ \text{nmol/mg protein/min}$, respectively (Fig. 4). Intrinsic clearance (V_{max}/K_m) for the glucuronide was estimated to be 0.16. In the absence of the UDPGA or

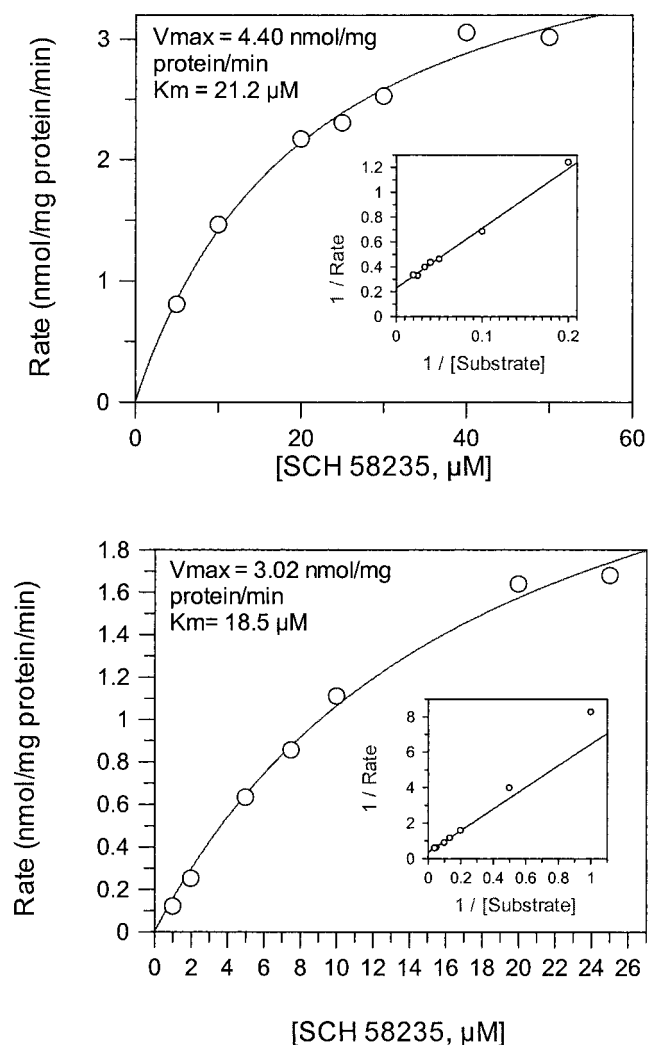


FIG. 4. Enzyme kinetics for the formation of phenolic glucuronide conjugate of ezetimibe (SCH 58235) incubated with pooled human liver microsomes (top panel) and human jejunum microsomes from single donor (HJ-004) (bottom panel).

Insert represents the transformation of Michaelis-Menten kinetics to a Lineweaver-Burk plot.

with boiled microsomes, no metabolite formation was observed. The recovery of metabolites from the HPLC column was on average greater than 98%.

Enzymatic hydrolysis (β -glucuronidase) of the two ezetimibe glucuronides generated from human jejunum microsomes was performed for 5, 10, 20, 30, and 180 min. The phenolic glucuronide peak observed at retention time ca. 7 min disappeared at a faster rate compared with the benzylic glucuronide peak at 9 min (Table 1). However, after 18 h of hydrolysis, only a single peak (ezetimibe) was observed, indicating a complete hydrolysis of both glucuronides (Fig. 5B). The enzyme β -glucuronidase is expressed in human intestine and liver, and it efficiently hydrolyzes glucuronides.

Activities of Human UGT Supersomes, Human Liver, and Jejunum Microsomes (Positive Control). The results of the activity determination of nine human UGT Supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) and human liver and jejunum microsomes are presented in Table 2. These results served as positive control for the Supersomes activities and demonstrated that the Supersomes and microsomes were active.

Screening with Human UGT Supersomes. Incubation of ezetimibe with nine human UGT Supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B7, and UGT2B15) revealed that only UGT1A1, UGT1A3, and UGT2B15 metabolized ezetimibe to its major phenolic glucuronide (Fig. 6). In contrast, UGT2B7 Supersomes, at a protein concentration of 1 mg/ml, metabolized ezetimibe primarily to the benzylic glucuronide (Fig. 6). No metabolism was observed with UGT1A6, UGT1A7, UGT1A10,

TABLE 1

Hydrolysis with β -glucuronidase after a 2-h incubation of ezetimibe with human jejunum and liver microsomes supplemented with UDPGA

Values represent means of duplicate determinations. Pooled human liver microsomes and jejunum microsomes from single donor (HJ-004) were used. Hydrolysis of incubation mixtures were performed by the methods as described under *Materials and Methods*.

Tissue	Conc. of Ezetimibe	Hydrolysis Time	% Phenolic Glucuronide	% Benzylic Glucuronide	% Ezetimibe
Jejunum	50 μ M	No hydrolysis	31.7	32.1	32.1
		5 min	2.23	19.3	68.1
		10 min	1.04	18.6	61.9
		20 min	0.77	15.1	66.0
		30 min	0	11.2	80.1
		180 min	0	6.50	84.7
Liver	5 μ M	18 h	0	0	95.5
		No hydrolysis	94.6	3.64	0
		18 h	0	0	100

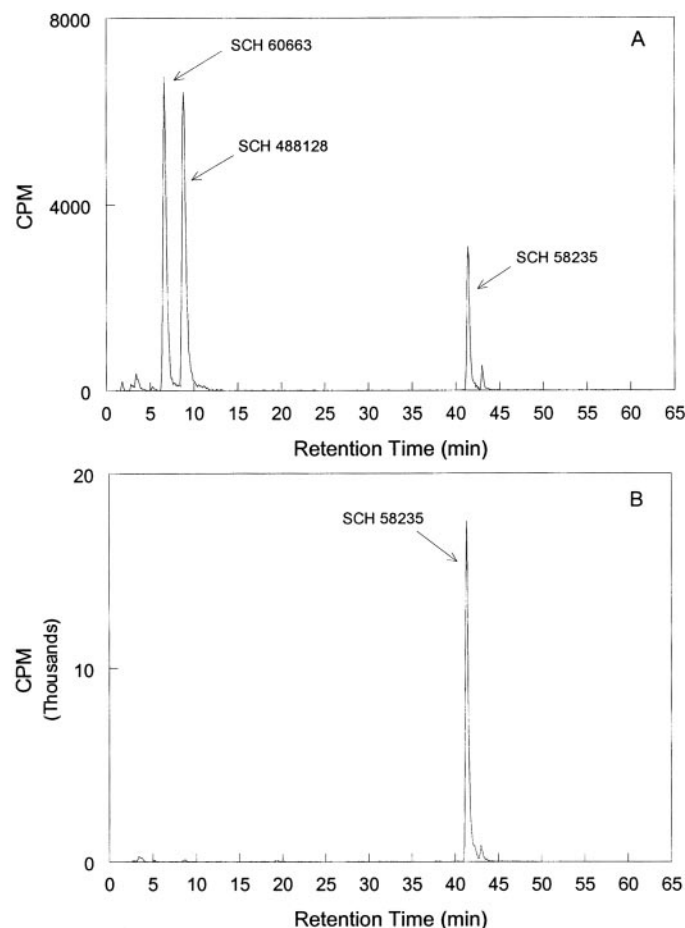


FIG. 5. Radiometric profile of drug-derived entities after 120-min incubation of 14 C-ezetimibe (100 μ M) with active human jejunum microsomes supplemented with UDPGA before hydrolysis (A) or with UDPGA followed by 18-h hydrolysis with β -glucuronidase (B).

TABLE 2

Determination of activities of cDNA-expressed human UGT Supersomes, human liver, and human jejunum microsomes (positive control)

Values represent means of duplicate determinations.

Supersomes	Substrates Used	Formation of Glucuronide
		nmol/mg protein/min
UGT1A1	7-HFC	1.26
UGT1A3	7-HFC	1.06
UGT1A4	Trifluoperazine	11.6
UGT1A6	7-HFC	3.14
UGT1A7	7-HFC	0.41
UGT1A9	7-HFC	3.18
UGT1A10	7-HFC	1.63
UGT2B7	7-HFC	2.13
UGT2B15	7-HFC	2.21
Insect control	7-HFC	0
Pooled liver microsomes (<i>n</i> = 10)	7-HFC	3.17
Pooled jejunum microsomes (<i>n</i> = 4)	7-HFC	3.11
Jejunum microsomes (HJ-004)	7-HFC	2.57
Jejunum microsomes (HJ-025)	7-HFC	3.10

and control insect microsomes. However, UGT1A9 yielded trace amounts of both glucuronides, and UGT1A4 formed a trace amount of the benzylic glucuronide. The metabolites formed in these incubations of ^{14}C -ezetimibe with UGT1A1, UGT1A3, UGT1A4, UGT1A9, UGT2B7, and UGT2B15 Supersomes were confirmed by LC/MS/radiometric analyses.

The metabolite profiles presented in Fig. 6 suggested that UGT1A1, UGT1A3, and UGT2B15 are primarily responsible for the *in vitro* glucuronidation of ezetimibe to SCH 60663 in liver and jejunum. In contrast, UGT2B7 may be responsible for the *in vitro* glucuronidation of ezetimibe to SCH 488128 in the jejunum. The recovery of metabolites from the HPLC column was on average greater than 96%.

The kinetic parameters of UGT Supersomes are presented in Table 3. Incubation of various concentrations of ezetimibe (1–150

μM) with UGT1A1 and UGT1A3 Supersomes demonstrated that the apparent K_m values for SCH 60663 were 64.3 and 41.7 μM , respectively. Corresponding V_{max} values for SCH 60663 were 0.71 and 0.48 nmol/mg protein/min, respectively. Intrinsic clearance (V_{max}/K_m) for SCH 60663 was estimated to be 0.011 in both cases. However, incubation of various concentrations of ezetimibe with UGT2B7 Supersomes demonstrated that the apparent K_m and V_{max} values for SCH 488128 were 20.9 μM and 0.05 nmol/mg protein/min, respectively. The summary of ezetimibe metabolite formation was presented in Table 4.

Inhibition Study. All inhibition studies were performed with pooled human liver and jejunum microsomes at a drug concentration of 25 μM . UGT Supersomes (UGT1A1, UGT1A3, UGT2B7, and UGT2B15) were also incubated with inhibitors to confirm the specificity of the inhibitors. The effect of incubations with UGT inhibitors (diclofenac and flunitrazepam) is presented in Table 5. In the present study, diclofenac (500 μM) was found to be a potent inhibitor of phenolic glucuronide formation by UGT2B15 (88%) as well as by human liver (>89%) and human jejunum microsomes (84%)(Table 5). The mean IC_{50} values of diclofenac for phenolic glucuronide formation from human liver and jejunum microsomes were 106 and 116 μM , respectively (Fig. 7). Another known UGT2B15 inhibitor, flunitrazepam (200 μM), inhibited phenolic glucuronide formation from UGT2B15 (87%) as well as from human liver (86%) and human jejunum microsomes (52%) (Table 5). These results supported the evidence that UGT2B15 is involved in the formation of phenolic glucuronide in human liver and jejunum microsomes.

Diclofenac (500 μM) was found to be a potent inhibitor of benzylic glucuronide formation by UGT2B7 (Table 5) with an IC_{50} value of 52.7 μM . In the case of human jejunum microsomes, however, diclofenac inhibited benzylic glucuronide formation by only 58% (Table 5). Flunitrazepam (200 μM) was a potent inhibitor of benzylic glucuronide formation from UGT2B7 (83%); however, it inhibited benzylic glucuronide formation by only 44% in

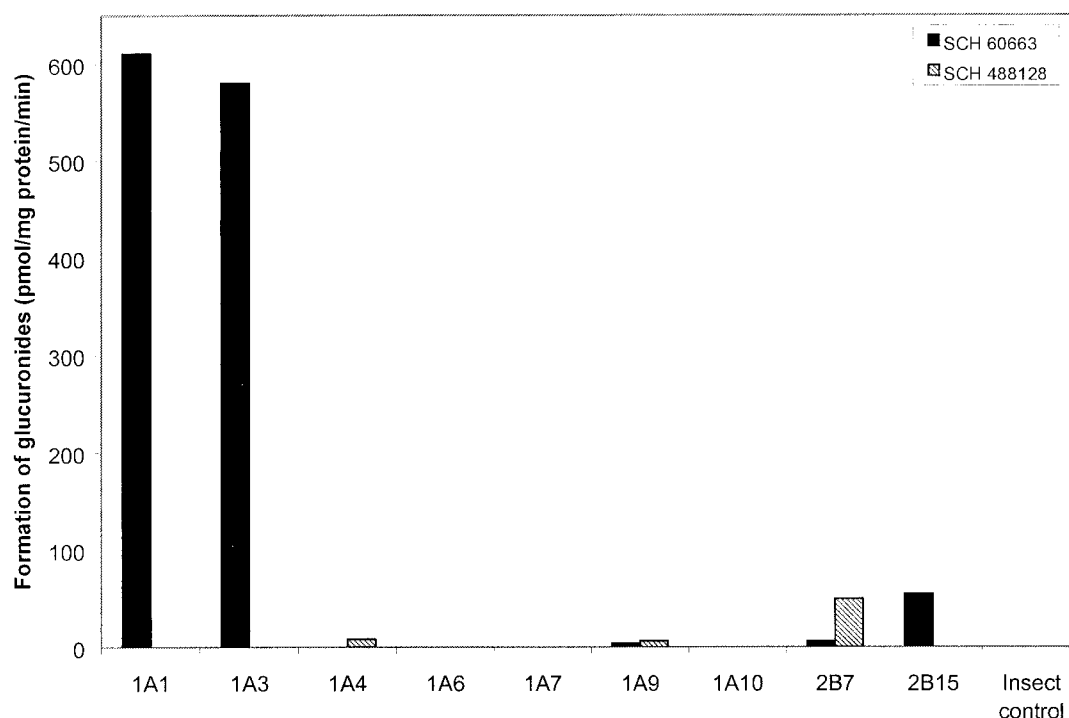


FIG. 6. Screening of UGT Supersomes for the formation of glucuronide conjugate from ezetimibe (100 μM).

TABLE 3

Kinetic parameters for the formation of glucuronides from ^{14}C -ezetimibe by human UGT Supersomes

Kinetic parameters were determined by GraFit 4.04 program (Erithacus Software).

Kinetic Parameter	UGT1A1	UGT1A3	UGT2B7
Phenolic/benzylic glucuronide	Phenolic	Phenolic	Benzylic
K_m (μM)	64.3 ± 17.2	41.7 ± 15.4	20.9 ± 4.05
V_{\max} (nmol/mg protein/min)	0.71 ± 0.09	0.48 ± 0.07	0.05 ± 0.004
V_{\max}/K_m (ml/mg protein/min) ^a	0.011	0.011	0.002

^a Intrinsic clearance.

TABLE 4

Summary of metabolites formation by human liver/jejunum microsomes and cDNA-expressed human UGT isozymes from ^{14}C -ezetimibe

Microsomes/UGT Enzymes + UDPGA	Ezetimibe Concentration (μM)	% Conversion (% Total Profiled Radioactivity)	
		Phenolic glucuronide (SCH 60663)	Benzylic glucuronide (SCH 488128)
Liver	5	100	ND
	25	47.8	2.32
Jejunum donor HJ-004	25	42.5	41.3
Jejunum donor HJ-025	100	41.8	41.4
UGT1A1	100	79.5	ND
UGT1A3	100	73.4	ND
UGT1A4	100	ND	0.78
UGT1A6	100	ND	ND
UGT1A7	100	ND	ND
UGT1A9	100	0.21	0.48
UGT1A10	100	ND	ND
UGT2B7	100	0.51	5.96
UGT2B15	100	6.09	ND
Insect control	100	ND	ND

ND, not detected.

TABLE 5

Inhibition of ezetimibe glucuronide formation after incubation of ezetimibe with human liver/jejunum microsomes and UGT Supersomes in the presence of various UGT inhibitors

Microsomes and expressed UGT enzymes were incubated with ezetimibe (25 μM) in the presence of UGT inhibitors as described under *Materials and Methods*.

Inhibitors	Conc. (μM)	% Control Activity Remaining ^a				
		SCH 60663 ^b			SCH 488128 ^c	
		Liver	Jejunum	UGT2B15	Jejunum	UGT2B7 ^b
Flunitrazepam	100	37.4	64.3	NP	78.7	NP
	200	13.9	47.4	13.1	55.8	17.2
Diclofenac	100	49.1	55.2	NP	92.4	27.5
	500	10.8	15.6	12.2	41.8	3.2

^a Activity remaining is calculated as percentage of vehicle control.^b Percentage of SCH 60663 activity.^c Percentage of SCH 488128 activity.

NP, not performed.

human jejunum microsomes. These findings suggested that UGT2B7 is involved in the formation of benzylic glucuronide in human jejunum microsomes. Of all the inhibitors tested, none of them was found to be specific inhibitor of UGT1A1 or UGT1A3 Supersomes.

Discussion

In the present study, incubation of ezetimibe with human liver microsomes resulted in the formation of one major metabolite (Fig. 2). This metabolite was identified as the phenolic glucuronide of ezetimibe (SCH 60663). However, incubation of ezetimibe with human jejunum microsomes showed that two glucuronides, i.e., phenolic and benzylic, were formed in vitro (Fig. 5) (Ghosal et al., 2002). The

apparent K_m and V_{\max} values for the formation of SCH 60663 were similar in both human liver and jejunum microsomes. The phenolic glucuronide is a well characterized major in vivo metabolite of ezetimibe that is pharmacologically active in inhibiting cholesterol absorption (Patrick et al., 2002). Formation of the phenolic glucuronide is mediated by UGT1A1, UGT1A3, and UGT2B15, based on the metabolite profiles after incubation of ezetimibe with recombinant human UGT Supersomes overexpressing UGT1A1, UGT1A3, or

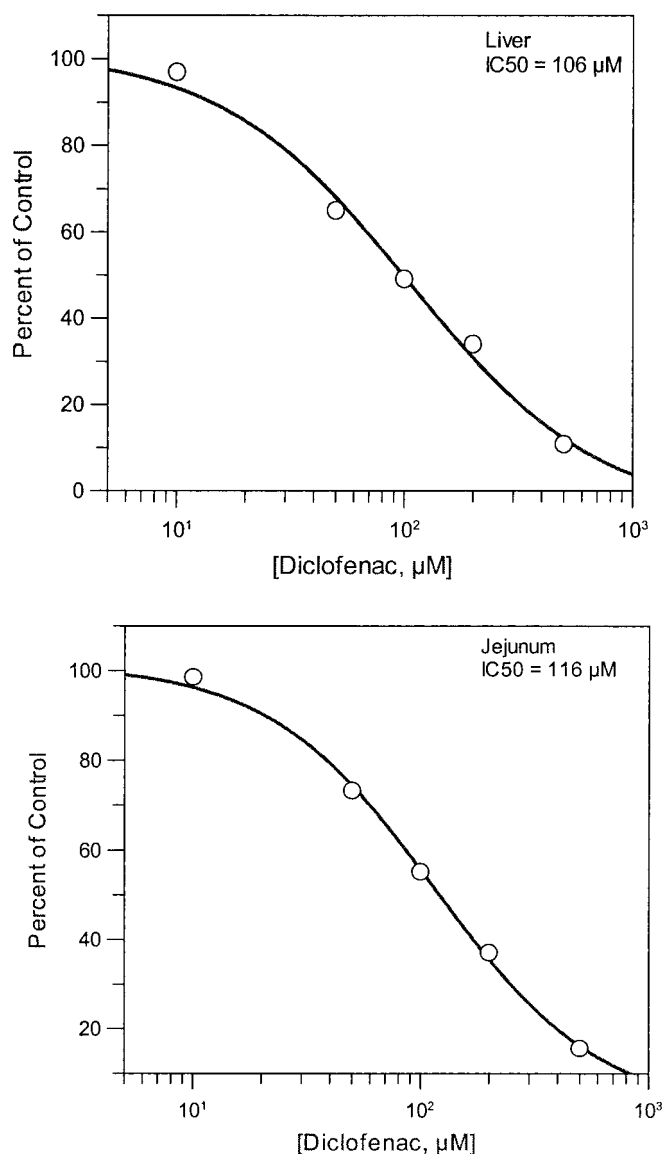


FIG. 7. Inhibition curve of diclofenac (IC_{50}) for the formation of phenolic glucuronide from ezetimibe incubated separately with pooled human liver (top panel) and human jejunum (bottom panel) microsomes.

UGT2B15. Comparison of the apparent kinetic constants with the recombinant UGT1A1 and UGT1A3 revealed that the K_m value of UGT1A3 was lower than that of UGT1A1, but intrinsic clearance was similar, suggesting that both enzymes were involved in the formation of SCH 60663. Formation of benzylic glucuronide was dependent on UGT2B7, based on the formation of the metabolite in the microsomes overexpressing UGT2B7 and inhibition by diclofenac (Miners et al., 1997; Kirkwood et al., 1998) and flunitrazepam (Cheng et al., 1998; King et al., 2000). Cheng et al. (1998) also reported that flunitrazepam inhibited catechol estrogen glucuronidation catalyzed by UGT2B7. Diclofenac (500 μ M) was reported to inhibit dihydrocodeine glucuronidation (Kirkwood et al., 1998) and morphine glucuronidation (King et al., 2001) catalyzed by UGT2B7. The findings of the present study are in agreement with the previous report that diclofenac is a potent inhibitor of UGT2B7 at 500 μ M.

In vitro incubation with nine different recombinant human UGT Supersomes showed that UGT1A1, UGT1A3, and UGT2B15 exhibited the most activity for the formation of the phenolic glucuronide (SCH 60663) followed by markedly less substrate conversion with UGT1A9 and UGT2B7 (Table 4). However, UGT2B7 exhibited the most activity for the formation of benzylic glucuronide (SCH 488128) followed by markedly less substrate conversion with UGT1A4 and UGT1A9 (Table 4). Diclofenac and flunitrazepam inhibited formation of the phenolic glucuronide by human liver and jejunum microsomes. These chemicals also inhibited formation of phenolic glucuronide from UGT2B15 Supersomes. These results suggest the involvement of UGT2B15 in the formation of phenolic glucuronide.

Diclofenac and flunitrazepam inhibited the formation of benzylic glucuronide by human jejunum microsomes as well as from UGT2B7 Supersomes. These results suggested the involvement of UGT2B7 in the formation of benzylic glucuronide. Since none of the inhibitors were close to being as inhibitory toward benzylic glucuronide in the jejunum microsomes as in UGT2B7, it is possible that some other UGT isoforms (unknown or not commercially available) can contribute to benzylic glucuronide formation in the intestine.

The formation of trace levels of phenolic glucuronide with recombinant UGT1A9 or UGT2B7 and trace levels of benzylic glucuronide with UGT1A4 or UGT1A9 suggested some involvement of these enzymes in the metabolism of ezetimibe. However, the contribution of these UGTs was judged to be minor based on their conversion percentage (Table 4). Since isozyme specific inhibitors of UGTs are not known in the literature and all the chemicals tested did not inhibit

UGT1A1 or UGT1A3, it was not possible to determine the relative contribution of each UGT in the metabolism of ezetimibe.

The results of this study suggest that the formation of phenolic glucuronide (SCH 60663) is mediated via UGT1A1, UGT1A3, and UGT2B15. The formation of the benzylic glucuronide (SCH 488128) observed after incubation of ezetimibe with human jejunum microsomes is mediated via UGT2B7.

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