In Vitro Glucuronidation of Fenofibric Acid by Human UDP-Glucuronosyltransferases and Liver Microsomes

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ABSTRACT:

Fenofibric acid (FA), the active moiety of fenofibrate, is an agonist of the peroxisome proliferator-activated nuclear receptor α that modulates triglyceride and cholesterol profiles. Lipid response to fenofibrate and FA serum concentrations is highly variable. Although FA is reported to be almost exclusively inactivated by UDP-glucuronosyltransferases (UGTs) into FA-glucuronide (FA-G), the contribution of UGT isoenzymes has never been systematically assessed. Heterologously expressed human UGT1A and UGT2B and their coding variants were tested for FA glucuronidation using liquid chromatography/mass spectrometry. Recombinant UGT2B7 presented the highest Vmax/Km value (2.10 μl/min/mg), 16-fold higher than the activity of other reactive UGTs, namely, UGT1A3, UGT1A6, and UGT1A9 (0.13, 0.09, and 0.02 μl/min/mg, respectively). UGT2B7.1 (His268) and UGT2B7.2 (Tyr268) enzyme activity was similar, whereas UGT1A3.2 (R134A), UGT1A3.3 (Trp71), and UGT1A9.3 (Thr258) showed 61 to 96% reduced Vmax/Km values compared with the respective (1) reference proteins. FA-G formation by a human liver bank (n = 48) varied by 10-fold, but the rate of formation was not associated with common genetic variations in UGT1A3, UGT1A6, UGT1A9, and UGT2B7. Correlation with activities for the probe substrates zidovudine (UGT2B7; r2 = 0.75), mycophenolic acid (UGT1A9; r2 = 0.42), fulvestrant (UGT1A3; r2 = 0.36), but not serotonin (UGT1A6; r2 = 0.06) indicated a primary role for UGT2B7 and lesser roles of UGT1A9 and UGT1A3 in hepatic FA glucuronidation. This was confirmed by a strong correlation of FA-G formation with UGT2B7 protein content and inhibition by fluconazole, a known UGT2B7 selective inhibitor. Additional studies are required to identify genetic factors contributing to the observed FA glucuronidation variability.

Fenofibrate is a widely prescribed drug approved for triglyceride-lowering in patients with mixed dyslipidemia (Guay, 2002). Fenofibrate is mostly known to reduce serum triglycerides (35–50%) and to elevate serum high-density lipoprotein-cholesterol (by 15–25%) (Staels et al., 1998). Lipid response to fenofibrate is highly variable.

Fenofibrin acid (FA), the active moiety of fenofibrate, is responsible for the primary pharmacodynamic effects of the drug, including decrease of triglycerides, cholesterol, and very low-density lipoproteins, as well as increase of high-density lipoprotein-cholesterol (Guay, 2002). These effects are modulated at the transcription level through the activation of peroxisome proliferator-activated receptor α. FA serum concentrations have been associated with lipid response, and they are characterized by high interindividual variability (Straka et al., 2007).

Most common adverse effects associated with fenofibrate treatment are dose-related increases of serum levels of liver-specific enzymes (3–13%), as well as headache (3%), abdominal pain (5%), and respiratory disorders (6%) (Steinmetz et al., 1996). Although infrequent, rhabdomyolysis is the most threatening adverse reaction. Fibrates are also frequently prescribed together with hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins) to treat patients with mixed hyperlipidemia, but this combination has been reported to increase risk of myopathy, including rhabdomyolysis (Murdoch et al., 1999). Fenofibrate and some statins are known to share common drug-metabolizing pathways. However, the underlying mechanisms and factors involved in such an adverse effect remain unclear.

Potential causes of variability in response to fenofibrate therapy

ABBREVIATIONS: FA, fenofibric acid; UGT, UDP-glucuronosyltransferase; HLM, human liver microsome; FA-G, fenofibric acid-glucuronide; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; HEK, human embryonic kidney; OHCE, hydroxy-catecholestrogen; CV, coefficient of variation; MPA, mycophenolic acid.
include genetic variations in drug-metabolizing pathways and transport. UGT is known to be exclusively metabolized by the phase II enzymes UDP-glucuronosyltransferases (UGTs) (Prueksaritanont et al., 2002), and no other phase II or cytochrome P450 drug-metabolizing enzymes have been implicated (Prueksaritanont et al., 2005). UGTs are responsible for clearance of various endobiotics, and this pathway is also critical for numerous drugs. Among known human UGTs, only six were previously evaluated for FA glucuronidation, of which UGT1A1, UGT1A3, UGT1A9, and UGT2B7 were able to glucuronidate FA (Prueksaritanont et al., 2002). However, the influence of common genetic variants on FA glucuronidation has yet to be addressed.

The aims of this study were 1) to perform a systematic study to identify human UGTs involved in FA glucuronidation using in vitro recombinant human UGTs and human liver microsome (HLM) samples, and 2) to explore the influence of common heterologous UGT variant allozymes and perform correlative studies concerning FA-glucuronide (FA-G) formation by HLMs. A specific and sensitive variant allozymes and perform correlative studies concerning FA-glucuronide (FA-G) formation by HLMs. A specific and sensitive dem mass spectrometry (MS/MS) method was developed to achieve these goals.

Materials and Methods

Chemicals and Reagents. FA was obtained from EDQM (Strasbourg, France). Preparation of FA-G standard is described below. All the other chemicals and reagents were of the highest grade commercially available.

Genomic DNA and Liver Samples. Human genomic DNA and liver samples were obtained as described previously (Hesse et al., 2004); approvals from human research institutional review boards were obtained. Available subject information included gender, age, race, ethnicity, and histories of smoking and alcohol use. The enzymatic quality of liver samples was confirmed with at least 10 other measures of glucuronidation activity with this sample set as previously stated in other studies (Hesse et al., 2004; Girard et al., 2006). UGT2B7 protein content measurements had been reported previously (Court et al., 2003).

Genotyping. UGT1A1 -537T to -3156G/A, and -3297T>G genotypes for all 48 subjects were determined in a previous study (Girard et al., 2005), as for those of UGT1A1 exon 1 polymorphisms at codons 5 (Tyr1) and 33 (Thr1); intron polymorphisms at positions 1143C>T, 1152G>A, 2014A>C, 2197T>A, 3131A>C, and 3993C>T (relative to the end of UGT1A1 exon 1); and 13 promoter polymorphisms from -87 to -5366 (Girard et al., 2004, 2006). UGT1A3 genotypes (12 polymorphisms in the first exon and 7 promoter polymorphisms from -66 to -758 relative to the ATG) were also available (Caillier et al., 2007). Finally, UGT2B7 and UGT1A3/UGT1A6, UGT1A9, and UGT2B7, and microsomes were incubated 1 h in the presence of substrate concentrations ranging from 5 to 2000 μM. Kinetic parameters were also assessed for the following allelic variant: UGT1A3.1, UGT1A3.2 (R143W), UGT1A3.3 (Arg143), UGT1A9.1, UGT1A9.2 (Tyr), UGT1A9.3 (Thr3), UGT2B7.1, and UGT2B7.2 (Tyr733). A specific and sensitive dem mass spectrometry (MS/MS) method was developed to achieve these goals.

Preparation of FA-G Standard. FA-G was obtained from enzymatic preparations of UGT-HEK293 (BD Gentest, Woburn, MA; PanVera Corp., Madison, WI). The level of UGT protein expression was determined using the same semiquantitative immuno blot analysis method. In addition, based on a previous report indicating higher activity of UGT1A10 in homogenates compared with microsomes, UGT1A10 cell homogenates were also prepared by resuspending pellets in phosphate buffer solution with 0.5 mM diethiothreitol and then stored at −80°C until use.

Glucuronidation Activity and Inhibition Assays. To assess which UGT(s) might be involved in the formation of FA-G, initial experiments with UGT1A and UGT2B microsomes, including commercial preparations consisting of 4-h incubation at 37°C with 0.2 mM FA, 10 to 50 μg of UGT membrane protein, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM UDP-glucuronic acid, 20 μg/ml amethicin, 10 μg/ml phosphatidylycholine, 5 μg/ml pepstatin, and 0.5 μg/ml leupeptin in a standard procedure, were performed in our laboratory (Gagné et al., 2002). Assays with liver samples and UGT1A10 homogenates, as well as inhibition assays, were performed in the same conditions. Inhibition assays were performed with varying concentrations of flunacazo (0–4 mM) and fixed FA (125 μM) concentration, 3′-azido-2′,3′-dideoxynucleoside (1.1 mM) was used as positive control of UGT2B7 activity. The assays were terminated by adding 100 μl of methanol + 0.02% formic acid and were centrifuged at 14,000g for 10 min before analysis. Before kinetics experiments with UGTs with a significant glucuronidation activity for FA, experiments were designed to assess the linearity of the glucuronidation reaction. Determination of kinetic parameters (Vmax and apparent Km) was then performed for UGT1A3, UGT1A6, UGT1A9, and UGT2B7, and microsomes were incubated 1 h in the presence of substrate concentrations ranging from 5 to 2000 μM. Kinetic parameters were also assessed for the following allelic variant: UGT1A3.1, UGT1A3.2 (R143W), UGT1A3.3 (Arg143), UGT1A9.1, UGT1A9.2 (Tyr), UGT1A9.3 (Thr3), UGT2B7.1, and UGT2B7.2 (Tyr733).

Mass Spectrometry Analysis. Detection of FA-G was performed by HPLC coupled with MS/MS. The analysis system consisted of an HPLC module (Agilent 1200 series; Agilent Technologies, Ville St-Laurent, QC, Canada) and a tripple-quadrupole mass spectrometer (API 3200; MDS Sciex, Concord, ON, Canada). Ten-micro liter samples, maintained at 4°C, were injected on a 100 × 4.6-mm (4.0-μm diameter) Synergic RP-Hydro C-18 reversed-phase column (Phenomenex, Torrance, CA). The mobile phase consisted of solution A (H2O containing 0.1% formic acid) and solution B (MeOH containing 0.1% formic acid). The Absolute glucuronidation activities were adjusted by UGT protein relative levels and expressed as relative glucuronidation activities. UGT protein levels were determined by Western blotting following a previously published procedure (Gagné et al., 2002). Visualization and expression level quantification were performed as previously published (Villeneuve et al., 2003). Statistical evaluation of best-fit model was used to select the enzyme kinetic model and confirmed by a visual inspection of fitted functions (V as a function of [S]) and Eadie-Hofstee plots (V as a function of [V]/[S]). Kinetic parameter calculations were performed with Sigma Plot 8.0 software assisted by Enzyme Kinetics 1.1 software (SPSS Inc., Chicago, IL). Values are expressed as the mean of at least two experiments performed in triplicate.

Preparation of FA-G Standard. FA-G was obtained from enzymatic assays using liver microsomes. In brief, media from enzymatic assays were diluted with 1% formic acid and loaded on Strata X cartridges (60 mg; Phenomenex) preconditioned with methanol followed by 1% formic acid. After loading the sample, the cartridge was washed with ultrapure water and chloroform to remove the unconjugated FA. FA-G was eluted with methanol, evaporated under nitrogen, and diluted in methanol and 0.02% formic acid, and the purity of the compound was confirmed by HPLC/MS. An aliquot was treated with β-glucuronidase, and the residue was quantified with a calibration curve of FA. The concentration of FA obtained from the aliquot digested by β-glucuronidase was then converted into a concentration of FA-G.

Statistical Analyses. All the statistical analyses were done using JMP 4.0.2 program (SAS Institute, Cary, NC). Correlation analyses between activity
values were done using Spearman’s $r$. The $r$ values greater than 0.50 and $p$ values less than 0.05 were considered significant. One-way analysis of variance and a comparison for each pair using Student’s $t$ test were used to determine the relationship between genotypes and glucuronosyltransferase activities. Normal distribution of expression and activity values was assessed with the Shapiro-Wilk W test. Raw data that were not normally distributed were transformed with a logarithm function to achieve a normal distribution. For all the analyses, a $p$ value less than 0.05 was considered significant.

**Results**

**Detection of FA-G.** A representative chromatogram and MS/MS spectra of FA-G are presented in Fig. 1. Retention time of FA-G was 3.32 min. The limit of quantification was 1 ng/ml using a signal-to-noise ratio of 10:1. The inter- and intraday precision (CV%) and accuracy (bias%) for the measurement of FA-G are presented. Signals were found to be linear from 1 to 1000 ng/ml for FA-G. All of the precision values (CV) were less than 10%, whereas accuracy was included in a range of 10% (Table 1).

**In Vitro Glucuronidation Assays with Recombinant Human UGT Enzymes.** Microsomes from recombinant UGT expressed in HEK293 cells were initially incubated with 200 μM FA for 4 h to detect formation of FA-G. UGT1A3, UGT1A6, UGT1A9, and UGT2B7 generated a significant amount of FA-G (>200 pmol/min/mg protein/UGT content), whereas other liver-expressed UGTs showed lower or undetectable activity (Fig. 2). From UGTs not expressed in liver, UGT1A8 was the most reactive (~600 pmol/min/mg/UGT). UGT1A10 homogenates were also tested based on recent observations of higher activity for 4-OH-tamoxifen, endoxifen, 2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine, and $N$-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine compared with microsomes (Dellinger et al., 2007; Sun et al., 2007). However, for UGT1A10, FA-G formation was 1.6-fold higher in microsomes than the activity observed with homogenates (data not shown). Our results were confirmed concomitantly performing assays with commercial preparations of UGTs (Supersomes), although they were less active than HEK293 recombinant UGTs in the assay conditions used (Fig. 2).

For subsequent studies, kinetic analyses were performed only for the most reactive UGTs expressed in the liver, namely, UGT1A3, UGT1A6, UGT1A9, and UGT2B7 (Tables 2 and 3). A first observation is that the kinetic profiles diverge from one enzyme to another. UGT1A6, UGT1A9, and UGT2B7 displayed a Michaelis-Menten profile, whereas UGT1A3 displayed a sigmoid profile (Hill’s) (Fig. 3). Kinetics for HLM reveals a Hill’s profile with an $S_{\infty}$ of 295 ± 68 μM and a $V_{\text{max}}$ of 1.8 ± 0.2 nmol/min/mg. Of these four enzymes, UGT2B7, UGT1A9, and UGT1A6 could be categorized as high-affinity enzymes with apparent $K_{m}$ values ranging from 131 to 222 μM, whereas UGT1A3 exhibited the lowest affinity for FA ($K_{m}$ of 724 μM). UGT2B7 showed the highest catalytic efficiency for FA-G formation, at least 16-fold higher than the other active UGTs, with a $V_{\text{max}}/K_{m}$ value of 2.1 μl/min/mg. UGT1A3, UGT1A9, and UGT1A6 had $V_{\text{max}}/K_{m}$ values of 0.13, 0.09, and 0.02 μl/min/mg, respectively.

**Glucuronidation by Recombinant Human UGT Variant Allozymes.** Variant UGT allozymes with common nonsynonymous polymorphisms were investigated in their ability to catalyze the formation
of FA-G (Tables 2 and 3). UGT2B7.1 (His268 or *1 allele) and UGT2B7.2 (Tyr268 or *2 allele) allozymes have similar apparent \( K_m \) values and no statistically different velocities, although a lower \( V_{\text{max}} \) value is observed for *2 (243 pmol/min/mg for *1 versus 136 pmol/min/mg for *2). Compared with UGT1A9.1, rates of FA-G formation were unaffected for UGT1A9.2 protein, with a tyrosine residue at codon 3, but were significantly reduced for UGT1A9.3 (Thr33) allozyme by 11-fold (1.1 versus 12 pmol/min/mg for UGT1A9.1; \( p = 0.0045 \)). Consequently, the \( V_{\text{max}}/K_m \) value of UGT1A9.3 (0.004 \( \mu \)l/min/mg) allele was 23-fold lower than that of the UGT1A9.1 protein (0.09 \( \mu \)l/min/mg) (\( p = 0.0049 \)). The two of the most frequent UGT1A3 variants were also tested: UGT1A3.2 (R11A47; frequency of 36% in whites) and UGT1A3.3 (Arg11; frequency of 6% in whites) (Caillier et al., 2007; Menard et al., 2009). A substantial decrease in the formation of FA-G was observed for both variants UGT1A3.2 and UGT1A3.3 compared with the reference UGT1A3.1 protein (by 61 and 76%, respectively; \( p = 0.005 \)), mainly explained by decreased velocities.

**FA-G Formation in a Liver Bank and Its Relationship to Probe Substrates, UGT Expression, and Common Genetic Variations.**

UGT activities for FA were measured, and those for common UGT marker substrates (zidovudine, mycophenolic acid, fulvestrant, and serotonin) were available for a set of HLMs (\( n = 48 \)) (Court et al., 2003; Girard et al., 2004; Krishnaswamy et al., 2004). Rates of
formation of FA-G ranged from 147 to 1413 pmol/min/mg and varied 10-fold (Fig. 4). Correlative studies with probe substrates revealed a significant correlation between formation of FA-G and glucuronidation of zidovudine (UGT2B7; \( r^2 = 0.75 \)), mycophenolic acid (UGT1A9; \( r^2 = 0.42 \)), and fulvestrant (UGT1A3; \( r^2 = 0.36 \)) (\( p \leq 0.01 \)), supporting the contribution of these UGTs in the hepatic glucuronidation of FA (Table 4). Inhibition assays with fluconazole, a known UGT2B7 selective inhibitor (Uchaipichat et al., 2006), indicate an IC\(_{50}\) of 36.4 \( \mu \)M on FA glucuronidation by liver microsomes and a maximum of 60% inhibition of FA glucuronidation at the highest concentration of fluconazole. FA-G formation was highly correlated with UGT2B7 protein content (\( r^2 = 0.736; p < 0.0001 \)). No significant relationship between the presence of genetic variations in the most reactive UGTs and FA-G formation was evidenced in the human liver bank (Fig. 5).

**Discussion**

Fenofibrate is approved for triglyceride-lowering in subjects with types IV and V hyperlipoproteinemia. Although fibrates have been associated with muscle toxicity (Magarian et al., 1991), an effect that is more pronounced in patients also treated with a statin (Pierce et al., 2007), fenofibrate remains the preferred fibrate in patients who require combined therapy (Rosenson, 2004). The extended use of fenofibrate emphasizes the importance of understanding its metabolism to explain and eventually prevent some drug-drug interactions and adverse reactions. Our results suggest that FA glucuronidation is mediated by multiple hepatic UGTs. Although UGT2B7 has a predominant role, UGT1A9 and UGT1A3 also contribute to FA-G formation (Fig. 6). The contribution of specific genetic polymorphisms in UGT2B7, UGT1A9, or UGT1A3 to variability in glucuronidation of FA by a human liver bank could not be confirmed.

Our observations, indicating that whereas UGT2B7 and UGT1A9 showed a high affinity for FA, UGT1A3 and UGT1A6 represent low-affinity enzymes for this substrate, are in agreement with a previous study concerning FA glucuronidation. The group of Prueksaritanont et al. (2002) tested six UGTs, and their data support a role for UGT2B7, UGT1A9, and UGT1A3 in FA glucuronidation. A more systematic characterization of enzyme kinetics presented here corroborated these results, including assays with commercial preparations of UGT enzymes. In contrast to a previous study that showed recombinant UGT1A10 homogenates have greater activity compared with UGT1A10 microsomes for a variety of substrates, including tamoxifen (Dellinger et al., 2007; Sun et al., 2007), we observed that microsomes are more active for fenofibrate than homogenates, suggesting that such differences could be substrate-specific. A higher activity with microsomes compared with Supersomes was also observed, as noted in other studies (Benoit-Biancamano et al., 2009). Thus, a primary role of UGT2B7 followed by UGT1A10 and possibly UGT1A3 in the hepatic clearance of FA is predicted and further supported by correlative studies using liver microsomes and UGT probe substrates. The substantial inhibition of hepatic glucuronidation with the UGT2B7 selective inhibitor fluconazole also supports a major contribution of UGT2B7 in the liver glucuronidation of FA. The role of UGT1A3 could also be relevant for FA glucuronidation in liver because both HLMs and UGT1A3 show sigmoidal kinetics, and the S\(_{50}\) value for UGT1A3 is only approximately 2-fold higher than the value for HLMs. Furthermore, UGT1A3 mRNA expression in the liver was reported to be higher than UGT1A6 and UGT1A9 mRNA, both having similar level of mRNA expression (Zhang et al., 2007). It is possible that the lower affinity of UGT1A3 for FA may be partially compensated for by its significant expression in the liver, which could also explain the kinetic profile similar to UGT1A3 observed for HLMs. UGT2B7 mRNA expression was also shown to be higher than that of UGT1A6 (Somers et al., 2007), suggesting it may also play an important role in FA metabolism.
High variability in the formation of FA-G was observed in the bank of 48 HLMs. Genetic polymorphisms that affect the metabolic activity or expression of UGT biotransformation enzymes may be important contributors to interindividual differences in FA pharmacokinetics. In line with this hypothesis, we explored the relationship between known polymorphisms of UGT2B7, UGT1A9, and UGT1A3 and the hepatic formation of FA-G using a bank of human liver microsomal fractions. The UGT2B7*2 (Tyr268) allele carried by 54% of whites (Lampe et al., 2000) was first studied. Previously reported data suggest a possible substrate-specific interaction for the mutation at codon 268. For example, a decreased activity for UGT2B7.2 (Tyr268) compared with the UGT2B7.1 (His268) allozyme for lithocholic acid, hyodeoxycholic acid, estradiol, and zidovudine (Gall et al., 1999; Barbier et al., 2000) increased activity for UGT2B7.2 compared with the UGT2B7.1 allozyme for 4-hydroxy-catecholestrogen (4-OHCE) (Thibaudeau et al., 2006) and showed similar activity for the two allozymes for 2-OHCE, 4-OHCE, androsterone, menthol, opioids, propranolol, and epirubicin (Cheng et al., 1998; Coffman et al., 1998; Bhasker et al., 2000; Innocenti et al., 2001). On the other hand, the UGT2B7*2 variant has no significant influence on the conjugation of mycophenolic acid (MPA), morphine, valproic acid (Court et al., 2003; Bernard et al., 2006; Chung et al., 2008), and FA, as described herein. However, the coding variation is in linkage disequilibrium with a series of promoter variations that were associated with a 2-fold reduced transcriptional activity compared with the reference allele (Duguay et al., 2004), thus leaving open the possibility of interactions that may be observed under other conditions. There was no evidence

![Eadie-Hofstee plots of recombinant UGT.](image1)

**Fig. 3.** Eadie-Hofstee plots of recombinant UGT. Whereas UGT1A6, UGT1A9, and UGT2B7 displayed a Michaelis-Menten profile, UGT1A3 was characterized by a sigmoid profile (Hill’s), as for liver microsomes.

![FA glucuronidation by microsomes from 48 human liver samples.](image2)

**Fig. 4.** FA glucuronidation by microsomes from 48 human liver samples.
of a relationship between UGT2B7 variations and rates of FA-G formation in the tested liver samples. In vitro correlation studies with rates of formation of FA-G were evidenced on stratification by UGT1A3, UGT1A6, or UGT1A9 genetic status for several known regulatory and coding functional variations. However, given the limited number of human liver bank samples, the contribution of genetic factors to the variability of FA glucuronidation cannot be ruled out based on our study.

Based on this study, we conclude that glucuronidation of FA is mainly mediated by a few UGT enzymes, primarily UGT2B7, but also by UGT1A9, which is a high-affinity and a low-capacity enzyme, and by UGT1A3, which is a low-affinity high-capacity enzyme. Additional studies either with in vivo experiments or in vitro studies with a sufficiently large number of subjects are required to determine whether the genetic status for the genes encoding these UGTs influence the metabolism of FA.

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