ISOLATION AND IDENTIFICATION OF NOVEL METABOLITES OF GEMFIBROZIL IN RAT URINE

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ABSTRACT:

Gemfibrozil (GEM) is a fibrate analog used to treat moderate to severe hypertriglyceridemias. In lab animals, GEM causes peroxisome proliferation, an effect that has been associated with hepatocarcinogenesis in rats. In humans, hepatobiliary disorders, but not carcinogenesis, have been associated with GEM therapy. In the present study [(14C)]GEM was administered orally to rats at a dose of 2000 mg/kg. At various time points, radioactivity in urine was analyzed by liquid scintillation spectrometry, high-pressure liquid chromatography, liquid chromatography/mass spectrometry, gas chromatography/mass spectroscopy, and nuclear magnetic resonance. Nine metabolites of GEM were identified, some that have not been reported previously. Although the majority of metabolites were glucurononated, some nonglucurononated metabolites were identified in urine, including a diol metabolite (both ring methyls hydroxylated), and the product of its further metabolism, the acid-alcohol derivative (ortho ring methyl hydroxylated, meta ring methyl completely oxidized to the acid). Hydroxylation of the aromatic ring also was a common pathway for GEM metabolism, leading to the production of two phenolic metabolites, only one of which was detected in the urine in the nonconjugated or free form. Also of interest was the finding that both acyl and ether glucuronides were produced, including both glucurononide forms of the same metabolite (e.g., 1-O-GlcUA, 5'-COOH-GEM, and 5'-COO-GlcUA-GEM); the positions and functionality of the glucurononide conjugates were identified using base hydrolysis or glucuronidase treatment, in combination with liquid chromatography/MS

Gemfibrozil (GEM) is a fibrate analog that is indicated for the treatment of hyperlipoproteinemias involving elevated triglycerides. At the present time, it is one of the primary lipid-lowering agents of the fibric acid class that is still marketed in the United States (e.g., Lopid, Parke-Davis). This is because of the concern caused by observations of rodents that fibric acid analogs (particularly clofibrate) cause an increase in malignancies (Childs and Girardot, 1992; Fitzgerald et al., 1981; Newman and Hulley, 1996) and produce peroxisome proliferation and hepatomegaly (Lalwani et al., 1983; Sausen et al., 1995). In humans, fibric acid derivatives have been shown to increase the risk of gallbladder disease and have been associated with an increased incidence of noncoronary mortality (The Committee of Principal Investigators, 1978; Huttenue et al., 1994). However, within this class of compounds, GEM appears to have a lower propensity for causing these untoward effects. Furthermore, GEM-induced hepatotoxicity is species-specific, with rats being far more susceptible than hamsters or humans to the hepatic peroxisome-proliferating effects of GEM (Gray and de la Iglesia, 1984). Thus, despite the clear indication for the usage of GEM in cases of severe triglyceride elevation, where the risk of xanthomas, pancreatitis, and coronary heart disease is clear, debate continues whether there is a benefit or a risk involved with the use of GEM as a prophylactic agent against coronary artery disease in conditions where triglycerides are not severely elevated or where other lipoprotein levels are adversely affected by GEM treatment (type I, IIa, and some type IIIb dyslipidaemia).

In rats as well as hamsters and humans, GEM undergoes extensive phase I and phase II metabolism (Okerholm, 1976; Okerholm et al., 1976). Phase I oxidations include hydroxylation of the benzylic carbons, oxidation to the para-phenol, and further oxidation of the meta methyl group to give a benzoic acid metabolite. The oxidized metabolites of GEM are also substrates for phase II conjugation reactions, resulting in a large pool of glucurononide conjugates. As is the case with other compounds, the acylglucurononides of GEM and its metabolites are reactive species that can either bind covalently to nucleophilic sites on macromolecules or be readily hydrolyzed to the free xenobiotic and glucuronic acid (Sallustio et al., 1997; Sallustio and Foster, 1995). Furthermore, acylglucurononides intramolecularly rearrange to give rise to metabolites with the xenobiotic at the 2-, 3-, and 4-C positions of the glucuronic acid ring and are no longer substrates for glucuronidases. Thus, acylglucuronidation is an important route of metabolism that may profoundly influence the pharmacokinetics and toxicity of GEM (Sallustio and Fairchild, 1995; Sallustio et al., 1996).

Because glucuronidation represents a major metabolic pathway of GEM, it is somewhat surprising that few studies have assessed quan-
titatively the production or elimination of these metabolites. Some species differences in the disposition and metabolism of GEM have been described; however, the importance of acylglucuronidation has been frequently underestimated, because either these molecules were intentionally hydrolyzed or their propensity for intramolecular rearrangements and facile hydrolysis was not controlled for. In the studies of Okerholm et al. (1976), rats and dogs eliminated GEM and its metabolites primarily in the feces, whereas in monkeys and humans, elimination of GEM was predominately via the urine. Despite the differing routes of elimination, enterohepatic recirculation has been suggested to play an important role in the metabolism of GEM in both laboratory rodents and primates. Finally, studies in isolated rat liver preparations perfused with GEM (Sallustio et al., 1996) clearly demonstrated that the reversible nature of GEM conjugation extends beyond enterohepatic recirculation. The 1-O-GEM-β-D-glucuronide conjugate was shown to be readily formed, transported across the canalicular membrane for excretion in the bile, transported across the sinusoidal membrane back into the perfusate, or hydrolyzed back into the parent acid.

It is reasonable to hypothesize that the formation of 1-O-GEM-β-D-glucuronide and other highly reactive metabolites from GEM and subsequent adduct formation with tissue proteins and DNA might be associated with hypersensitivity reactions and hepatotoxicity. However, to identify any relationship between these events and GEM toxicity, it is important to identify both the cellular targets for adduct formation, the absolute structural identity of metabolites of GEM, and the kinetics of the formation and elimination of these metabolites. This study, in combination with the pharmacokinetic and biodisposition data of Dix et al., 1999, further investigates phase I and phase II metabolism of GEM.

Materials and Methods

Nonradiolabeled GEM was obtained from Sigma Chemical Company (St. Louis, MO). Identity of the nonradiolabeled GEM was confirmed by infrared spectroscopy and nuclear magnetic resonance (NMR) spectrometry, and the purity was determined to be >99% by high performance liquid chromatography (HPLC). Radiolabeled [14C]GEM, randomly radiolabeled with carbon-14 on the benzylic carbons (40.0 mCi/mmol, 10.18 mCi), was received from Wizard Laboratories (West Sacramento, CA) and found to be ca. 90 to 93% radiochemically pure by HPLC at Research Triangle Institute. Metabolite standards (metabolites I, II, III, and IV as reported by Todd and Ward, 1988) were obtained from Parke-Davis Pharmaceutical Research (Ann Arbor, MI). The reported purity of these standards was ~100%.

The oral dose formulations contained [14C]- and nonradiolabeled-GEM suspended in 0.5% aqueous methylcellulose. These formulations were prepared on the day of dosing or 1 day before dosing. The dose levels for this single-dose oral study was 2000 mg GEM/kg b.wt. administered in a dose volume of 5 ml/kg. The 2000-mg/kg dose was selected because of previous toxicity studies performed in rats and hamsters (Mike Cunningham, personal communication). Specifically, the 2000-mg/kg dose is approximately equal to the daily dose of GEM that hamsters in the high-dose group (24,000 ppm) received in a 13-week feed study. Rats only received ca. 1.300 mg/kg/day because the high dose for rats was only 16,000 ppm.

After oral dosing the rats were placed in metabolic chambers. Citric acid (400–500 mg) was placed in the urine collection vessels to stabilize any acyl glucuronides of GEM that were present. Urine collected between 24 and 48 h was selected for further analyses aimed at metabolite identification. This time interval was selected because the largest portion of radioactivity excreted in the urine (23%) was eliminated during this time and also because the majority of rat metabolites were present during this time (Dix et al., 1999). The urine was centrifuged before analysis by HPLC, a procedure that did not affect the recovery of total radioactivity (data not shown). For quantitative analysis, the chromatography system consisted of a Zorbax XDB-C8 analytical column (5-mm particle size, 250 × 4.6 mm; MAC-MOD Analytical Inc., Chadds Ford, PA) with 0.1% trifluoroacetic acid (TFA) in acetonitrile (Solvent A, pH 2) and 0.1% TFA in water (Solvent B, pH 2) at a flow rate of 1.5 ml/min and the following gradient program: 30% Solvent A for 3 min, ramped to 100% Solvent A in 12 min, and then held at 100% Solvent A for 5 min. To quantitate the various radiolabeled metabolites, fractions of each run were collected and analyzed for carbon-14 content by liquid scintillation spectrometry. The results of these analyses, as well as a complete description of the biodisposition of GEM in male and female Sprague-Dawley rats and Syrian Golden Hamsters, are provided by Dix et al. (1999). Sufficient quantities of urinary metabolites for structural elucidation were purified using a semipreparative Zorbax XDB-C8 (5-μm particle size, 250 × 9.4 mm, MAC-MOD Analytical Inc.) with 0.1% TFA in acetonitrile (Solvent A, pH 2) and 0.1% TFA in water (Solvent B, pH 2) as the mobile phase at a flow rate of 5.0 ml/min. Elution was effected using the following gradient program: 30% Solvent A for 15 min, changed linearly to 100% Solvent A in 10 min, and then held at 100% Solvent A for 10 min. These isolated metabolites were concentrated and purified further using Bond Elut C18 solid-phase extraction cartridges (500 mg/6 ml reservoir, Varian, Harbor City, CA).

Purified metabolites were analyzed by liquid chromatography/mass spectrometry (LC/MS) using a Finnigan LCQ quadrupole ion trap mass spectrometer and either atmospheric pressure chemical ionization (APCI) or electrospray ionization. For APCI, a syringe pump was used to infuse (at a rate of 2 μl/min) the purified metabolite samples into the mobile phase of an HPLC. This mobile phase was composed of 70:30 water/acetonitrile set at a flow rate of 0.5 ml/min. Select metabolites were derivatized with trimethylsilyl (TMS) and analyzed further by gas chromatography/MC (GC/MS) with electron impact and chemical ionization. The GC/MS system consists of an HP-5890 capillary gas chromatograph (Hewlett Packard) and HP5989A MS Engine.

NMR samples were prepared by dissolving the metabolite in approximately 100 μl of acetonitrile-d3 and 10 μl of methanol-d4. NMR spectra were obtained on a Bruker AMX-500 spectrometer operating at 500.13 MHz for protons. The spectra were obtained at 300K using a Nararac inverse broadband microprobe tuned for 1H and 13C. Phase-sensitive, double quantum-filtered COSY spectra, heteronuclear multiple quantum coherence (HMQC), and het-
LC/MS and LC/MS/MS analysis of GEM metabolites

Purified HPLC fractions were analyzed and found to produce the following ionization profiles. In some instances, MS/MS was used to increase the amount of fragmentation occurring with a particular compound.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Calculated M-H m/z</th>
<th>Observed M-H m/z (% Relative Abundance)</th>
<th>Fragment Ions m/z (% Relative Abundance)</th>
<th>MS/MS Ions m/z (% Relative Abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEM</td>
<td>249</td>
<td>249 (100)</td>
<td>121 (15)</td>
<td></td>
</tr>
<tr>
<td>1-O-GlcUA-GEM</td>
<td>425</td>
<td>425 (10)</td>
<td>N.D.*</td>
<td>249 (100), 175 (80), 121 (20)</td>
</tr>
<tr>
<td>2',3'-CH\textsubscript{2}OH-GEM</td>
<td>281</td>
<td>281 (10)</td>
<td>N.D.</td>
<td>152.5 (100)</td>
</tr>
<tr>
<td>2',5'-CH\textsubscript{2}OH, 5'-COOH-GEM</td>
<td>295</td>
<td>295 (10)</td>
<td>121 (5)</td>
<td>166.1 (100)</td>
</tr>
<tr>
<td>4'-O-SO\textsubscript{3} -GEM</td>
<td>345</td>
<td>345 (100)</td>
<td>137 (2)</td>
<td>265 (45), 216 (100), 137 (86)</td>
</tr>
<tr>
<td>1-O-GlcUA, 5'-CH\textsubscript{2}OH-GEM</td>
<td>441</td>
<td>441 (2)</td>
<td>265 (100), 137 (8)</td>
<td></td>
</tr>
<tr>
<td>1-O-GlcUA, 4'-OH-GEM</td>
<td>441</td>
<td>441 (10)</td>
<td>265 (100)</td>
<td></td>
</tr>
<tr>
<td>4'-O-GlcUA-GEM</td>
<td>441</td>
<td>441 (10)</td>
<td>265 (100)</td>
<td>265 (20), 175 (80)</td>
</tr>
<tr>
<td>1-O-GlcUA, 5'-COOH-GEM</td>
<td>455</td>
<td>455 (100)</td>
<td>279 (15), 151 (2)</td>
<td>279 (100), 175 (30)</td>
</tr>
<tr>
<td>5'-COO-GlcUA-GEM</td>
<td>455</td>
<td>455 (100)</td>
<td>279 (15), 151 (2)</td>
<td></td>
</tr>
</tbody>
</table>

* N.D., not determined.


carbon spectra were obtained as weighted projections from the HMQC and HMBC spectra. All chemical shifts were reported in ppm from tetramethyl silane at 0.0 ppm.

Metabolites that had been identified tentatively as glucuronide acid (GlcUA) conjugates by mass and NMR spectroscopy were subjected to enzyme and base hydrolysis. Purified metabolites were incubated overnight with β-glucuronidase (Sigma Chemical Company) from Helix pomatia (89,400 units/ml β-glucuronidase and 3,300 units/ml sulfatase) and Escherichia coli (Type VII; 2000 units/ml β-glucuronidase in 0.1 M sodium acetate buffer, pH 6.8). Because under certain conditions, acyl glucuronides undergo intramolecular rearrangements and the resulting isomers are not substrates for β-glucuronidase, base hydrolysis also was performed on purified metabolites in acetonitrile by the addition of 20 μl of 5 M NaOH. The incubates were heated at 37°C for 1 to 2 h and then acidified with 20 to 50 μl of 5 M TFA before analysis by HPLC.

Results

Metabolite Separation and Purification. The preparative chromatography system that was developed had sufficient loading capacity and resolution to allow the baseline separation of a large number of radiolabeled substances (Fig. 2). Indeed, the increased loading capacity and resolution of the preparative column enabled further separation of peaks that appeared to represent one substance on an analytical-scale HPLC column. Thus, a single peak in the quantitative analysis of rat urine using an analytical column (peak F in Fig. 5) was resolved into two peaks (Fig. 2, this manuscript) labeled as 5'-O-GlcUA, 5'-COOH-GEM and 1-O-GlcUA, 5'-CH\textsubscript{2}OH-GEM metabolite (data not shown). The other signals in this spectrum designated with letters were found to be the most significant peaks for determining the structures of all of the GEM metabolites and are more fully described in Table 2. In this instance, these proton signals indicated that the aromatic ring and both aromatic methyls were intact. HMBC analysis clearly indicated the linkage of the glucuronide moiety in that the carbonyl carbon correlated to both the anomic proton and the protons of the geminal dimethyl groups (data not shown). These data indicate that the glucuronide ring system has not undergone migration to other carbon positions, a conclusion supported by the observation that this metabolite was readily cleaved by Helix pomatia β-glucuronidase (Table 3).

Identification of 1-O-GlcUA Conjugate of GEM. The predominant peak in the chromatographic analysis of urine revealed a mass spectrum with a base ion at m/z 539 (Fig. 4, top panel). This ion was believed to represent adduct formation between TFA (used to stabilize any acyl glucuronides during the purification of urinary metabolites) and an M-H ion at m/z 425. Isolation of the ion at m/z 539 and MS/MS analysis resulted in the production of a single, intense daughter ion at m/z 425, which would be expected if the ion at m/z 539 was the result of adduct formation. Isolation and MS/MS analysis of the ion at m/z 425 resulted in the production of daughter ions at m/z 121 and 249, which could be rationalized in part by the cleavage patterns shown (Fig. 4, bottom panel).

The proton NMR spectrum of this metabolite was similar to that recorded for GEM, with the notable exception of a doublet at 5.60 ppm and several multiplets around 3.5 ppm (Fig. 5). The proton at 5.6 ppm is indicative of glucuronide conjugates, representing the anomic proton of the glucuronide ring system (designated as proton “α” in Fig. 5). The other signals in this spectrum designated with letters were found to be the most significant protons for determining the structures of all of the GEM metabolites and are more fully described in Table 2. In this instance, these proton signals indicated that the aromatic ring and both aromatic methyls were intact. HMBC analysis clearly indicated the linkage of the glucuronide moiety in that the carbonyl carbon correlated to both the anomic proton and the protons of the geminal dimethyl groups (data not shown). These data indicate that the glucuronide ring system has not undergone migration to other carbon positions, a conclusion supported by the observation that this metabolite was readily cleaved by Helix pomatia β-glucuronidase (Table 3).

Isolation and Identification of 2',5'-CH\textsubscript{2}OH-GEM. The mass spectrum recorded for this metabolite contained an apparent M-H ion at m/z 281 and a simple cleavage of the molecule during MS/MS that gave rise to an ion at m/z 153 (Table 1, Fig. 6). This ionization and fragmentation could be produced by a 2',5'-CH\textsubscript{2}OH-GEM metabolite as shown. Mass spectral analysis of the trimethylsilyl derivative of this metabolite using GC/MS and chemical ionization (methane) further supported the structural assignment. Specifically, an apparent M + 1 at m/z 499 was detected with other diagnostic ions at m/z 527 (M + 29), 483 (M − 15), and 409 (M − 89), which would be anticipated from the trimethylsilyl derivative of a dihydroxylated GEM metabolite (data not shown).

The 1H NMR analysis of this isolated metabolite showed only the movement of the ring methyl protons down-field from 2.3 to 4.8 ppm as would be expected because of their carbon atom’s hydroxylation (Table 2; Fig. 7). Similarly, new carbon atoms at 60 and 64 ppm were detected, indicative of CH\textsubscript{2}OH groups, with the disappearance of the aromatic methyls at 14 ppm. HMBC analysis indicated that the
**Fig. 3.** Metabolic scheme postulated for GEM and mass spectral fragmentation pathways proposed for metabolites.

**TABLE 2**

NMR analysis of GEM metabolites

NMR data for the significant protons (a–f, as denoted in the structure shown in Fig. 5) in the purified GEM metabolites are indicated below [shift (δ), multiplicity, number of protons].

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEM</td>
<td>N.D.</td>
<td>2.38, s, 3H</td>
<td>2.23, s, 3H</td>
<td>7.09, d, 1H</td>
<td>6.76, d, 1H</td>
<td>6.81, s, 1H</td>
</tr>
<tr>
<td>2'-, 5'-CH_2OH-GEM</td>
<td>N.D.</td>
<td>4.64, s, 2H</td>
<td>4.66, s, 2H</td>
<td>7.38, d, 1H</td>
<td>6.98, d, 1H</td>
<td>7.01, s, 1H</td>
</tr>
<tr>
<td>2'-CH_2OH, 5'-COOH-GEM</td>
<td>N.D.</td>
<td>4.62, s, 2H</td>
<td>N.D.</td>
<td>7.97, d, 1H</td>
<td>7.58, d, 1H</td>
<td>7.46, s, 1H</td>
</tr>
<tr>
<td>4'-O-SO_3-GEM</td>
<td>N.D.</td>
<td>2.22, s, 3H</td>
<td>2.29, s, 3H</td>
<td>7.10, s, 1H</td>
<td>N.D.</td>
<td>6.65, s, 1H</td>
</tr>
<tr>
<td>1-O-GlcUA-GEM</td>
<td>5.60, d, 1H</td>
<td>2.24, s, 3H</td>
<td>2.38, s, 3H</td>
<td>7.10, d, 1H</td>
<td>6.76, d, 1H</td>
<td>6.82, s, 1H</td>
</tr>
<tr>
<td>1-O-GlcUA, 5'-CH_2OH-GEM</td>
<td>5.54, d, 1H</td>
<td>2.47, s, 3H</td>
<td>4.64, s, 2H</td>
<td>7.27, d, 1H</td>
<td>6.82, d, 1H</td>
<td>6.85, s, 1H</td>
</tr>
<tr>
<td>1-O-GlcUA, 4'-OH-GEM</td>
<td>5.46, d, 1H</td>
<td>2.08, s, 3H</td>
<td>2.09, s, 3H</td>
<td>6.62, s, 1H</td>
<td>N.D.</td>
<td>6.53, s, 1H</td>
</tr>
<tr>
<td>4'-O-GlcUA-GEM</td>
<td>4.89, d, 1H</td>
<td>2.22, s, 3H</td>
<td>2.29, s, 3H</td>
<td>6.96, s, 1H</td>
<td>N.D.</td>
<td>6.77, s, 1H</td>
</tr>
<tr>
<td>1-O-GlcUA, 5'-COOH-GEM</td>
<td>5.44, d, 1H</td>
<td>2.22, s, 3H</td>
<td>N.D.</td>
<td>7.45, d, 1H</td>
<td>7.20, d, 1H</td>
<td>7.44, s, 1H</td>
</tr>
<tr>
<td>5'-COO-GlcUA-GEM</td>
<td>5.69, d, 1H</td>
<td>2.22, s, 3H</td>
<td>N.D.</td>
<td>7.58, d, 1H</td>
<td>7.25, d, 1H</td>
<td>7.47, s, 1H</td>
</tr>
</tbody>
</table>

* N.D., not detected.
protons of both CH₂OH groups' protons correlated to aromatic pro-
tons, providing further confirmation of the location of the hydroxyl
groups. No anomeric proton was detected, confirming the absence of
a glucuronide conjugate.

Identification of 2⁹-CH₂OH, 5⁹-COOH-GEM. The APCI/MS
analysis of this isolated metabolite resulted in a base ion detected at
m/z 295, with weak fragment ions detected at m/z 166 and 121. Isolation of the apparent M-H ion at m/z 295 and MS/MS analysis
resulted in the fragmentation and production of a daughter ion at m/z
166. One plausible explanation for this ionization and fragmentation
would be provided by further oxidation of the 2',5'-CH₂OH-GEM
metabolite to a 2'-CH₂OH, 5'-COOH-GEM. The cleavage of the
molecule, as shown in Fig. 3, would explain the detection of the
daughter ion at m/z 166 (albeit the simple cleavage as shown in Fig.
3 gives rise to a fragment of m/z 167, and not m/z 166, it is probable
that fragmentation at the location shown produces a rearrangement ion
at m/z 166).

Further elucidation of this structure was provided by NMR spec-
troscopy wherein one of the signals produced by the ring methyl
groups' protons was noted to be absent (Table 2). The 'H NMR also
revealed the absence of an anomeric proton (i.e., no glucuronide
present), that the aromatic protons were intact, and the presence of one
CH₂OH group. Both of the aromatic methyl groups appeared oxidi-
dized, suggesting that one must have been converted to a carboxyl
group. The identification of which methyl group had been oxidized to
a carboxylic acid was provided by HMBC experiments, which re-
vealed that the 2'-CH₂OH was correlated to an oxygenated quaternary
carbon atom (Fig. 8).

Identification of 4'-O-SO₃⁻GEM. Tentative identification of this
isolated metabolite was based on APCI/MS data wherein an intense
signal (base ion) was detected at m/z 345 and much weaker signals
were detected at m/z 137 and 216. MS/MS analysis of the ion at m/z
345 resulted in several daughter ions at m/z 137, 216, and 265. This
ionization and fragmentation might be expected from a sulfate con-
jugate of a hydroxylated metabolite of GEM as described in Fig. 3. Again, the simple cleavage as shown in Fig. 3 to produce m/z 136 is not sufficient to explain the formation and detection of the ion at m/z 137; however, it is reasonable to propose that fragmentation at the locations shown followed by rearrangement yields an ion at m/z 137. Consistent with this hypothesis, NMR analysis revealed the absence of an anomeric proton, that one of the aromatic protons (the 4’-H, denoted as “e” in Table 2) was absent, and that both aromatic methyls were intact, indicating that the sulfate conjugate was present at a 4’-phenolic hydroxyl. The chemical shifts of the aromatic carbons and the protons are different from those of other 4’-OH metabolites, which also could be explained by the presence of a sulfate conjugate at this position.

Identification of 1-O-GlcUA, 5’-CH₂OH-GEM. Mass spectrometric analysis of this purified metabolite resulted in an apparent M-H ion at m/z 441, with fragment ions occurring at m/z 265 (base ion) and 137 (Table 1). This M-H ion would be anticipated with a monohydroxylated GEM metabolite that had been conjugated with glucuronic acid. The fragmentation of this compound to give rise to ions at m/z 265 and 137 could be rationalized by a glucuronidated metabolite, with hydroxylation occurring somewhere on the ring system or the benzylic carbons (Fig. 3).

NMR analysis of this metabolite (Table 2) revealed a doublet at 5.54 ppm that has been shown to be indicative of the anomeric proton of a glucuronide conjugate. Furthermore, the signal for one of the ring methyl protons was shifted down-field to approximately 4.64 ppm, which is in agreement with hydroxylation at this position. Therefore, the data are consistent with the identification of this metabolite as a monohydroxylated, glucuronidated metabolite of GEM. The position of the glucuronide moiety was determined by HMBC analysis, which revealed that the attachment site was at the 1 position (the carbonyl carbon was found to be coupled to the protons on both of the GEM dimethyl groups and the anomeric proton of the glucuronide’s anomeric proton (e.g., see data described for the identification of the 1-O-GlcUA, 5’-COOH-GEM metabolite described below)].

Identification of 1-O-GlcUA, 5’-COOH-GEM. The predominant ion detected with APCI/MS analysis of this metabolite was at m/z 455, which is consistent with a glucuronidated metabolite of GEM that had undergone complete oxidation of one of the ring methyl groups to the carboxylic acid (Fig. 3). The detection of fragment ions at m/z 279 and 151 also supports this tentative identification.

Proton NMR analysis revealed the absence of one of the ring methyl groups, suggesting complete oxidation to CO₂H, and the presence of an anomeric proton from a glucuronic acid conjugate (Table 2). The presence of all aromatic protons indicated that the aromatic ring was not substituted further. Two-dimensional NMR experiments (HMBC) confirmed that the conjugation had occurred at the 1-O position. Specifically, the protons of the geminal dimethyl groups and the anomeric proton of the glucuronide correlated to the...
same carbonyl, thereby indicating the acyl glucuronide was at the 1-O position (Fig. 9). The singlet aromatic proton at position 6' correlated to the benzylic carbonyl group. Therefore, the benzylic carbonyl groups was shown to be located at the 5' position. This conclusion was confirmed by the correlation of the proton at 3' to the intact aromatic methyl group (the 3'-H assigned by correlation to the oxygenated quaternary carbon atom at the 1' position). Further confirmation of this structure was afforded by enzyme and base hydrolysis studies (Table 3). This metabolite was cleaved by base and E. coli β-glucuronidase, supporting the acyl (ester) linkage.

Identification of 5'-COO-GlcUA–GEM. Similar to the APCI/MS spectrum recorded for the 1-O-GlcUA, 5'-COOH-GEM metabolite described above, LC/MS analysis of this metabolite also resulted in the detection of an ion at m/z 455 (Table 1). This metabolite also produced fragment ions at m/z 279 and 151, which could be explained by the oxidation of a ring methyl group to a carboxylic acid (Fig. 3).
As was seen with the previously described GEM metabolite, proton NMR analysis revealed the absence of one of the ring methyl groups and the presence of an anomic proton from a GlcUA conjugate (Table 2). One aromatic methyl group again was absent, suggesting complete oxidation to CO₂H. HMBC analysis revealed that the geminal dimethyl protons and the glucuronide anomic proton did not correlate to the same carbonyl. Instead, the nonoxidized aromatic methyl and the 3’ proton correlated to an oxygenated quaternary carbon, and the 4’ and 5’ protons correlated to a benzylic carbonyl. Therefore, the HMBC spectrum of this metabolite indicated that the glucuronidation had occurred at the ring methyl group, specifically the 5’ position (Fig. 10). This metabolite also was cleaved by base, supporting the presence of an ester linkage between the xenobiotic and the glucuronide.

**Identification of 1-O-GlcUA, 4’-OH-GEM.** A weak ion at m/z 441 was detected upon APCI/MS analysis of this metabolite (Table 1), which, as has been described previously, is a predicted mass for a monohydroxylated metabolite of GEM that has been conjugated with glucuronic acid. MS/MS analysis of this ion resulted in the production of daughter ion at m/z 265, indicating further the presence of a glucuronidated, monohydroxylated GEM metabolite. MS/MS of the ion at m/z 265 resulted in the production of a daughter ion at m/z 137, indicating that the hydroxylation had occurred on the ring system or the ring methyl groups of GEM and not on the alkyl side chain.

Proton NMR indicated the presence of a glucuronide (anomeric proton detected at 5.46 ppm), the absence of an aromatic proton, and the presence of both aromatic methyl groups (Table 2). Two-dimensional HMBC analysis showed that both aromatic methyl groups correlated to an oxygenated quaternary carbon. In addition, the two remaining aromatic protons also correlated to an oxygenated quaternary carbon. Together, this symmetry indicates that the metabolite is 4’-substituted. Finally, the HMBC data indicated that the anomic proton of the glucuronide and the geminal dimethyl group protons correlated to the same carbonyl, indicating that this metabolite was also an acyl glucuronide (data not shown). In support of this conclusion, treatment of this metabolite with either base or β-gluc-
uronidase (albeit the glucuronidase cleavage did not go to completion) resulted in the cleavage of the metabolite (Table 3).

Identification of 4'-O-GlcUA-GEM. APCI/MS analysis of this metabolite resulted in a spectrum with an apparent M-H ion at m/z 441 and a base ion at m/z 265 (Table 1). Again, a molecular weight of 442 would be consistent with a monohydroxylated GEM glucuronide. MS/MS analysis of the M-H ion at 441 resulted in the detection of a fragment ion at m/z 265, indicating a glucuronide conjugate of a GEM metabolite hydroxylated on the ring or on the ring methyl groups (Fig. 3).

The 1H NMR spectrum recorded for this metabolite confirmed the presence of a glucuronide (anomeric proton detected as a doublet at 4.89 ppm, which is shifted considerably from the anomeric protons of 1-O-GlcUA metabolites; see Table 2). Because one of the aromatic ring protons was absent, this spectrum also indicated that oxidation of the aromatic ring had occurred. Both aromatic methyl groups were seen to be intact. HMBC analysis showed a correlation of both methyl groups to an oxygenated quaternary carbon, as well as a correlation of the remaining aromatic protons to an oxygenated quaternary carbon. This symmetry demonstrates that the hydroxyl group resides at the 4' position. The position of the glucuronide was not revealed conclusively with the HMBC data. However, β-glucuronidase from E. coli cleaved this metabolite, whereas base did not (Table 3), indicating that the glucuronide linkage was via a 4’ ether.

Discussion

In these studies, the combination of APCI/MS and microprobe NMR techniques facilitated the identification of metabolites whose lability or nonvolatility previously would have made their structural elucidation much more intractable. In previous studies by Okerholm (1976) and others (Nakagawa et al., 1991; Okerholm et al., 1976; Sallustio and Fairchild, 1995), only the 1-O-acyl glucuronide and the hydrolyzed, nonconjugated metabolites of GEM could be determined. However, in these studies the careful collection, isolation, and analysis of GEM metabolites in rat urine allowed the identification of several novel metabolites, as well as the direct, structural determina-
tion of glucuronidated metabolites other than the 1-O-acyl glucuronide. In particular, the inclusion of citric acid in the urine-collection vessels of the metabolic chambers and the use of TFA in the HPLC purification procedures proved sufficient to stabilize the wide variety of acyl glucuronides present. Although the TFA led to the production of adduct ions with glucuronides in the APCI analyses (TFA$^+$GlcUA$^-$GEM), it was possible to remove most of this acid immediately before MS analysis and still retain the metabolites with their glucuronide conjugates intact. In this way it was possible to use negative-ionization techniques for detection and identification of all of the GEM metabolites reported herein.

Although the 1-O-GlcUA of GEM was the predominant metabolite isolated in rat urine collected between 24 and 48 h postdosing, other metabolites of GEM were also present in considerable concentrations. Their rank order of abundance for this collection interval as determined by their approximate proportion of total radioactivity, in decreasing order, was found to be 1-O-GlcUA-GEM > 1-O-GlcUA, 4'-OH-GEM > 2',5'-CH$_2$OH-GEM > 2'-CH$_2$OH, 5'-COOH-GEM = 1-O-GlcUA, 5'-COOH-GEM = 5'-COO-GlcUA-GEM > 4'-O-SO$_3$-GEM = 4'-O-GlcUA-GEM = 1-O-GlcUA, 5'-CH$_2$OH-GEM > GEM (see Fig. 2 and Dix et al., 1999). The presence of the parent compound at this time point indicates that some GEM is still being excreted unchanged. Whether this is a result of its extended presence in plasma due to enterohepatic recirculation, conjugate hydrolysis, or the direct excretion of nonmetabolized parent compound is unknown.

Novel metabolites of GEM that were identified in these studies include the 2',5'-CH$_2$OH-GEM and the 4'-O-SO$_3$-GEM. The identification of acyl glucuronides at ring carbonyl groups, as well as the identification of ether glucuronides at the 4'-OH position, also represent previously unreported metabolic products of GEM. The relevance of these novel metabolites, particularly with regard to their presence or absence in the metabolic profile of GEM in humans, remains to be determined. However, because of the hypothesis of Sallustio et al.

**Fig. 10.** HMBC spectrum recorded for the isolated metabolite believed to be the 5'-COO-GlcUA-GEM.

The correlation of the protons to the extrapolated carbon data is shown by the arrows and by the highlighted bonds in the metabolites' structure provided within the figure (compare with Fig. 4).
(1997) that the 1-O-acyl glucuronide of GEM can form adducts with proteins and DNA, and that this may represent a mechanism of mutagenesis, the identification of numerous acyl glucuronide metabolites other than the 1-O-acyl glucuronide indicates that the number of potentially adduct-forming metabolites of GEM is much greater than previously thought. This observation also leads to the conclusion that the number of metabolites of GEM that can undergo enterohpatic recirculation is similarly increased.

As mentioned above, acyl glucuronides of GEM are highly reactive species that can undergo nonenzymatic reactions with the hydroxyl groups on the glucuronic acid moiety resulting in intramolecular rearrangement and migration of the GEM moiety to positions 2, 3, and 4 of the glucuronic acid ring (Sallustio and Foster, 1995). The process of intramolecular ring migration allows opening of the glucuronic acid ring, forming an open-chain conjugate with a free aldehyde group. These open-ring aldehyde forms are able to covalently bind to N-terminal groups or nucleophilic lysines on proteins as well as DNA residues. In this case, the entire conjugate is covalently bound to the protein or nucleic acid. Alternatively, acyl glucuronides of GEM can bind covalently to proteins through a simple nucleophilic attack of a protein functional group (NH2, SH, or OH) at the 1-O-β-GlcUA moiety or its rearrangement isomers. In this mechanism, only the xenobiotic is left bound to the protein (Sallustio et al., 1997).

Whereas O-glucuronides (either ether or ester linkages) are cleaved by β-glucuronidase, acyl migration of the conjugate moiety leads to the formation of β-glucuronidase-resistant glucuronides, a phenomenon that was first described for clofibrate by Faed and McQueen (1978). These ring-migrated conjugates are, however, labile to alkaline hydrolysis, as are 1-O-β-glucuronides. The rates of acyl migration and hydrolysis are different for different molecules (Spahn-Langguth and Benet, 1992) and thus may be different between the 1-O-GlcUA, 5'-COOH-GEM and the 5'-COO-GlcUA-GEM. Glucuronide stability is also dependent on pH, temperature, and even the composition of the medium. Although it is known that cold-acidic conditions such as those used in this study are suitable to stabilize most acyl glucuronides, the hydrolysis data provided in Table 2 must be interpreted cautiously, particularly because only a negative (heat-deactivated) control was performed during these studies.

Despite their reactive nature, acyl glucuronides are stable enough to circulate in plasma, and covalently bound protein adducts of GEM have been detected in rat kidney, liver, and heart (Sallustio and Foster, 1995). It appears that by covalently binding to proteins, these metabolites may be able to function as haptens, resulting in hypersensitivity reactions. It is also believed that the formation of adducts with other organ macromolecules can produce additional cellular dysfunctions, possibly including cancer. It has not been possible to discern whether all adducts were being formed primarily by the 1-O-acyl glucuronide of GEM or other metabolites. However, our studies demonstrate that there are several forms of glucuronides that are formed from GEM, many of which would be anticipated to be as reactive as the 1-O-β-GlcUA-GEM, particularly because many additional acyl glucuronides were identified, a form of glucuronide conjugate that has been reported to be more chemically reactive than other forms of Phase II conjugates (Sallustio et al., 1997).

Does the identification of these novel metabolites of GEM in rat urine provide any further explanation for the species-specific toxicity seen in rats but not in hamsters or humans? Experimentation comparing the DNA-adducting properties of these other acyl glucuronides needs to be performed to determine whether specific metabolites are as reactive (or more so) as the 1-O-acyl glucuronide. It seems more likely that the renal elimination of GEM conjugates that predominates in hamsters (Coleman et al., 1997; Dix et al., 1999) allows a greater percentage of a given GEM dose to be eliminated without reabsorption, and that this may be the greatest contributor to these species’ differences in GEM hepatotoxicity. Although it is true that, compared with hamsters, rats excrete less GEM and its metabolites via the urine, it is still an important metabolic pathway for GEM removal. Indeed, at low doses the clearance of GEM and GEM metabolites in the urine was only 30%, but at the high dose (2000 mg/kg) the clearance of GEM-derived radioactivity increased to approximately 55 to 70% (K.J.D. et al., submitted). Therefore, other considerations, including the target proteins that serve as substrates for adduct formation, the rate of formation of the protein adducts, and the rate of the repair of the adducts also may be critical determinants of GEM sensitivity.

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