

**Glucuronidation of the Aspirin Metabolite Salicylic Acid  
by Expressed UGTs and Human Liver Microsomes**

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**ABBREVIATIONS:** ESI, electrospray ionization; HPLC, high performance liquid chromatography; HLM, human liver microsomes; LC, liquid chromatography; MS, mass spectrometry; NSAID, nonsteroidal anti-inflammatory drug; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; SAAG, salicylic acid acyl glucuronide; SAPG, salicylic acid phenolic glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.

## ABSTRACT

Acetylsalicylic acid (aspirin) is a common nonsteroidal anti-inflammatory drug (NSAID) 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 (HLM) and to develop a liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method to quantify glucuronidation activity of UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 Supersomes<sup>TM</sup>. 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 HLM and in expressed UGTs, UGT2B7 was suggested as a likely catalyst of salicylic acid acyl glucuronidation while multiple UGTs were suggested as catalysts of phenolic glucuronidation. The results of this UGT screening may help target future evaluation of effects of UGT polymorphisms on response to aspirin in clinical and population-based studies.

## INTRODUCTION

Acetylsalicylic acid (aspirin) is a common nonsteroidal anti-inflammatory drug (NSAID) used in the treatment of pain and inflammation and in the secondary prevention of cardiovascular disease. Several studies have suggested that aspirin may be an effective chemopreventive agent against cancer, especially colorectal cancer [for review see (Baron and Sandler, 2000)].

The rate of elimination of aspirin and its metabolite salicylic acid is likely a major factor determining drug efficacy among users. In the body, aspirin is rapidly de-acetylated to form salicylic acid (Hutt et al., 1986). Salicylic acid is further metabolized by conjugation, with either glycine or glucuronic acid, or by oxidation. The glycine conjugate, salicyluric acid, is the most abundant metabolite in the urine of individuals dosed with aspirin. However, the glucuronide conjugates of salicylic acid, salicylic acid phenolic glucuronide (SAPG) and salicylic acid acyl glucuronide (SAAG), account for between 0.8% and 42% of the administered aspirin dose (Hutt et al., 1986). The wide range in urinary levels of SAPG, SAAG, and salicyluric acid among individuals dosed with aspirin may, in part, explain differences in therapeutic response within a population.

Glucuronidation activity may be a critical determinant in aspirin efficacy; however, the enzymes catalyzing this conjugation have yet to be identified and characterized. Polymorphisms in many of the UGT enzymes have been identified (Ciotti et al., 1997; Lévesque et al., 1997; Lampe et al., 1999; Lévesque et al., 1999; Lampe et al., 2000; Balram et al., 2002; Gagné et al., 2002; Huang et al., 2002; Villeneuve et al., 2003; Ehmer et al., 2004; Iwai et al., 2004; Verlaan et al., 2004; Yamanaka et al., 2004). Therefore, variability in glucuronidation activity among aspirin users is probably a result of specific expression levels and/or functional polymorphisms present in the UGTs catalyzing the conjugation of salicylic acid. Among aspirin users, carrying

a UGT1A6 variant allele has been associated with decreased risk of colorectal adenoma (Bigler et al., 2001; Chan et al., 2005). Consistent with these findings, Ciotti and coworkers demonstrated that UGT1A6 was able to catalyze the glucuronidation of salicylic acid, with expressed UGT1A6\*2 demonstrating a 2-fold lower salicylic acid glucuronidation compared with UGT1A6\*1 (Ciotti et al., 1997).

We aimed to identify the UGT enzymes catalyzing the glucuronidation of salicylic acid. We measured the ability of individually expressed UGTs to catalyze the glucuronidation of salicylic acid using a liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method and measured the apparent kinetic parameters of salicylic acid glucuronidation catalyzed by selected UGTs. This is the first study to systematically screen all UGTs for salicylic acid glucuronidation activity and to identify the role of individual UGTs in the catalysis of glucuronidation in human liver microsomes.

## 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, 2B17 Supersomes<sup>TM</sup> and pooled human liver microsomes (HLM) were purchased from BD Biosciences Gentest (Bedford, MA). A crude solution of  $\beta$ -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 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 3-4 using concentrated phosphoric acid. Urinary metabolites were concentrated using C18 SepPaks SPE cartridges (Waters Corporation, Beverly, 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  $\beta$ -glucuronidase or 2 M NaOH at 37° C in a shaking water bath for 1 hour. Although  $\beta$ -glucuronidase cleaved both SAPG and SAAG, base treatment cleaved only SAAG. Treated aliquots were separated on a Waters Nova-Pak C18 column (5  $\mu$ m, 3.9 x 300 mm) using a gradient of 0.1% TFA, A, and acetonitrile, B, as follows: 100% A held for 10 minutes, to 40% B at 40 minutes. The HPLC system consisted of an HP 1050 solvent delivery pump, an HP 1050 autosampler, and an HP 1050 photodiode array detector monitoring at wavelength 235 nm. SAPG and SAAG eluted at 19 and 28 minutes, respectively. SAPG and SAAG were purified, the eluate dried under nitrogen, and resuspended in nanopure water. Aliquots of the purified glucuronide standards were treated with  $\beta$ -glucuronidase and concentrations determined by HPLC analysis of the salicylic acid released. Residual glucuronide peaks were not detected by HPLC-UV after treatment with  $\beta$ -glucuronidase or base. MS 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 with a gradient controller and a Shimadzu SIL-10ADvp autoinjector or a Waters Alliance 2695 Separation Module. Glucuronides were eluted from a Phenomenex (Torrence, CA) Luna C<sub>5</sub> column (100 x 2 mm, 5  $\mu$ m) isocratic at 70% TFA (0.05%)/30%

acetonitrile at a flow rate of 0.15 ml/min. Ammonium hydroxide (7%) was added post-column at a rate of 1  $\mu$ l/min. Glucuronides were detected by MS/MS analysis using a Micromass Quattro II Mass Spectrometer or a Micromass Quattro Micro Mass Spectrometer (Waters Corporation, Milford, MA) 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) following 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 Quattro 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/hr. Instrument response for the glucuronide concentrations examined was linear over the range of 0.2 – 50 ng. Rates of glucuronidation were determined based on comparison with standard curves. SAPG and SAAG eluted at 4 and 7 minutes, respectively. Instrument response when analyzing salicylic acid glucuronides was more than 7-fold higher with SAAG than SAPG at the same concentrations. Samples were spiked with equal amounts of glucuronide standard and re-analyzed to confirm the accuracy of quantification.

*Screening UGT Supersomes<sup>TM</sup> for Salicylic Acid Glucuronidation Activity:* Glucuronidation activity by UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 Supersomes<sup>TM</sup> was measured. The assay conditions were as follows: 0.05-2 mg/ml protein, 50  $\mu$ g alamethicin/mg protein, 2 mM UDP-glucuronic acid, 500  $\mu$ M salicylic acid, and 10 mM  $MgCl_2$  in 50 mM potassium phosphate (pH 6). Reactions were carried out for 2 hours in a shaking water bath at 37° C before being terminated with equal volumes of methanol. Samples

were vortexed and centrifuged at 13,000 x g for 5 minutes. The supernatants were removed, dried under nitrogen, and the dried supernatant was resuspended in nanopure water before glucuronides were quantified by LC-MS/MS. Rates of salicylic acid glucuronidation were linear from 30 to 120 minutes over the protein-concentration range indicated previously. To examine the pH-dependence of salicylic acid glucuronidation by UGT1A6 and UGT1A9, reactions were carried out in 50 mM potassium phosphate (pH 5.5 – 7.5).

*Kinetics of Salicylic Acid Glucuronidation by Selected UGTs:* Rates of salicylic acid glucuronidation were measured as described above except that 1 mM phenylmethylsulfonyl fluoride (PMSF) and 50 mM potassium phosphate (pH 7) and 10-5000  $\mu$ M concentrations of salicylic acid were used. Reactions were allowed to progress for 30 minutes before being terminated with equal volumes of ice-cold methanol. Mixtures were vortexed and centrifuged at 13,000 x g for 5 minutes. Supernatants were transferred to vials for analysis by LC-MS/MS as described above.

*Glucuronidation of Salicylic Acid by Pooled HLM:* Reactions were carried out with 1 mg/ml protein, 50  $\mu$ g alamethicin/mg HLM protein, 10-5000  $\mu$ M salicylic acid, 2 mM UDP-glucuronic acid, 1 mM PMSF, 8.5 mM saccharolactone, and 10 mM  $\text{MgCl}_2$  in 50 mM potassium phosphate (pH 7). Assays were allowed to progress for 1 hour at 37° C in a shaking water bath and terminated with equal volumes of methanol. Mixtures were vortexed and centrifuged at 13,000 x g for 5 minutes. 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 (Figure 1A). Maximal acyl glucuronidation activity by UGT1A6 and UGT1A9 was at pH 5.5 and pH 6, respectively (Figure 1B). Glucuronidation rates decreased from pH 6 to pH 7.5 for both UGT enzymes investigated. Thus, pH 6 was chosen as the pH at which to screen the UGT Supersomes<sup>TM</sup>.

All UGTs, except 1A4, 2B15, and 2B17, formed acyl and phenolic glucuronides of salicylic acid at detectable levels (Figure 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 HLM were 5-fold to 17-fold higher than acyl glucuronidation 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 (Figure 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 HLM, 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<sup>TM</sup> 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 HLM also suggest that UGT2B7 may be an important catalyst of salicylic acid acyl glucuronidation and comparison of HLM and expressed UGTs suggest 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 Supersomes<sup>TM</sup> 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 HLM 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<sup>TM</sup> and UGTs in pooled HLM limit our ability to compare directly between the systems. Differences in membrane environment between the artificial Supersomes<sup>TM</sup> expression system and pooled HLM 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 same pH, it is very unlikely that the activities would be similar because of activity differences in the different UGTs at a specific pH (figure 1) and differences in access of salicylic acid to the UGTs in the two systems. Further, because of protein overexpression in Supersomes<sup>TM</sup> as well as the other cellular differences, the specificity of the enzymes is not likely to be the same between Sf9 Supersomes<sup>TM</sup> and pooled HLM. The expressed UGT data support a 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; Lampe et al., 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 may 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  $\mu$ M with a single 80 mg dose (baby aspirin) to 2800  $\mu$ M with a chronic dose of 1300 mg qid (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, 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.

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(UGT1A9\*22) and its effects on the transcriptional activity. *Pharmacogenetics* **14**:329-332.



## FOOTNOTES

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## LEGENDS FOR FIGURES

Figure 1. Effect of pH on (A) salicyl-phenolic glucuronidation and (B) salicyl-acyl glucuronidation by UGT1A6 and UGT1A9.

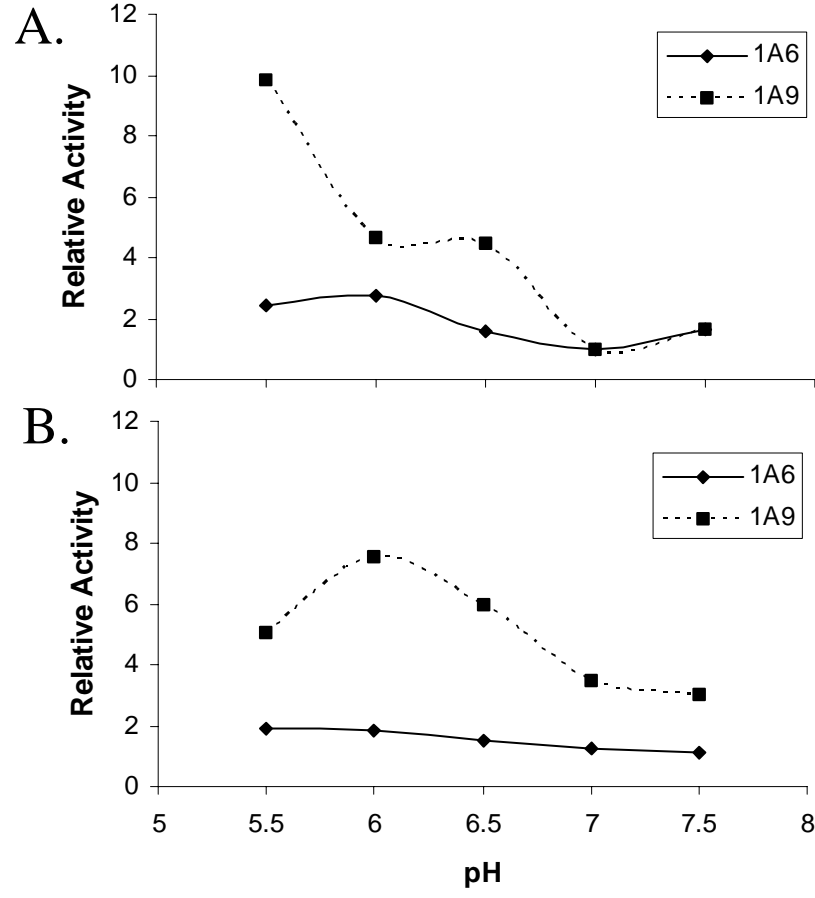
Figure 2. Rates of glucuronidation catalyzed by UGT Supersomes<sup>TM</sup> at pH 6 with 500  $\mu$ M salicylic acid. Rates of glucuronidation were not adjusted for the amount of expressed enzyme in each preparation.

Table 1. Kinetics of Salicylic Acid Glucuronidation by Selected UGT Supersomes<sup>TM</sup> and pooled human liver microsomes.

UGT	Phenolic Glucuronidation				Acyl Glucuronidation			
	Apparent $K_m$ ( $\mu M$ )	Apparent $V_{max}$ ( $pmol/min/mg$ protein)	$V_{max}/K_m \times e^{-2}$ ( $ul/min/mg$ protein)	Fit ( $R^2$ )	Apparent $K_m$ ( $\mu M$ )	Apparent $V_{max}$ ( $pmol/min/mg$ protein)	$V_{max}/K_m \times e^{-2}$ ( $ul/min/mg$ protein)	Fit ( $R^2$ )
1A1	683.1 (77.8)	52.7 (2.0)	7.7	0.99	256.5 (71.5)	2.1 (0.1)	0.8	0.93
1A3	2337 (326.4)	29.8 (1.8)	1.3	0.99	2659 (542.1)	4.7 (0.4)	0.2	0.98
1A6	40.7 (9.8)	1.9 (0.1)	4.7	0.85	78.0 (5.2)	1.8 (0.0)	2.3	0.99
1A7	278.6 (39.9)	14.3 (0.7)	5.1	0.98	373.7 (36.4)	5.4 (0.2)	1.4	0.99
1A8	NA	NA	NA		NA	NA	NA	
1A9	94.2 (18.8)	149.5 (6.7)	158.7	0.94	334.3 (92.8)	31.8 (2.4)	9.5	0.92
1A10	698.2 (59.1)	8.8 (0.2)	1.3	0.99	348.6 (58.1)	1.1 (0.1)	0.3	0.98
2B4	NA	NA	NA		NA	NA	NA	
2B7	1242 (252.9)	28.4 (2.2)	2.3	0.97	1640 (271.4)	18.4 (1.2)	1.1	0.98
HLM	345.5 (81.4)	68.7 (4.5)	19.9	0.96	1720 (173.4)	15.6 (0.6)	0.9	0.99

NA, not available (rates of glucuronidation were not detected below 500  $\mu M$  concentrations of salicylic acid). Reported as value (standard error) as determined by Prism 3.03 software. Reactions were carried out under the following conditions; 1 mg/ml protein, 50  $\mu g$  alamethicin/mg 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 10-5000  $\mu M$  salicylic acid. Reactions with expressed UGT Supersomes<sup>TM</sup> were carried out for 120 minutes while reactions containing pooled HLM were carried out for 30 minutes.

**Figure 1**



**Figure 2**

