Acetylsalicylic acid (aspirin) is a common nonsteroidal anti-inflammatory drug used for treatment of pain and arthritis. In the body, acetylsalicylic acid is rapidly deacetylated to form salicylic acid. Both compounds have been proposed as anti-inflammatory agents. Major metabolites of salicylic acid are its acyl and phenolic glucuronide conjugates. Formation of these conjugates, catalyzed by UDP-glucuronosyltransferases (UGTs), decreases the amount of pharmacologically active salicylic acid present. We aimed to identify the UGTs catalyzing the glucuronidation of salicylic acid using both heterologously expressed enzymes and pooled human liver microsomes (HLMs) and to develop a liquid chromatography-tandem mass spectrometry method to quantify glucuronidation activity of UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes. All UGTs tested, except 1A4, 2B15, and 2B17, catalyzed salicylic acid phenolic and acyl glucuronidation. Ratios of salicylic acid phenolic to acyl glucuronide formation varied more than 12-fold from 0.5 for UGT1A6 to 6.1 for UGT1A1. These results suggest that all UGTs except 1A4, 2B15, and 2B17 might be involved in the glucuronidation of salicylic acid in vivo. From comparisons of apparent $K_m$ values determined in pooled HLMs and in expressed UGTs, UGT2B7 was suggested as a likely catalyst of salicylic acid acyl glucuronidation, whereas multiple UGTs were suggested as catalysts of phenolic glucuronidation. The results of this UGT screening may help target future evaluation of the effects of UGT polymorphisms on response to aspirin in clinical and population-based studies.

Abbreviations: SAPG, salicylic acid phenolic glucuronide; ESI, electrospray ionization; HPLC, high performance liquid chromatography; HLM, human liver microsome; LC, liquid chromatography; MS/MS, tandem mass spectrometry; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; SAAG, salicylic acid acyl glucuronide; UGT, UDP-glucuronosyltransferase.
Materials and Methods

Materials. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated and were of the highest grade commercially available. UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 Supersomes and pooled human liver microsomes (HLMs) were purchased from BD Gentest (Bedford, MA). A crude solution of β-glucuronidase from Helix pomatia was used (134,400 units/ml).

Preparation of SAPG and SAAG Standards. Phenolic and acyl glucuronides of salicylic acid were isolated from urinary metabolites from individuals dosed with 650 mg of aspirin. The Institutional Review Board of the Fred Hutchinson Cancer Research Center approved the study, and informed written consent was obtained from each participant. The pH of urinary aliquots was adjusted to 3 to 4 using concentrated phosphoric acid. Urinary metabolites were concentrated using C18 SepPak SPE cartridges (Waters, Milford, MA) and eluted with increasing concentrations of 0.1% trifluoroacetic acid (TFA) in methanol. Eluates were dried under nitrogen and resuspended in water. Eluate fractions were purified by high performance liquid chromatography (HPLC) before and after treatment with 1340 units of β-glucuronidase or 2 M NaOH at 37°C in a shaking water bath for 1 h. Although β-glucuronidase cleaved both SAPG and SAAG, base treatment cleaved only SAAG. Treated aliquots were separated on a Waters Nova-Pak C18 column (5 μm, 3.9 × 300 mm) using a gradient of 0.1% TFA (A) and acetonitrile (B), as follows: 100% A held for 10 min, to 40% B at 40 min. The HPLC system consisted of an HP 1050 solvent delivery pump (Hewlett Packard, Palo Alto, CA), an HP 1050 autosampler, and an HP 1050 photodiode array detector monitoring at wavelength 235 nm. SAPG and SAAG eluted at 19 and 28 min, respectively. SAPG and SAAG were purified, and the eluate was dried under nitrogen and resuspended in nanopure water. Aliquots of the purified glucuronide standards were treated with β-glucuronidase, and concentrations were determined by HPLC analysis of the salicylic acid released. Residual glucuronide peaks were not detected by HPLC-UV after treatment with β-glucuronidase or base. Mass spectrometry and MS/MS analysis was used to confirm the identification of the SAPG and SAAG standards.

LC-MS/MS Analysis of Salicylic Acid Glucuronides. The HPLC system consisted of two Shimadzu LC-10AD pumps (Shimadzu Scientific Instruments Inc., Columbia, MD) with a gradient controller and a Shimadzu SIL-10ADvp autoinjector or a Waters Alliance 2695 Separation Module. Glucuronides were eluted from a Phenomenex (Torrance, CA) Luna C5 column (100 × 2 mm, 5 μm) isocratic at 70% TFA (0.05%)/30% acetonitrile at a flow rate of 0.15 ml/min. Ammonium hydroxide (7%) was added postcolumn at a rate of 1 μl/min. Glucuronides were detected by MS/MS analysis using a Micromass Quattro II Mass Spectrometer or a Micromass Quattro Micro Mass Spectrometer (Waters) with electrospray ionization (ESI) in negative ion mode. We monitored the release of salicylic acid (m/z 137) from SAPG or SAAG (m/z 313) after the neutral loss of the sugar moiety, 176. ESI source conditions on the Quattro II Mass Spectrometer were set as follows: capillary voltage, 3.5 kV; cone voltage, −30 eV; collision energy, 15; source block temperature, 100°C; and desolvation temperature, 250°C. ESI source conditions on the Quatro Micro Mass Spectrometer were set as follows: capillary voltage, 3.5 kV; cone voltage, 20 V; collision energy, 10; source temperature, 120°C; desolvation temperature, 350°C, desolvation gas, 700 l/h. Instrument response when analyzing salicylic acid glucuronides was more than 5000 units/mg of alamethicin/mg of HLM protein, 10 to 5000 μM salicylic acid, 2 mM UDP-glucuronic acid, 1 mM PMSF, 8.5 mM saccharolactone, and 10 mM MgCl2 in 50 mM potassium phosphate (pH 7). Assays were allowed to progress for 30 min before being terminated with equal volumes of ice-cold methanol. Mixtures were vortexed and centrifuged at 13,000g for 5 min. Supernatants were transferred to vials for analysis by LC-MS/MS as described above.

Glucuronidation of Salicylic Acid by Pooled HLMs. Reactions were carried out with 1 mg/ml protein, 50 μg of alamethicin/mg of HLM protein, 10 to 5000 μM salicylic acid, 2 mM UDP-glucuronic acid, 1 mM PMSF, 8.5 mM saccharolactone, and 10 mM MgCl2 in 50 mM potassium phosphate (pH 7). Assays were allowed to progress for 1 h at 37°C in a shaking water bath and terminated with equal volumes of methanol. Mixtures were vortexed and centrifuged at 13,000g for 5 min. Supernatants were transferred to fresh tubes and prepared for LC-MS/MS analysis.

Data Handling and Analysis. All rates of salicylic acid glucuronidation were determined in duplicate and reported as the mean of these determinations, which varied by less than 10%. Duplicates discrepant by more than 10% were rerun. Glucuronidation rates at increasing concentrations of salicylic acid were fit to the Michaelis-Menten equation using GraphPad Prism 3.03 software (GraphPad Software Inc., San Diego, CA).

Results

Glucuronidation activity catalyzed by UGTs 1A6 and 1A9 was initially measured at increasing pH from 5.5 to 7.5 to optimize conditions for screening the remaining UGTs. The pH profiles of phenolic glucuronidation were similar with both UGTs 1A6 and 1A9 (Fig. 1A). Maximal acyl glucuronidation activity by UGT1A6 and
expressed UGT Supersomes were carried out for 120 min, whereas reactions containing pooled HLMs were carried out for 30 min.

K had the highest affinity for salicylic acid phenolic glucuronidation with a $K_m$ of 78 $\mu$M. However, the $K_m$ for salicylic acid acyl glucuronidation in HLM was 1720 $\mu$M, which was closer to the $K_m$ value determined for acyl glucuronidation by UGT2B7. These results suggest that multiple UGT enzymes expressed in human liver are involved in both phenolic and acyl glucuronidation.

FIG. 2. Rates of glucuronidation catalyzed by UGT Supersomes at pH 6 with 500 $\mu$M salicylic acid. Rates of glucuronidation were not adjusted for the amount of expressed enzyme in each preparation.

All UGTs, except 1A4, 2B15, and 2B17, formed acyl and phenolic glucuronides of salicylic acid at detectable levels (Fig. 2). UGT1A9 had the highest rate of both SAPG and SAAG formation: 105 and 19 pmol/min/mg protein, respectively; rates of salicylic acid glucuronidation by the remaining UGTs were significantly lower. Rates of phenolic glucuronidation in pooled HLMs were 5-fold to 17-fold higher than acyl glucuronidation rates, and the ratio of SAPG to SAAG decreased as the concentration of salicylic acid in reaction mixtures increased.

The ratios of salicylic acid phenolic and acyl glucuronides formed were enzyme-specific (Fig. 2). The ratio of SAPG to SAAG formation varied more than 12-fold from 0.5 for UGT1A6 to 6.1 for UGT1A1. UGTs 1A1, 1A3, 1A8, 1A9, 1A10, and 2B4 catalyzed phenolic glucuronidation at faster rates and UGTs 1A6 and 2B7 catalyzed acyl glucuronidation at faster rates.

Apparent kinetic parameters were determined for salicylic acid acyl and phenolic glucuronidation catalyzed by all UGTs except 1A4, 2B15, and 2B17. Rates of glucuronidation were fit to Michaelis-Menten kinetics and are reported in Table 1. Except for the acyl glucuronidation for UGT1A1 and UGT1A9, acyl and phenolic glucuronide formation fit Michaelis-Menten-type kinetics. UGT1A6 had the highest affinity for salicylic acid phenolic glucuronidation with a $K_m$ of 40.7 $\mu$M. However, this affinity was more than 8-fold higher than that determined for HLMs, suggesting that a combination of enzymes expressed in liver is responsible for phenolic glucuronidation. UGT1A6 also had the highest affinity for acyl glucuronidation with a $K_m$ of 78 $\mu$M. However, the $K_m$ for salicylic acid acyl glucuronidation in HLM was 1720 $\mu$M, which was closer to the $K_m$ value determined for acyl glucuronidation by UGT2B7. These results suggest that multiple UGT enzymes expressed in human liver are involved in both phenolic and acyl glucuronidation.

**Discussion**

All UGT Supersomes except 1A4, 2B15, and 2B17 catalyzed the formation of SAPG and SAAG at rates and ratios that varied substantially by UGT enzyme. UGT1A9 had the highest rate of salicylic acid phenolic and acyl glucuronidation, suggesting a role for this enzyme in in vivo metabolism. Affinity measurements in HLMs also suggest that UGT2B7 may be an important catalyst of salicylic acid acyl glucuronidation, and comparison of HLMs and expressed UGTs suggests that a combination of enzymes is probably involved in salicylic acid phenolic glucuronidation in liver. We were unable to adjust glucuronidation rates for the amount of active UGT enzyme in each Supersome preparation because of the lack of a common substrate or antibody to carry out these measurements. Thus, given our results, we can conclude only that all UGT enzymes except UGTs 1A4, 2B15, and 2B17 are potential catalysts of salicylic acid phenolic and acyl glucuronidation.

With increasing concentrations of salicylic acid, the ratios of SAPG to SAAG formed in pooled HLMs varied between 10 and 22. These results demonstrated that, within the context of the present assay conditions, hepatic UGT enzymes responsible for salicylic acid conjugation preferentially formed the phenolic glucuronide. However, the ratio of SAPG to SAAG formed by expressed UGTs varied from 0.5 to 6.1 by UGTs 1A6 and 1A1, respectively. Several differences between the UGT Supersomes and UGTs in pooled HLMs limit our ability to compare directly between the systems. Differences in membrane environment between the artificial Supersome expression system and pooled HLMs and differences in optimal assay conditions used may play an important role in determining substrate affinity and product formation. Even if we had tested the HLMs and UGTs at the same pH, it is very unlikely that the activities would be similar because of activity differences in the different UGTs at a specific pH (Fig. 1) and differences in access of salicylic acid to the UGTs in the two systems. Furthermore, because of protein overexpression in Supersomes as well as the other cellular differences, the specificity of the enzymes is not likely to be the same between Sf9 Supersomes and HLMs.

**TABLE 1**

Kinetics of salicylic acid glucuronidation by selected UGT Supersomes and pooled human liver microsomes

<table>
<thead>
<tr>
<th>UGT</th>
<th>Apparent $K_m$ (pmol/min/mg protein)</th>
<th>Apparent $V_{max}$ (pmol/min/mg protein)</th>
<th>$V_{max}/K_m \times 10^{-2}$</th>
<th>Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>683.1 (77.8)</td>
<td>52.7 (2.0)</td>
<td>7.7</td>
<td>0.99</td>
</tr>
<tr>
<td>1A3</td>
<td>2337 (326.4)</td>
<td>29.8 (1.8)</td>
<td>1.3</td>
<td>0.99</td>
</tr>
<tr>
<td>1A6</td>
<td>40.7 (9.8)</td>
<td>1.9 (0.1)</td>
<td>4.7</td>
<td>0.85</td>
</tr>
<tr>
<td>1A7</td>
<td>278.6 (39.9)</td>
<td>14.3 (0.7)</td>
<td>5.1</td>
<td>0.98</td>
</tr>
</tbody>
</table>
| 1A8 | N.A.                                | N.A.                                   | N.A.                        | N.A.
| 1A9 | 94.2 (18.8)                         | 149.5 (6.7)                            | 158.7                       | 0.94|
| 1A10| 698.2 (59.1)                        | 8.8 (0.2)                              | 1.3                         | 0.99|
| 2B4 | 1242 (252.9)                        | 28.4 (2.2)                             | 2.3                         | 0.97|
| 2B7 | 345.5 (81.4)                        | 68.7 (4.5)                             | 19.9                        | 0.96|
| HLMs| 345.5 (81.4)                        | 68.7 (4.5)                             | 19.9                        | 0.96|

N.A., not available (rates of glucuronidation were not detected below 500 $\mu$M concentrations of salicylic acid).

Results are reported as value (standard error) as determined by Prism 3.03 software. Reactions were carried out under the following conditions: 1 mg/ml protein, 2 mM UDP-glucuronic acid, 1 mM PMSF, 8.5 mM saccharolactone, and 10 mM MgCl$_2$ in 50 mM potassium phosphate (pH 7) with 10 to 5000 $\mu$M salicylic acid. Reactions with expressed UGT Supersomes were carried out for 120 min, whereas reactions containing pooled HLMs were carried out for 30 min.
pooled HLMs. The expressed UGT data support the role of many of the UGTs in salicylic acid glucuronidation, but they do not inform specifically as to the hepatic UGTs involved.

In vivo, urinary concentrations and ratios of SAPG and SAAG vary widely among individuals (Hutt et al., 1986; J. W. Lampe, J. D. Potter, J. Bigler, and G. E. Kuehl, unpublished data). Results of several studies (Ciotti et al., 1997; Bigler et al., 2001; Chan et al., 2005) suggest that UGT1A6 might be an important catalyst of salicylic acid glucuronidation and that UGT1A6 polymorphisms influence the process. Our present results indicate that although UGT1A6 is capable of catalyzing the glucuronidation of salicylic acid, it is unlikely to be the only catalyst. In vivo, depending on oral aspirin dose and assuming an intermediate hepatic extraction ratio of 0.5 (Rowland, 1978), hepatic portal vein salicylic acid concentrations could range from approximately 30 μM with a single 80-mg dose (baby aspirin) to 2800 μM with a chronic dose of 1300 mg q.i.d. (Benedek et al., 1995; Liu and Smith, 1996). Consequently, the binding affinities (Table 1) are likely within the range of physiologically relevant concentrations and do not rule out further the role of any of the UGTs studied. This would suggest that the effects of other UGT polymorphisms on the glucuronidation of salicylic acid warrant investigation.

In conclusion, in this screening study, we observed that UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, and 2B7 catalyzed the formation of SAPG and SAAG. Metabolic studies comparing genotypes and glucuronidation profiles using microsomes from human tissues, including liver and colon, may help to resolve the discrepancy between in vivo and in vitro studies.

References


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