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Effect of the β-glucuronidase inhibitor saccharolactone on glucuronidation by human tissue microsomes and recombinant UDP-glucuronosyltransferases

Lauren Oleson and Michael H. Court

Abstract

Glucuronidation studies using microsomes and recombinant uridine diphosphoglucuronosyltransferases (UGTs) can be complicated by the presence of endogenous β-glucuronidases, leading to underestimation of glucuronide formation rates. Saccharolactone is the most frequently used β-glucuronidase inhibitor, although it is not clear whether this reagent should be added routinely to glucuronidation incubations. Here we have determined the effect of saccharolactone on eight different UGT probe activities using pooled human liver microsomes (pHLMs) and recombinant UGTs (rUGTs). Despite the use of buffered incubation solutions, it was necessary to adjust the pH of saccharolactone solutions to avoid effects (enhancement or inhibition) of lowered pH on UGT activity. Saccharolactone at concentrations ranging from 1 to 20 mM did not enhance any of the glucuronidation activities evaluated that could be considered consistent with inhibition of β-glucuronidase. However, for most activities, higher saccharolactone concentrations resulted in a modest degree of inhibition. The greatest inhibitory effect was observed for glucuronidation of 5-hydroxytryptamine and estradiol by pHLMs, with a 35% decrease at 20 mM saccharolactone concentration. Endogenous β-glucuronidase activities were also measured using various human tissue microsomes and rUGTs with estradiol-3-glucuronide and estradiol-17-glucuronide as substrates. Glucuronide hydrolysis was observed for pHLMs, lung microsomes and insect-cell expressed rUGTs, but not for kidney, intestinal or human embryonic kidney HEK293 microsomes. However, the extent of hydrolysis was relatively small, representing only 9–19% of the glucuronide formation rate measured in the same preparations. Consequently, these data do not support the routine inclusion of saccharolactone in glucuronidation incubations. If saccharolactone is used, concentrations should be titrated to achieve activity enhancement without inhibition.

Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Ave, M + V Rm 308, Boston, MA 02111, USA

Lauren Oleson, Michael H. Court

Correspondence: Michael Court, Tufts University School of Medicine, 136 Harrison Ave, M + V Rm 308, Boston, MA 02111, USA.
E-mail: Michael.Court@tufts.edu

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Introduction

Glucuronidation is one of the main conjugation reactions responsible for converting lipophilic xenobiotics and endogenous compounds into metabolites that are more water soluble than the parent compound and are thus more readily excreted in the urine or bile. Conjugation with glucuronic acid is catalysed by uridine diphosphoglucuronosyl transferases (UGTs) (Miners & Mackenzie 1991). To date, at least 16 different isoforms of human UGT have been identified, with different but overlapping substrate specificities (Miners et al 2002). Expression of various UGT isoforms is tissue-dependent in humans: UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, 2B17 are primarily expressed in the liver, whereas UGT1A7, 1A8, and 1A10 are extrahepatic isoforms (Tukey & Strassburg 2000).

Conjugation with glucuronic acid results in the inactivation of many compounds and is not limited to drugs – it also applies to environmentally toxic chemicals, carcinogens, steroid hormones, bile acids and bilirubin (Miners & Mackenzie 1991). UGTs are predominately localized in the smooth endoplasmic reticulum of liver cells, but have also been found in a variety of other organs, including the lung, kidney and gastrointestinal tract (Mulder 1992).

Microsomal glucuronidation studies have shown a wide range in variability between laboratories. One possible explanation for such variation is latency in UGT activity, which is seen in in-vitro assays. Because the active site of the UGT is located in the lumen of the endoplasmic reticulum, a rate-limiting step in the in-vitro glucuronidation reaction is the

transport of substrates, cofactors and products through the intact membrane of the liver microsome (Meech & Mackenzie 1997). In order to achieve maximum enzymatic activity, the membrane barrier must be disrupted in some fashion. In the past, detergents and sonication have been used to disrupt the integrity of the membrane; more recently, the pore-forming peptide, alamethicin, has been used (Fisher et al 2000; Soars et al 2003). Fisher et al (2000) found that microsomes in the presence of alamethicin at a concentration of $50\,\mu g\,mg^{-1}$ microsomal protein yielded a 2–3 times faster conjugation rate than that observed in the absence of alamethicin.

In addition to latency, enzyme-catalysed hydrolysis of the newly formed glucuronide by β-glucuronidase might also affect UGT activity in microsomal incubations. Human β-glucuronidase has been found in all mammalian tissues and body fluids, with the highest activity in kidney, spleen, epididymis, liver, cancer tissue and the gastrointestinal tract, which is distinct from the β-glucuronidase produced by gastrointestinal tract microorganisms (Marsh et al 1952; Levvy & Marsh 1960; Wakabayashi 1970). One-third of the β-glucuronidase found in liver cells is localized in the endoplasmic reticulum; the remaining two-thirds is found in the lysosome (Swank et al 1986). The close proximity of the enzymes responsible for the formation (UGT) and degradation (β-glucuronidase) of the glucuronide has the potential to result in a futile cycle, which would greatly affect the apparent glucuronidation rate of the UGT.

In order to evaluate the effect of β-glucuronidase on glucuronide formation rates, researchers have used different β-glucuronidase inhibitors. The primary β-glucuronidase inhibitor used for in-vitro assays is D-saccharic acid 1,4-lactone (saccharolactone; Figure 1), which was discovered by Levvy in 1952 to competitively inhibit the enzyme (Levvy 1952). Several other β-glucuronidase inhibitors have been identified, including polymeric phosphates of diethystibestrol, dienestrol, hexesterol and benzeterol, and some metals (Cu^{2+}, Ag^{+}, Hg^{2+}) (Wakabayashi 1970). However, these inhibitors are not selective for β-glucuronidase and are not used in routine microsomal glucuronidation assays.

Some investigators routinely add saccharolactone to incubation mixtures, presumably because of previous reports showing enhanced activities with certain substrates. Brunelle and Verbeeck (1993) investigated the effect of saccharolactone on the acyl glucuronidation of diflunisal in rat liver microsomes and found that the maximum rate increased 2-fold with the addition of 4 mM saccharolactone. Follow-up experiments showed similar results both in other microsomal systems (human) (Brunelle & Verbeeck 1996), and in-vivo (rat) (Brunelle & Verbeeck 1997). Other investigators have found similar results, supporting the use of saccharolactone in in-vitro glucuronidation experiments (Gigon & Bickel 1979; Haaz et al 1997; Kemper & Nabb 2005). One study, however, showed opposite results, in that 5 mM saccharolactone decreased the formation of acetaminophen glucuronide (Alkharfy & Frye 2001). Consequently, it is unclear whether saccharolactone is needed routinely in microsomal experiments, or whether inclusion of this compound in incubations could adversely affect UGT function. Therefore, in this study we investigated the effect of increasing concentrations of saccharolactone on eight different glucuronidation activities representing the majority of human hepatic UGTs, using liver microsomes and recombinant enzymes. We also quantified endogenous β-glucuronidase activities in microsomes prepared from various human tissues and recombinant enzyme preparations.

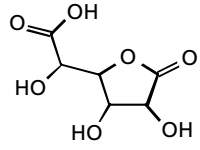

Figure 1 Chemical structure of saccharolactone.

Materials and Methods

Chemicals and reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), including alamethicin, UDP-glucuronic acid (UDPGA), D-saccharic acid 1,4-lactone (saccharolactone), β-glucuronidase (*Helix pomatia*), paracetamol, phenacetin, 3′-azido-3′-deoxythymidine (AZT), AZT glucuronide, codeine, codeine glucuronide, estradiol, estradiol-3-glucuronide, estradiol-17-glucuronide, trifluoperazine, salicylic acid, salicylic acid phenol glucuronide, e-4-hydroxy-tamoxifen, and 5-hydroxytryptamine. Analytical-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ, USA).

Liver, kidney, intestinal and lung microsomes and recombinant enzymes

Pooled human livers microsomes (pHLM) from 54 individuals were obtained from a frozen bank maintained by the Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA, USA. The microsomes were prepared from the frozen livers using differential centrifugation (Court & Greenblatt 1997). The resultant pellet was reconstituted in 20% glycerol/100 mM phosphate buffer pH 7.5, aliquoted, and stored at −80°C at a final concentration of $4\,mg\,mL^{-1}$. Human kidney microsomes (pooled from six donors) and intestinal microsomes (pooled from ten donors) were purchased from In Vitro Technologies Inc. (Baltimore, MD, USA). Human lung microsomes (pooled from six donors) were obtained from Human Biologics International (Scottsdale, AZ, USA). Human embryonic kidney HEK293 cells expressing UGT1A6 were prepared in our laboratory as described previously (Krishnaswamy et al 2005). Microsomes from insect cells infected with baculovirus containing cDNA of human UGT isoforms 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7 and 2B15 were purchased from BD Biosciences (Woburn, MA, USA).

Glucuronidation assays

Glucuronidation of seven different substrates – estradiol, trifluoperazine, 5-hydroxytryptamine, e-4-hydroxy-tamoxifen, AZT, codeine and salicylic acid – were measured to assess the effect of saccharolactone on UGT activities by human

tissue microsomes and recombinant enzymes. Assays for estradiol, trifluoperazine, 5-hydroxytryptamine, AZT and codeine glucuronidation have been described previously and were performed with minor modifications (Court et al 2002, 2003; Krishnaswamy et al 2003; Court 2005). The salicylic acid glucuronidation assay had a substrate concentration of 5 mM, a protein concentration of 0.5 mg mL^{-1}, incubation time of 6 h and paracetamol as the internal standard. Although salicylic acid formed both phenol and acyl glucuronides that could be detected by HPLC, we measured only phenol glucuronide formation, since the acyl glucuronide peak was relatively small, and a quantitation standard was not commercially available. The e-4-hydroxy-tamoxifen glucuronidation assay had a substrate concentration of 25 μM, protein concentrations of 0.04 mg mL^{-1} for pHLMs and 0.5 mg mL^{-1} UGT2B15, an incubation time of 30 min and phenacetin as the internal standard. For each glucuronidation assay, preliminary experiments were conducted to confirm that the glucuronide formation rate was proportional to protein concentration and incubation time under the conditions that were used to obtain final data.

The basic incubation mixture consisted of the dried-down substrate, pHLMs or recombinant UGTs (rUGTs) and various concentrations of saccharolactone dissolved in 50 mM phosphate buffer (pH 7.5). Saccharolactone solutions at final concentrations of 0, 1, 2, 5, 10 and 20 mM were prepared in 50 mM phosphate buffer adjusted to pH 7.5 with potassium hydroxide. The pore-forming antibiotic, alamethicin, was also added to incubations at a final concentration of 50 μg mg^{-1} protein to eliminate latency. Although in previous work, Fisher et al (2000) incubated alamethicin and microsomes on ice for 15 min before the incubation at 37°C, preliminary experiments with and without this additional incubation period showed that this pre-incubation was not necessary. Immediately before incubation, a UDPGA solution was added to the incubation mixture, consisting of 5 mM UDPGA, 5 mM $MgCl_2$ and 50 mM pH 7.5 phosphate buffer to yield a final incubation volume of 100 μL. Samples were then incubated in a 37°C water bath and the reaction stopped with 100 μL acetonitrile containing the appropriate internal standard. After centrifugation at 13000 *g* for 10 min, the supernatants were dried down in a vacuum oven at 45°C, reconstituted with 100 μL deionized water and analysed by HPLC as indicated below.

A model 1100 system (Agilent, Palo Alto, CA, USA) was used for HPLC, consisting of an autoinjector, binary pump, column, and serially connected UV absorbance and fluorescence detectors. The mobile phase was delivered at a flow rate of 1 mL min^{-1} through a 4.6 mm × 25 cm 10 μm C_{18} column (Synergi Hydro-RP; Phenomenex, Torrance, CA, USA). Unless stated otherwise, the mobile phase consisted of 20 mM potassium phosphate buffer in water (pH 2.2) (solution A) and acetonitrile (solution B). The solvent gradient for salicylic acid consisted of 10 to 50% solution B over 30 min and was detected by UV absorbance at a wavelength of 237 nm. The solvent gradient for e-4-hydroxy-tamoxifen consisted of 35 to 45% solution B over 15 min and the glucuronide was detected by UV absorbance at 280 nm. Samples were analysed in triplicate. Glucuronide formation was quantified by calculating the peak height ratio of the glucuronide to the internal standard. E-4-hydroxy-tamoxifen glucuronide was identified by showing sensitivity to inclusion of cofactor, inactivation of enzyme and treatment by β-glucuronidase. E-4-hydroxy-tamoxifen glucuronide was quantified from a standard curve using various concentrations of substrate, assuming similar UV absorbance of parent and glucuronide.

β-glucuronidase activity assay

The effect of saccharolactone on β-glucuronidase activity was measured using 100 μM estradiol-3- and 200 μM estradiol-17-glucuronides as substrates. The incubation mixture consisted of various tissue microsomes or recombinant enzymes, 5 mM $MgCl_2$, alamethicin and 50 mM phosphate buffer (pH 7.5) without UDPGA. The effects of saccharolactone were determined by comparing the endogenous β-glucuronidase activity of the protein source in the presence and absence of pH-adjusted 10 mM saccharolactone. The protein concentrations of the UGT2B7, UGT1A1, HEK293 vector control and the insect vector control cells were 0.25 mg mL^{-1}. The protein concentration of the pHLMs and intestine, kidney and lung microsomes was 0.5 mg mL^{-1}. The incubation mixture was placed in a water bath at 37°C and the reaction was stopped by the addition of 50 μL of an acetonitrile and phenacetin solution at 0 and 6 h. Samples were prepared as described above in triplicate and measured using the estradiol glucuronidation HPLC assay.

Statistical analysis

Results are expressed as the mean ± s.d. Statistical analysis was performed using the SigmaStat 3.0 software (Systat, San Jose, CA, USA). A P value of less than 0.05 was considered significant using an unpaired Student's t-test.

Results

Effect of saccharolactone and incubation pH on e-4-hydroxy-tamoxifen glucuronidation

The effect of increasing concentrations of saccharolactone on glucuronidation of e-4-hydroxy-tamoxifen by pHLMs or UGT2B15 was evaluated in preliminary studies using saccharolactone dissolved directly into the incubation buffer (50 mM potassium phosphate, pH 7.5). As shown in Figure 2A, there was a significant increase (by 100%, $P < 0.05$) in glucuronidation activity of pHLMs at a saccharolactone concentration of 5 mM, followed by a concentration-dependent decrease in activity, such that at 20 mM saccharolactone the activity was 15% of that of the control activity (i.e. without saccharolactone) ($P < 0.05$). The effect on glucuronidation by UGT2B15 was somewhat different: activity was unchanged up to 5 mM saccharolactone but was essentially abolished at 20 mM (Figure 2A).

Since saccharolactone is a carboxylic acid derivative (Figure 1), we next examined the effect of this compound on the pH of the incubation buffer system (i.e. 50 mM potassium phosphate pH 7.5). As shown in Figure 2B, there was minimal effect on pH until the saccharolactone concentration exceeded 5 mM, at which point there was a concentration-dependent

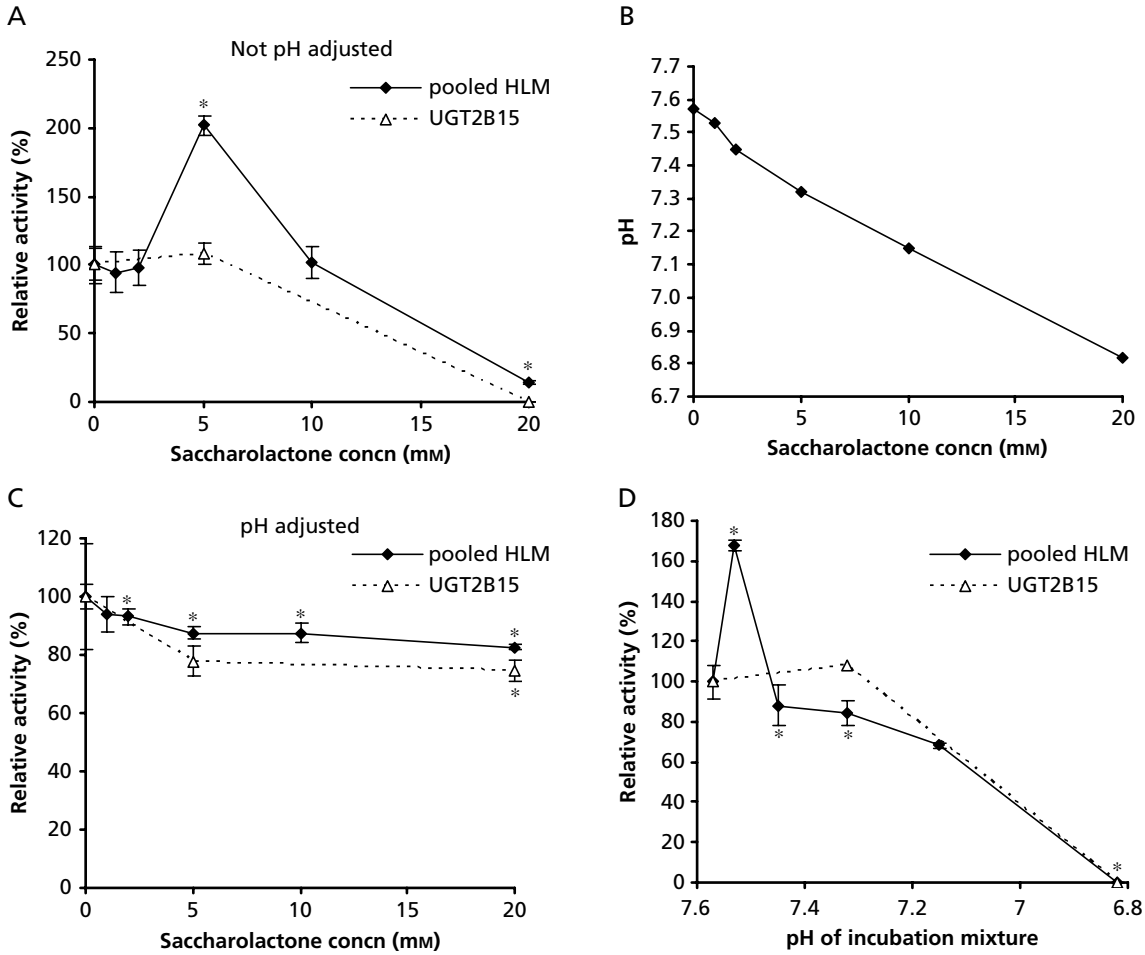

Figure 2 Effect of saccharolactone and incubation pH on glucuronidation of e-4-hydroxy-tamoxifen by pHLMs and insect cell-expressed UGT2B15. Glucuronidation was measured in the presence of increasing concentrations of pH-unadjusted (A) or pH-adjusted (to pH 7.5) saccharolactone (C). (B) shows the effect of adding increasing amounts of saccharolactone on the pH of 50 mM phosphate buffer (initially at pH 7.5 without added saccharolactone); (D) shows the direct effect of decreasing incubate pH (to match pH values determined in (B)) on e-4-hydroxy-tamoxifen glucuronidation. Glucuronide formation rates were expressed relative to control values determined without added saccharolactone (or values obtained at pH 7.5 for data shown in (B)). Individual data points are mean ± s.d. of triplicate measurements. $*P < 0.05$ vs control values obtained without added saccharolactone (or values obtained at pH 7.5 for (B)) by unpaired *t*-test.

decrease in pH to 6.8 at the 20 mM concentration. Stock solutions of saccharolactone were subsequently adjusted with potassium hydroxide to the pH of the buffer system (7.5) and glucuronidation experiments were repeated (Figure 2C). Unlike the previous results, both pHLMs and UGT2B15 did not show enhancement in activities with increasing saccharolactone concentrations; instead a modest inhibition was observed (17% decrease in pHLMs and 25% decrease in UGT2B15) at 20 mM saccharolactone.

The direct effect of pH on e-4-hydroxy-tamoxifen glucuronidation activities was then evaluated by adjusting the pH of the incubation buffer using phosphoric acid, to the levels observed with the different saccharolactone concentrations (up to 20 mM), but without adding any saccharolactone. As shown in Figure 2D, the effect of decreasing pH on glucuronidation activity by both pHLMs and UGT2B15 was similar to that observed for increasing concentrations of unbuffered saccharolactone (Figure 2A), since there was a small increase in glucuronidation activity for UGT2B15. In contrast, the pHLMs exhibited a substantial increase in activity at the pH of the lower saccharolactone concentrations; activity was essentially abolished at the lowest pH evaluated (equivalent to 20 mM saccharolactone). Consequently, the remaining studies were conducted using pH-adjusted saccharolactone solutions so that the effect of saccharolactone could be evaluated independent of pH.

Effect of saccharolactone on seven other glucuronidation activities

We also investigated the effect of saccharolactone on seven other glucuronidation activities, reflecting the majority of UGT isoforms expressed in liver, using pHLMs and rUGTs. We first compared the effects of saccharolactone on glucuronidation of 5-hydroxytryptamine by pHLMs and rUGT1A6 expressed in both insect cells and HEK293 cells, to determine

Table 1 Effect of saccharolactone on various UGT activities measured using pooled human liver microsomes (pHLMs) and recombinant UGTs. Substrate concentrations were 4 mM 5-HT, 200 μM trifluoperazine, 1 mM codeine, 500 μM AZT, 5 mM salicylic acid and 100 μM estradiol

	Saccharolactone concentration (mM)					
	0	1	2	5	10	20
5-HT glucuronidation						
pHLM	100 ± 3	104 ± 8	93 ± 5	86 ± 4[a]	87 ± 4[a]	63 ± 5[a]
UGT1A6 (insect)	100 ± 2	–	–	88 ± 7	–	96 ± 0
UGT1A6 (HEK293)	100 ± 4	–	–	91 ± 6	–	90 ± 7
Trifluoperazine glucuronidation						
pHLM	100 ± 5	98 ± 5	101 ± 9	95 ± 3	93 ± 4	97 ± 5
UGT1A4	100 ± 10	–	–	90 ± 4	–	82 ± 5[a]
Codeine glucuronidation						
pHLM	100 ± 2	98 ± 7	97 ± 4	91 ± 2[a]	95 ± 5	88 ± 8
UGT2B4	100 ± 11	–	–	91 ± 4	–	88 ± 2
UGT2B7	100 ± 9	–	–	90 ± 7	–	90 ± 3
AZT glucuronidation						
pHLM	100 ± 11	96 ± 2	98 ± 2	94 ± 2	94 ± 3	94 ± 1
UGT2B7	100 ± 3	–	–	102 ± 2	–	102 ± 8
Salicylic acid glucuronidation						
pHLM	100 ± 8	101 ± 5	111 ± 5	83 ± 3[a]	90 ± 1	85 ± 9[a]
UGT1A9	100 ± 13	–	–	96 ± 7	–	99 ± 7
Estradiol-3 glucuronidation						
pHLM	100 ± 18	68 ± 5	84 ± 1	76 ± 9[a]	67 ± 5[a]	67 ± 7[a]
UGT1A1	100 ± 12	–	–	92 ± 2	–	87 ± 5
UGT1A3	100 ± 25	–	–	92 ± 18	–	97 ± 10
Estradiol-17 glucuronidation						
pHLM	100 ± 17	71 ± 4[a]	81 ± 8	78 ± 7[a]	68 ± 2	66 ± 6[a]
UGT1A3	100 ± 12	–	–	90 ± 14	–	98 ± 10
UGT2B7	100 ± 7	–	–	97 ± 2	–	87 ± 7[a]

Data are presented as mean ± s.d. (n = 3) of the relative activity (% of activity in absence of saccharolactone).
[a] $P < 0.05$ vs activity in absence of saccharolactone.

whether there might be differences in effect related to enzyme source (Table 1). In pHLMs, the presence of saccharolactone resulted in a concentration-dependent decrease in glucuronidation activity. This effect first became apparent at the 5 mM concentration and was greatest at the 20 mM concentration (33% decrease in activity). In contrast, saccharolactone at concentrations up to 20 mM had no effect on UGT1A6 expressed in either insect or mammalian cells (Table 1).

Glucuronidation of other substrates, including trifluoperazine, salicylic acid, codeine and AZT, were similar, in that saccharolactone had essentially no effect on glucuronidation by either pHLMs or rUGTs (Table 1). Exceptions to this were glucuronidation of trifluoperazine by rUGT1A4, which was inhibited by 18% at the 20 mM concentration ($P < 0.05$); glucuronidation of codeine by pHLMs was inhibited by 9% at the 5 mM concentration ($P < 0.05$); glucuronidation of salicylic acid by pHLMs was inhibited by 25% at the 5 mM concentration ($P < 0.05$) (Table 1).

The use of estradiol as a substrate enabled the simultaneous investigation of the effects of saccharolactone on two different glucuronidation activities – at the 3- and 17-hydroxy positions. Both estradiol-3 and estradiol-17 glucuronidation activities by pHLMs were significantly inhibited at 1, 10 and 20 mM saccharolactone, with the greatest effect observed at the 20 mM concentration (33% decrease in estradiol-3 glucuronidation and 35% decrease in estradiol-17 glucuronidation; $P < 0.05$) (Table 1). In contrast, saccharolactone had no effect on estradiol glucuronidation by rUGT1A1 and rUGT1A3; in rUGT2B7, it caused a small but significant (13%; $P < 0.05$) reduction in activity in estradiol-17 glucuronidation at the 20 mM concentration.

Quantitation of endogenous β-glucuronidase activity in different enzyme systems

Since we did not find any enhancement of the different glucuronidation activities with saccharolactone, it is possible that endogenous β-glucuronidase activity is minimal in the in-vitro systems used for glucuronidation assays. We therefore quantified the endogenous β-glucuronidase activity in pHLMs and microsomes from human lung, kidney and intestine, and in the recombinant enzyme preparations. Estradiol-3-glucuronide (100 μM) and estradiol-17-glucuronide (200 μM) were used as substrates in separate incubations, and the loss of glucuronide was measured by HPLC. Phosphate buffer (50 mM, pH 7.5) was used as a negative control; bovine liver β-glucuronidase (Sigma Aldrich G-0376) was used as a positive control, which completely hydrolysed both glucuronides in less than 6 h. The effect of adding 10 mM saccharolactone was also evaluated.

As shown in Table 2, similar results were exhibited with both glucuronides, with low but variable β-glucuronidase

Table 2 Effect of 10 mM saccharolactone on the hydrolysis of estradiol-3-glucuronide (E3G; 100 μM) and estradiol-17-glucuronide (E17G; 200 μM). Estradiol glucuronide concentrations were measured by HPLC before and after 6 h' incubation with human liver, intestine, kidney and lung microsomes, insect-cell-expressed UGTs and control cells

Glucuronide	Saccharo lactone	pHLM	Intestine	Kidney	Lung	UGT2B7	UGT1A1	Insect control	HEK293 control	Buffer
E3G	–	82 ± 1[a,b]	104 ± 3	104 ± 1[a,b]	85 ± 6	77 ± 9	77 ± 2	90 ± 5	102 ± 4	101 ± 5
	+	84 ± 2[a]	101 ± 3	110 ± 3	80 ± 5[a]	91 ± 5	87 ± 7	89 ± 7	98 ± 10	95 ± 4
E17G	–	86 ± 2[b]	110 ± 4[b]	104 ± 3	92 ± 6	75 ± 5	71 ± 4	88 ± 3	104 ± 3	102 ± 13
	+	84 ± 3	106 ± 2	116 ± 2	86 ± 3	89 ± 5	85 ± 3	88 ± 4	101 ± 8	95 ± 3

Data are mean ± s.d. of percent glucuronide remaining after 6 h, n = 3. [a]$P < 0.01$ 0 h vs 6 h timepoint. [b]$P < 0.05$ 6 h timepoint with and without saccharolactone.

activity depending on the enzyme source. The greatest hydrolysis (about 25% degradation over 6 h) was observed with rUGT2B7 and rUGT1A1, followed by pHLM (15–20% degradation over 6 h), control SF9 insect cells and lung microsomes (10–15% degradation each). The remaining tissues (intestine and kidney) and HEK293 control cell microsomes did not exhibit glucuronide degradation. Saccharolactone (10 mM) had only a minimal effect in preventing degradation by the tissue microsomes, but was effective in inhibiting hydrolysis by rUGT1A1 and rUGT2B7.

Apparent formation rate versus degradation rate of estradiol glucuronides

Apparent formation rates of estradiol-3- and estradiol-17-glucuronide (nmols formed in 6 h with 0.5 mg mL^{-1} protein and 100 μM estradiol) for pooled intestinal, kidney and liver microsomes were also measured under incubation conditions similar to the β-glucuronidase assay. Both assays were performed under conditions that resulted in maximum formation and degradation rates. Since these values represent the net of the amount of glucuronide formed minus the amount degraded, a predicted actual formation rate was calculated by adding the amount of glucuronide determined in the previous experiments to be hydrolysed over the same time (Table 3). All tissues evaluated were capable of forming both estradiol glucuronides. Apparent formation rates for extrahepatic tissues were close to rates measured in hepatic microsomes; in the case of 3-hydroxy glucuronidation by intestinal microsomes, the rate substantially exceeded that of hepatic microsomes. Despite these similarities, only hepatic microsomes were capable of degrading estradiol glucuronides, with the amounts degraded relative to amounts formed ranging from 9% for the 3-hydroxyglucuronide to 19% for the 17-hydroxyglucuronide (Table 3).

Discussion

Saccharolactone is added to glucuronidation reaction mixtures to minimize the hydrolysis of glucuronide products by endogenous β-glucuronidases. Resultant activity measurements should therefore directly reflect UGT function rather than being a balance between glucuronide formation and hydrolysis associated with futile enzymatic cycling. In the present study we systematically investigated the effect of saccharolactone on eight different glucuronidation activities known to be mediated by distinct UGT isoenzymes (i.e. UGT probe activities) using both human tissue microsomes and recombinant enzyme preparations. We had expected that inhibition of endogenous β-glucuronidase activity would result in a saccharolactone-concentration-dependent increase in glucuronidation activity. Our preliminary experiments with e-4-hydroxy-tamoxifen as a substrate appeared to fit this hypothesis, since there was a two-fold increase in glucuronidation activity at the 5 mM saccharolactone concentration, followed by inhibition at higher concentrations (Figure 2A). Upon closer inspection,

Table 3 Rate of formation and hydrolysis of estradiol glucuronides. Pooled human liver microsomes (pHLMs), intestinal and kidney microsomes (0.5 mg protein mL^{-1}) were incubated in the presence of β-estradiol (100 μM) and UDPGA (apparent formation rate) or estradiol glucuronide without UDPGA (hydrolysis rate) for 6 h. The gross amount of glucuronide formed was estimated by adding the amount of glucuronide present after a 6 h incubation (formed minus degraded) to the amount that was determined to be degraded from hydrolytic activity after 6 h. Data are the mean of triplicate measurements

	nmol formed	nmol degraded	Predicted formation	Degradation (% of formation)
Estradiol-3-glucuronide				
pHLMs	3.5	0.36	3.87	9.30%
Intestine	16.7	0	16.7	0%
Kidney	3.0	0	2.99	0%
Estradiol-17-glucuronide				
pHLMs	1.23	0.29	1.52	19%
Intestine	0.58	0	0.58	0%
Kidney	1.11	0	1.11	0%

however, this result is most likely attributable to an indirect non-specific effect of saccharolactone on incubate pH thereby affecting UGT activity (increased activity with slight pH decrease; decreased activity with greater pH decrease). Adjusting the pH of the stock saccharolactone solutions abolished this enhancement in activity and instead resulted in modest inhibition up to the 20 mM concentration (Figure 2C). Various studies have established that UGTs have distinct pH optima for glucuronidation activity depending on the substrate and UGT isoform, with values ranging from as low as 5.5 to as high as 8.5 (Basu et al 2004). However, for most reported studies, incubation pH is fixed close to physiological pH (~7.5) since this is close to that found in liver tissue, the most common tissue used in glucuronidation studies.

Relatively few studies have specifically investigated the effects of saccharolactone on glucuronidation. Brunelle and Verbeeck (1993) investigated the effects of saccharolactone on phenolic and acyl glucuronidation of diflunisal by rat liver microsomes. Using 4 mM saccharolactone, they found a 2-fold increase in acyl glucuronide formation, but no change in the phenol glucuronide formation, suggesting different susceptibilities of the acyl and phenol glucuronides to β-glucuronidase. Brunelle and Verbeeck (1997) also investigated the effect of saccharolactone on acyl and phenol glucuronidation of diflunisal in rats in-vivo. Again, they saw an increase in partial clearance of the acyl glucuronide but little effect on the phenol glucuronide. Of note, they also observed a pH-dependent stabilization effect of the acyl glucuronide in urine (i.e. the lower the pH, the lower the acyl degradation). However, it was not appreciated in either report that acyl glucuronides can undergo spontaneous non-enzymatic hydrolysis, particularly at higher pH, and that the pH-lowering effect of saccharolactone may have been responsible for the apparent effect through stabilizing the acyl glucuronide rather than by inhibition of β-glucuronidase. Our study focused on phenolic glucuronides and did not include any acyl glucuronides. Other than high pH and β-glucuronidase, acyl glucuronides can also be degraded by esterases found in blood and tissues.

Several other investigators have reported that saccharolactone either has no effect or may even inhibit glucuronidation in-vitro. Boase and Miners (2002) found that 8.5 mM saccharolactone had no effect on glucuronidation of zidovudine by HLMs. Alkharfy and Frye (2001), however, observed that 5 mM saccharolactone decreased acetaminophen glucuronidation by HLMs by 45%, which was further supported by Kemp and colleagues' study in which 5 mM saccharolactone inhibited raloxifene glucuronidation by HLMs (Kemp et al 2002).

In the present study, saccharolactone solutions were pH-adjusted to near neutrality by the addition of potassium hydroxide. Higher incubation buffer strength (i.e. more than the 50 mM used here) could have been used instead to minimize alterations in incubate pH. Brunelle and Verbeeck (1993) found that saccharolactone lowered the pH of the incubation medium and corrected for this in part by using higher Tris buffer concentrations (100–300 mM). They noted, however, that such additional buffering capacity was insufficient for saccharolactone concentrations above 8 mM. High buffer concentrations also have the potential to adversely affect glucuronidation – Boase and Miners (2002) showed that increasing phosphate buffer concentrations from 50 to 200 mM decreased zidovudine glucuronidation in HLMs by about 25%.

Since we did not see any enhancement of UGT activity by saccharolactone, we decided to investigate the amount of glucuronide hydrolysis in relation to glucuronide formation using a variety of different enzyme sources and estradiol glucuronides as substrates (Table 3). These studies confirmed that hydrolysis of estradiol glucuronide does occur in both tissue and recombinant enzyme systems, but would only decrease the apparent estradiol glucuronidation rates by at most 9–19%. Confirming previous observations (Bracey & Paigen 1987; Paigen 1989), β-glucuronidase activity was primarily observed in hepatic microsomes, but also in lung microsomes and insect cell preparations. We found no evidence for β-glucuronidase activity in our intestinal epithelial cell microsomes. Although the intestine is the main site of β-glucuronidase-mediated hydrolysis of the glucuronides of drugs that undergo enterohepatic recycling, this β-glucuronidase originates from intestinal bacteria rather than the intestinal epithelial cells. This appears to be the first report of β-glucuronidase activity in recombinant enzymes expressed in insect cells. Since these preparations are generally crude cell homogenates, such activity may represent combined microsomal and lysosomal enzymes. Saccharolactone was somewhat effective in inhibiting this activity in insect cells but not in liver microsomes. The reason for this difference is not immediately apparent. Different glucuronides are likely to differ in susceptibility to hydrolysis by tissue β-glucuronidase, although this has not been studied systematically. Consequently, our findings with estradiol glucuronides will need to be confirmed and extended by study of other glucuronide substrates.

Conclusions

The results of this study indicate that endogenous β-glucuronidase activity can have a measurable, albeit relatively small, influence on a range of glucuronidation activities measured in human tissue microsomes and recombinant enzymes. We recommend that investigators conduct preliminary experiments to assess the effect (either enhancement or inhibition) of saccharolactone on glucuronidation activities measured with different substrates and enzyme sources. Moreover, the pH of aqueous saccharolactone solutions should be adjusted to that of the desired incubation pH before addition, in order to minimize the potentially substantial effects of pH on UGT activity.

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