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The Role of pH in the Glucuronidation of Raloxifene, Mycophenolic Acid and Ezetimibe

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Abstract: The UDP-glucuronosyltransferase (UGT) active site faces the lumen of the endoplasmic reticulum and is enclosed behind a lipid bilayer. Consequently, observed UGT activity is latent in microsomal preparations, and thus, mechanical and/or chemical disruptions of the vesicle membrane are commonly employed to better expose the active site. The aim of the present investigation was to explore the impact of incubation pH on the glucuronidation of raloxifene, mycophenolic acid (MPA) and ezetimibe, which are basic, acidic and neutral compounds, respectively. Their glucuronidation was examined in human liver microsomal incubations by monitoring for the production of the glucuronide metabolites at pHs ranging between 5.4 and 9.4. Compared to physiological pH, unbound intrinsic clearance ($CL_{int,u}$) was 11- and 12-fold higher at pH 9.4 for raloxifene 4′-glucuronide (R4G) and raloxifene 6-glucuronide (R6G), respectively; whereas a 10-fold increase was observed at pH 5.4 for MPA glucuronide (MPAG). In contrast, ezetimibe glucuronidation did not vary as the pH deviated from 7.4. Kinetic analysis revealed that increases in $CL_{int,u}$ were accompanied by less than a 2-fold change in V_{max}. Instead, $K_{m,u}$ decreased 8-, 13- and 5-fold for R4G, R6G and MPAG, respectively. Similar pH dependency on glucuronidation was observed in experiments utilizing recombinant UGT enzymes (recUGT). Particularly, recUGT1A9 was one of the major isoforms involved in the glucuronidation of raloxifene and MPA. While the highest rate of glucuronidation was found at pH 9.4 for raloxifene, the pH for optimal glucuronidation of MPA was between 5.4 and 7.4. In summary, these results suggest that microsomal glucuronidation may be enhanced for acidic and basic compounds by altering the incubation pH, perhaps by improving substrate membrane permeability.

Keywords: Glucuronidation; microsomes; pH; permeability; UGT; raloxifene; mycophenolic acid; ezetimibe

Introduction

Glucuronidation catalyzed by UDP-glucuronosyltransferase (UGT) is the predominant phase II biotransformation reaction in human. UGT mechanism is hypothesized to involve S_N2 attack of the nucleophilic substrate on the cofactor UDP-glucuronic acid (UDPGA).[1] The reaction comes to an end as UDP is displaced to release the glucuronide metabolite. The newly formed glucuronide metabolite is highly hydrophilic and, thus, is able to be efficiently excreted into the urine and/or the bile. Prevalence of glucuronidation may be attributed to the large availability of UDPGA present in human cells. Mean concentrations measured in human tissues such as the liver and ileum are

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approximately 280 and 19 μmol/kg wet tissue, respectively.[2] Another reason may be that UGTs are expressed almost ubiquitously throughout the body, with high expression the liver and the intestines.[3] Finally, UGTs accept a broad spectrum of endogenous and exogenous compounds.[4,5] Indeed, approximately 40% of drugs undergoing phase II metabolism are glucuronidated,[6] which is widely observed in tissues including the liver and the intestines.[7]

At the molecular level, UGTs are integral membrane proteins localized to the endoplasmic reticulum (ER). Yet, unlike cytochrome P450s (CYPs) whose active sites face the cytosolic compartment, the UGT catalytic site is concealed within the lumen of the ER.[8,9] As a result, UGT activity becomes compartmentalized and enzyme latency emerges in *in vitro* subcellular fractions such as microsomes because they are unable to overcome the biological barrier.[10,11] Microsomes are closed vesicles derived from the ER. They are one of the most commonly used tools to characterize metabolism because they are relatively inexpensive, widely available, easy to use and their enzyme activities have been well characterized. However, because microsomes are artificial systems composed of ER fragments, they are not self-sufficient, requiring the addition of cofactors to catalyze metabolism. Furthermore, since microsomes only express enzymes associated with the ER, the metabolites that are produced *in vitro* may not be representative of metabolites formed *in vivo*. Moreover, since the microsomal system itself is not competent to overcome the ER membrane barrier, latency is observed with glucuronidation. There have been many efforts to circumvent this constraint with mechanical (e.g., sonication, freeze/thaw and vortexing)[12] and/or chemical (e.g., detergents and alamethicin) disruptions.[12−15] Although some of these methods have been partially successful in improving glucuronidation, they do not seem to completely remove UGT latency.

One reason for the residual latency may be because the ER membrane cannot be entirely removed if the native protein conformation is to be maintained to preserve enzyme function. In fact, it has been demonstrated that activating agents such as alamethicin do not eliminate the ER membrane but create channels within the lipid bilayer to enable more substrate to enter the microsomal lumen.[16,17] Therefore, although the ER lumen may become more accessible due to increased number of channels or pores created by the activating agents, the ER membrane can continue to limit substrates from entering the catalytic site. The ER membrane is semi-permeable, which permits uncharged small molecules (up to 600 in molecular weight) to freely traverse the lipid membrane via a passive process. However, the permeability of large and charged compounds is limited.[18,19] It has been

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suggested that channels or pores serve as gates and may be responsible for this selectivity.[20] Since channels that facilitate the passage of small molecules express ion selectivity, the charged state of the substrate as well as of the channels may affect substrate permeability to impact glucuronidation.

The aim of the present study was to examine the effect of incubation pH on microsomal glucuronidation. The effect of pH has been examined previously by several groups who have attributed increased glucuronidation to changes in the charge state of the amino acids at the catalytic site to yield optimal UGT activity.[12,21−23] However, in addition to the modifications at the catalytic site, this work hypothesizes that the pH effect on glucuronidation may also be to enhance membrane permeability. This is accomplished by not only neutralizing the substrate charge but by altering the affinity and selectivity of the membrane channels as well. Raloxifene, mycophenolic acid (MPA) and ezetimibe are basic, acidic and neutral drugs undergoing extensive glucuronidation, respectively (Figure 1). The impact of incubation pH on the microsomal glucuronidation of these substrates with differing charge states was investigated by altering the pH of the *in vitro* incubation.

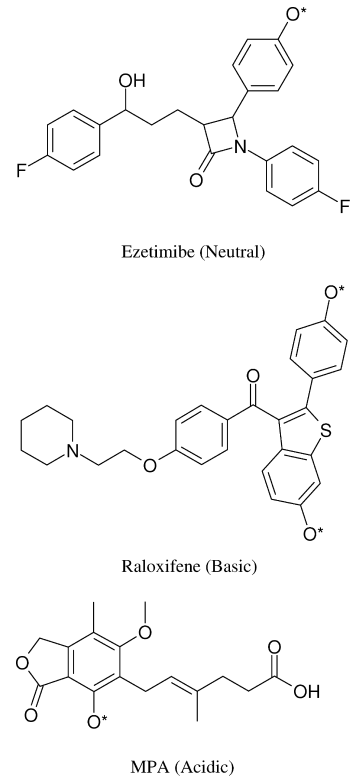

Figure 1. Structures of ezetimibe, MPA and raloxifene. Site of glucuronidation monitored for each compound is marked with a star (*).

Materials and Methods

Materials. Raloxifene, UDPGA, alamethicin and $MgCl_2$ were purchased from Sigma-Aldrich Co. (St. Louis, MO). Ezetimibe, ezetimibe-glucuronide (EZG), MPA, MPA glucuronide (MPAG), raloxifene 4′-glucuronide (R4G) and raloxifene 6-glucuronide (R6G) were purchased from Toronto Research Chemicals (Ontario, Canada). All solvents were of HPLC grade purchased from Sigma-Aldrich Co. Pierce Rapid Equilibrium Dialysis (RED) device was purchased from Thermo Scientific (Rockford, Il). Human liver microsomes (HLM) pooled from 50 donors (Lot 88114), recUGT1A1 (Lot 99972), recUGT1A3 (Lot 99974), recUGT1A4 (Lot 95375), recUGT1A6 (Lot 04294), recUGT1A9 (Lot 11), recUGT2B4 (Lot 04182), recUGT2B7 (Lot 03362), recUGT2B15 (Lot 93815), recUGT2B17 (Lot 00431) and insect control (Lot 05614) were purchased from BD Biosciences (San Jose, CA).

Microsomal Incubations. Glucuronidation studies utilizing pooled HLM or recUGTs in the presence or absence of alamethicin were performed as described previously with slight modifications.[14,24] Briefly, the concentration of microsomal protein in the incubation reactions and the length of incubation were optimized at physiological pH to ensure linear kinetics and that the amount of parent consumption did not exceed 15%. Final conditions included 0.05 mg/mL microsomes, 1 mM $MgCl_2$ and 0.1 M phosphate buffer (pH ranging between 5.4−9.4) being chilled on ice in the absence or presence of 50 μg/mg protein alamethicin. After approximately 15 min, ezetimibe (0.5−50 μM), raloxifene (0.1−100 μM) or MPA (5−750 μM) was spiked into the reaction mixtures (0.1% DMSO, v/v) and preincubated at 37 °C for 15−20 min. Following preincubation, the reactions were initiated with the addition of 5 mM UDPGA. The total incubation volume was 200 μL. Reactions were terminated after 6 min (or 7.5 min for recUGT incubations) with an equal volume of acetonitrile containing 0.1 μM cortisone as the internal standard. Each sample was centrifuged, and the supernatant was analyzed using LC−MS/MS as described below. Formation of glucuronide metabolites was quantitated using a standard curve prepared in a similar manner as outlined above. All experiments were done in triplicate.

Nonspecific Binding in HLM. Equilibrium dialysis using the Pierce RED device was used to evaluate microsomal binding ($f_{u,inc}$). Preliminary results showed that equilibrium was reached with the RED by 4 h for all compounds that were examined (data not shown). Microsomal mixtures similar to the ones used in the microsomal incubations were prepared by mixing 0.05 mg/mL HLM, 1 mM $MgCl_2$ and 1 μM drug into 0.1 M phosphate buffer (pH range 5.4−9.4).

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The RED inserts were loaded with the microsomal mixture on one side and 0.1 M phosphate buffer on the other side. After incubating at 37 °C for 4 h, samples from the two compartments were collected and extracted by adding an equal volume of acetonitrile containing 1 μM cortisone as the internal standard. Each sample was centrifuged and the supernatant was injected onto LC−MS/MS. Concentration of compound in the microsome and buffer compartments was determined using a standard curve prepared in a similar manner as outlined above. All experiments were performed in triplicate. Binding data were not obtained for ezetimibe at pH 8.4 and pH 9.4 because it was not stable for the duration of the experiment (i.e., 4 h). However, preliminary work showed that ezetimibe was stable to at least 6 min in pH 8.4 buffer at 37 °C (data not shown), suggesting that the kinetic analysis was not affected. For the purposes of kinetic calculations, it was assumed that binding at pH 7.4 was comparable to pH 8.4 since $f_{u,inc}$ did not change at varying acidic pHs.

LC−MS/MS Conditions. The parent and the glucuronide metabolites were quantitated with a Sciex API4000 triple quadrupole mass spectrometer using electrospray (Applied Biosystems, Foster City, CA) equipped with Shimadzu LC 10AVP HPLC (Shimadzu Scientific Instruments, Columbia, MD) coupled to a CTC LEAP HTS autosampler with temperature controlled racks (LEAP Technologies, Carrboro, NC). The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) through a TARGA C18 50 × 2.1 mm column (Higgins Analytical, Mountain View, CA). The initial condition was set at 5% B for 0.5 min. For raloxifene, R4G and R6G, 5% B was held for 0.5 min followed by a quick ramp to 20% B over 0.1 min. This was ensued by another set of gradients of 25% B over 0.9 min, 40% B over 0.3 min and 90% B over 0.1 min. After holding at 90% B for 0.9 min, the gradient was changed back to the initial conditions. The flow rate was 0.8 mL/min. Raloxifene and its glucuronide metabolites were chromatographically separated and monitored in the positive multiple reaction monitoring mode with transitions of *m/z* 474 → 112 and *m/z* 650 → 474, respectively. The mass transitions for cortisone in the positive mode was 361 → 163. The accuracy and precision of R4G and R6G were within ±20%, with lower limit of quantitation (LOQ) of 0.5 nM and 0.1 nM, respectively. R4G and R6G recoveries in 0.05 mg/mL HLM were 96 ± 3% and 100 ± 4%, respectively. For ezetimibe and EZG, 5% B was held for 5 min followed by a quick ramp to 40% B over 0.1 min. This was held for 0.2 min after which a gradient to 90% B over 0.8 min took place. After holding at 90% B for 0.2 min, the gradient was changed back to the initial conditions. The flow rate was 0.8 mL/min. Ezetimibe and EZG were chromatographically separated and monitored in the negative multiple reaction monitoring mode with transitions of *m/z* 408 → 271 and *m/z* 584 → 271, respectively. The accuracy and precision of MPAG were within ±20%, with LOQ of 1 nM. MPAG recovery in 0.05 mg/mL HLM was 100 ± 3%. For MPA and MPAG, 5% B was held for 5 min followed by a quick ramp to 40% B over 0.25 min. This was held for 0.25 min, after which a gradient to 90% B over 0.3 min took place. After holding at 90% B for 0.2 min, the gradient was changed back to the initial conditions. The flow rate was 1 mL/min. MPA and MPAG were chromatographically separated and monitored in the negative multiple reaction monitoring mode with transitions of *m/z* 319 → 191 and *m/z* 495 → 319, respectively. The mass transition for cortisone in the negative mode was 359 → 329. Accuracy and precision of EZG were within ±20%, with LOQ of 1 nM. EZG recovery in 0.05 mg/mL HLM was 91 ± 4%.

Data Analysis. GraFit 5.0.6 (Erithacus Software Limited, Horley, Surrey, U.K.) was utilized to fit the following kinetic models to untransformed experimental data.[25]

Michaelis−Menten model (MPA at pH 6.4 and pH 7.4):

$$v = \frac{V_{max}S}{K_m + S}$$

where v is the rate of reaction, V_{max} is the maximum velocity, K_m is the Michaelis constant and S is the substrate concentration.

Substrate inhibition model (raloxifene and ezetimibe at all pHs; MPA at pH 5.4):[25]

$$v = \frac{V_{max}}{1 + (K_m/S) + (S/K_{si})}$$

where K_{si} is the substrate inhibition constant.

Kinetic parameters were corrected for nonspecific binding observed in microsomes ($f_{u,inc}$) so that unbound Michaelis constant ($K_{m,u}$) and unbound intrinsic clearance ($CL_{int,u}$) are reported. Kinetic curves are transformed data presented as Eadie−Hofstee plots.

$K_{m,u}$ and V_{max} values generated were utilized to calculate $CL_{int,u}$ defined as

$$CL_{int,u} = V_{max}/K_{m,u}$$

$f_{u,inc}$ was calculated using the following equation:

$$f_{u,inc} = [\text{compound}]_{buffer}/[\text{compound}]_{microsomes}$$

Results

Glucuronidation in HLM. Rates of formation of the glucuronide metabolites for ezetimibe, MPA, and raloxifene were examined in HLM at various pHs ranging from 5.4 to 9.4. There were less than 2-fold differences in the rate of EZG formation across the pH ranges examined, suggesting that the rate of glucuronidation was independent of the incubation pH (Figure 2A). On the contrary, rate of MPA and raloxifene glucuronidation increased as the incubation pH became acidic and basic, respectively. Specifically, compared to physiological pH when alamethicin is absent, rate of MPAG formation was 8-fold greater at pH 5.4 (Figure

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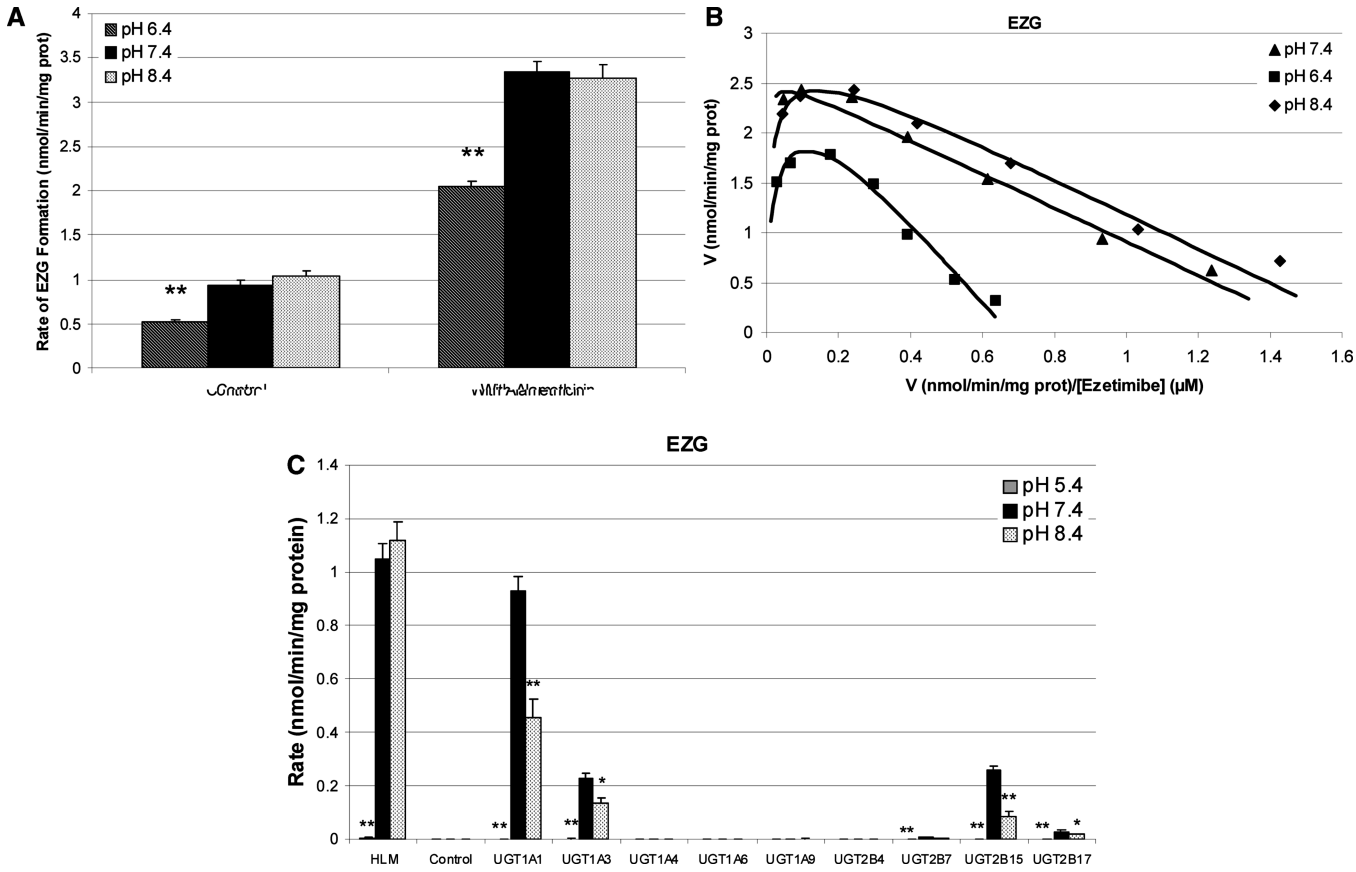

Figure 2. Ezetimibe glucuronidation. (A) Rate of EZG formation in HLM when ezetimibe was incubated at 1 μM in 0.05 mg/mL HLM at 37 °C for 6 min, in the absence (Control) or presence of alamethicin. *In vitro* conditions were controlled to reflect incubation at pH 6.4 (cross-hatched bars), pH 7.4 (solid black bars) or pH 8.4 (dotted bars). No glucuronidation was observed at pH 5.4, and ezetimibe was not chemically stable at pH 9.4. The results represent the mean of $N = 3$ with standard error bars. (B) Representative Eadie−Hofstee plots (v versus V/S) for the formation of EZG by HLM in the absence of alamethicin at pH 7.4 (triangle), pH 6.4 (square) and pH 8.4 (diamond). Points are experimentally determined values and curves are fitted using the substrate inhibition model at all pHs. (C) Rate of EZG formation in recUGT when ezetimibe was incubated at 0.5 μM in 0.05 mg/mL recUGT at 37 °C for 7.5 min. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 7.4 (solid black bars) or pH 8.4 (dotted bars). The results represent the mean of $N = 6$ with standard error bars. For all graphs, one star (*) represents $p < 0.01$ and two stars (**) represent $p < 0.001$, compared against physiological pH.

3A); whereas the rates of R4G and R6G formation were 4- and 3-fold higher at pH 9.4, respectively (Figure 4A and Figure 5A). A similar trend was observed when alamethicin was supplemented in the incubation.

The effect of pH shift was further investigated by examining glucuronidation kinetics in HLM in the presence or absence of alamethicin. Eadie−Hofstee plot of the transformed data was used to select the appropriate kinetic model to estimate kinetic parameters as described in Materials and Methods. Kinetic parameters were corrected for nonspecific binding to microsomes ($f_{u,inc}$) so that unbound Michaelis constant ($K_{m,u}$) and unbound intrinsic clearance ($CL_{int,u}$), along with V_{max}, are reported. MPAG at pH 7.4 and 6.4 followed standard kinetics (Figure 3B). However at pH 5.4, an atypical kinetic profile indicative of substrate inhibition kinetics was observed, with K_{si} value of 1860 μM. MPA did not bind to microsomes at any pH ranges examined, and Table 1 summarizes MPAG kinetic parameters. In the absence of alamethicin, $K_{m,u}$, V_{max} and $CL_{int,u}$ at physiological pH were 186 μM, 2.81 nmol/min/mg prot and 15.1 μL/min/mg prot, respectively. The $K_{m,u}$ value was comparable to the previously reported literature values.[26,27] As the incubation pH became more acidic, $CL_{int,u}$ increased to 145 μL/min/mg prot at pH 5.4. Changes in $CL_{int,u}$ were accompanied by 5-fold decrease in $K_{m,u}$ to 40.6 μM and 2-fold increase in V_{max} to 5.90 nmol/min/mg prot at pH 5.4.

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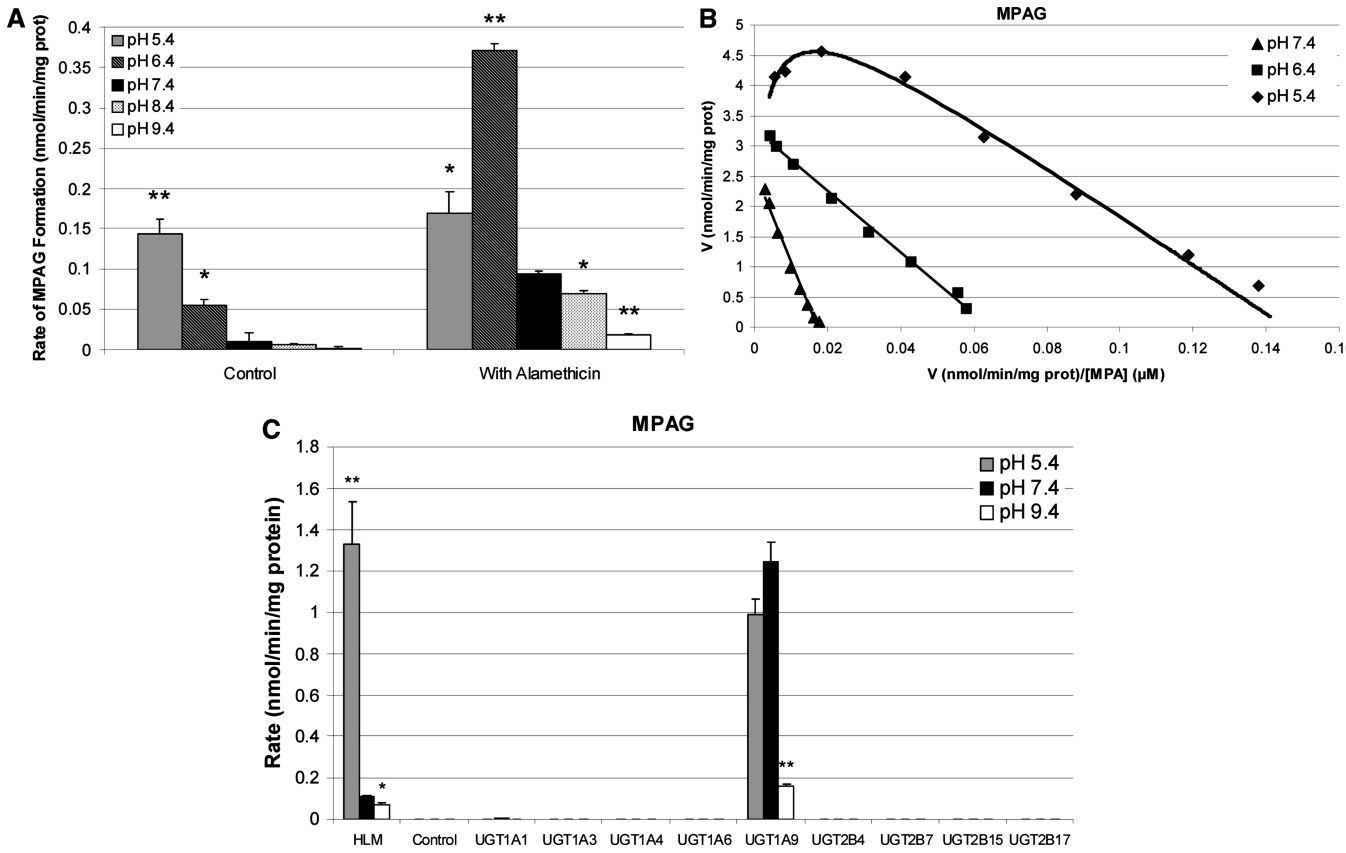

Figure 3. MPA glucuronidation. (A) Rate of MPAG formation in HLM when MPA was incubated at 1 μM in 0.05 mg/mL HLM at 37 °C for 6 min, in the absence (Control) or presence of alamethicin. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 6.4 (cross-hatched bars), pH 7.4 (solid black bars), pH 8.4 (dotted bars) or pH 9.4 (open bars). The results represent the mean of $N = 3$ with standard error bars. (B) Representative Eadie−Hofstee plots (v versus V/S) for the formation of MPAG by HLM in the absence of alamethicin at pH 7.4 (triangle), pH 6.4 (square) and pH 5.4 (diamond). Points are experimentally determined values, and curves are fitted using the substrate inhibition model at pH 5.4 and Michaelis–Menten model at pH 7.4 and pH 6.4. (C) Rate of MPAG formation in recUGT when MPA was incubated at 0.5 μM in 0.05 mg/mL recUGT at 37 °C for 7.5 min. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 7.4 (solid black bars) or pH 9.4 (open bars). The results represent the mean of $N = 6$ with standard error bars. For all graphs, one star (*) represents $p < 0.01$ and two stars (**) represent $p < 0.001$, compared against physiological pH.

Raloxifene bound moderately to microsomes with $f_{u,inc}$ of 0.674 ± 0.089, 0.335 ± 0.038 and 0.369 ± 0.047 at pH 7.4, 8.4 and 9.4, respectively. Raloxifene glucuronidation followed substrate inhibition kinetics at all pHs examined (Figure 4B and Figure 5B). K_{si} at pH 7.4 was 45.1 μM and 454 μM for R4G and R6G, respectively, and became less potent as the pH became more basic. Kinetic parameters accounting for $f_{u,inc}$ are summarized in Table 2. In the absence of alamethicin, $K_{m,u}$ and V_{max} at physiological pH were 1.72 μM and 192 pmol/min/mg prot for R4G; and 2.56 μM and 80.7 pmol/min/mg prot for R6G. The differences in $K_{m,u}$ values compared to the previously reported studies may be because the earlier studies did not consider atypical kinetics and ignored $f_{u,inc}$.[28,29] $CL_{int,u}$ for R4G and R6G were 112 μL/min/mg prot and 31.5 μL/min/mg prot, respectively. As the incubation reached pH 9.4, R4G and R6G $CL_{int,u}$ increased 8- and 10-fold, respectively. Similar to MPAG kinetics, increases in $CL_{int,u}$ were due to decreases in $K_{m,u}$ but with less than a 2-fold change in V_{max} for both raloxifene glucuronide pathways.

Ezetimibe followed substrate inhibition kinetics (Figure 2B) with K_{si} value of 276 μM at physiological pH. Estimated kinetic parameters corrected for $f_{u,inc}$ of 0.734 ± 0.097 are summarized in Table 3. Consistent with earlier observations, kinetic parameters for ezetimibe glucuronidation were not altered with changing pH. In the absence of alamethicin at pH 7.4, $K_{m,u}$, V_{max} and $CL_{int,u}$ were 1.51 μM, 2.86 nmol/min/mg prot and 1.89 mL/min/mg prot, respectively. As with raloxifene, the different $K_{m,u}$ value compared to the previously reported studies may be because atypical kinetics was not considered and $f_{u,inc}$ ignored.[30] For all compounds examined, supplementing the incubation reaction with alame-

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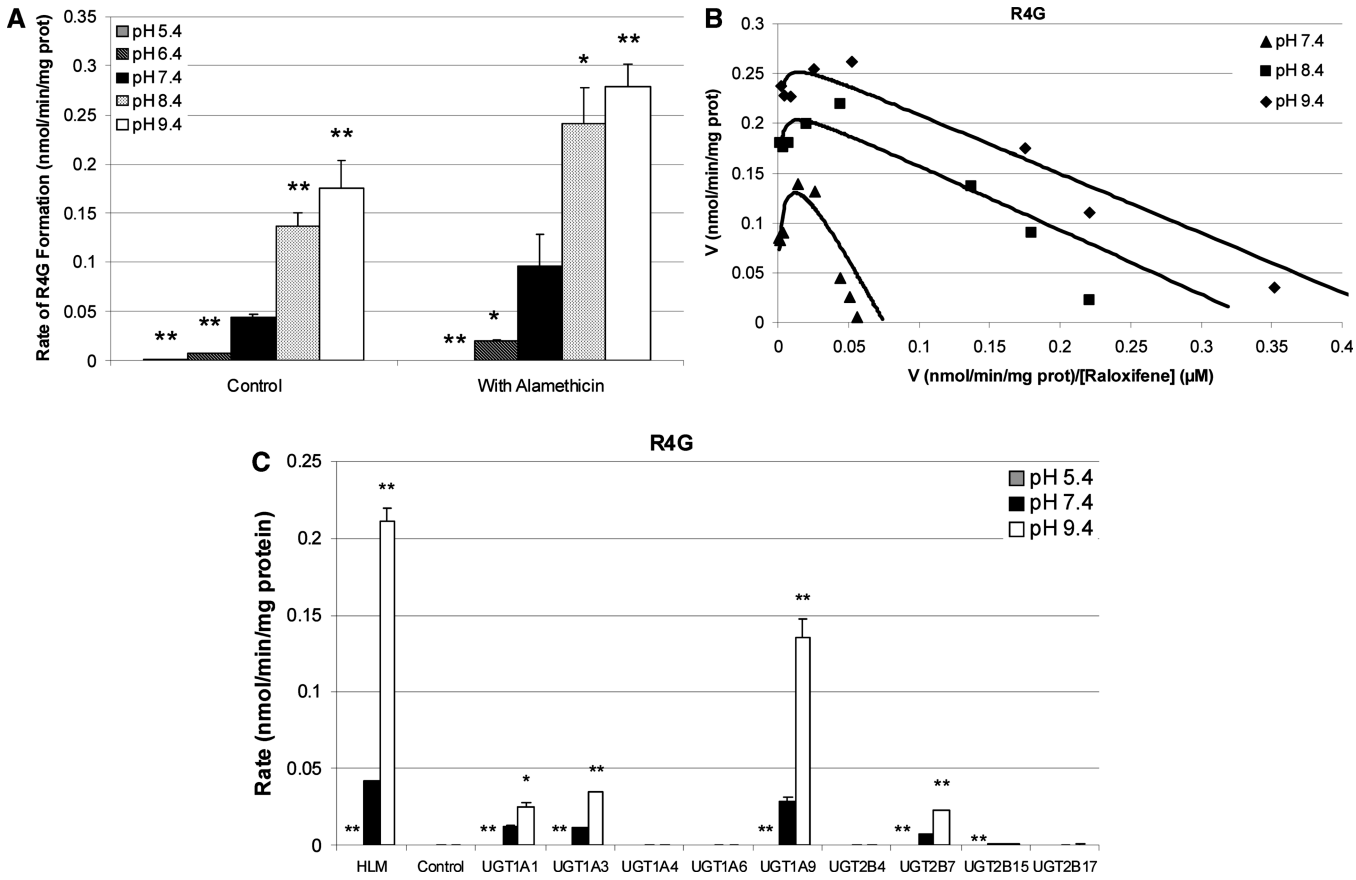

Figure 4. Raloxifene glucuronidation to R4G. (A) Rate of R4G formation in HLM when raloxifene was incubated at 1 μM in 0.05 mg/mL HLM at 37 °C for 6 min, in the absence (Control) or presence of alamethicin. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 6.4 (cross-hatched bars), pH 7.4 (solid black bars), pH 8.4 (dotted bars) or pH 9.4 (open bars). The results represent the mean of $N = 3$ with standard error bars. (B) Representative Eadie−Hofstee plots (v versus V/S) for the formation of R4G by HLM in the absence of alamethicin at pH 7.4 (triangle), pH 8.4 (square) and pH 9.4 (diamond). Points are experimentally determined values, and curves are fitted using the substrate inhibition model at all pHs. (C) Rate of R4G formation in recUGT when raloxifene was incubated at 0.5 μM in 0.05 mg/mL recUGT at 37 °C for 7.5 min. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 7.4 (solid black bars) or pH 9.4 (open bars). The results represent the mean of $N = 6$ with standard error bars. For all graphs, one star (*) represents $p < 0.01$ and two stars (**) represent $p < 0.001$, compared against physiological pH.

thicin yielded similar trends in the kinetic parameters. Meanwhile, as reported previously on the effect of alamethicin,[14,29] rates of glucuronidation were enhanced by 2- to 3-fold for all substrates across all pHs when compared to controls.

Effect of pH on Recombinant UGT (recUGT) Activity. There are currently 17 human UGT isoforms identified to date. Of these, 12 isoforms are expressed in the liver,[3,31] but only recUGT1A1, recUGT1A3, recUGT1A4, recUGT1A6, recUGT1A9, recUGT2B4, recUGT2B7, recUGT2B15 and recUGT2B17 are available commercially. To examine if the pH effect can be ascribed to the function of a particular isoform, glucuronidation in liver-specific recUGTs were monitored at pH 5.4, pH 7.4 and pH 9.4. UGT1A1, UGT1A3 and UGT2B15 were the major isoforms involved in the formation of EZG (Figure 2C). Rate of glucuronidation was not dependent on the incubation pH. UGT1A9 was the major isoform for MPAG and the highest rate was observed at neutral and acidic pHs (Figure 3C). UGT1A1, UGT1A3, UGT1A9 and UGT2B7 were the major isoforms for the formation of R4G (Figure 4C); whereas UGT1A1, UGT1A3 and UGT1A9 were the major isoforms for R6G (Figure 5C). Incubating raloxifene at pH 9.4 gave the best activity in these recombinant enzymes. These results indicate that the effect of pH was strongly associated with the substrate but to a lesser extent with a specific isoform. In particular, although

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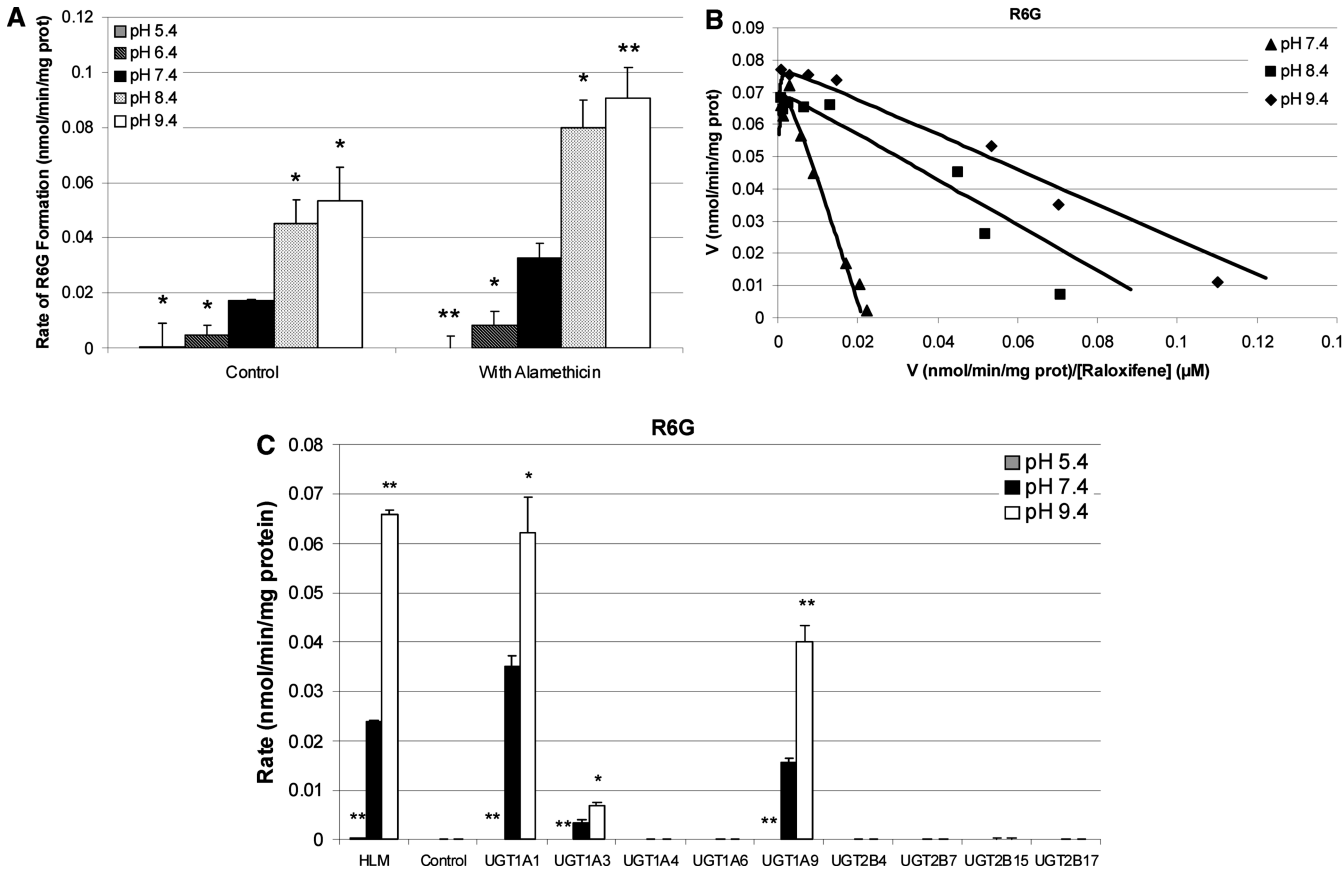

Figure 5. Raloxifene glucuronidation to R6G. (A) Rate of R6G formation in HLM when raloxifene was incubated at 1 μM in 0.05 mg/mL HLM at 37 °C for 6 min, in the absence (Control) or presence of alamethicin. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 6.4 (cross-hatched bars), pH 7.4 (solid black bars), pH 8.4 (dotted bars) or pH 9.4 (open bars). The results represent the mean of $N = 3$ with standard error bars. (B) Representative Eadie−Hofstee plots (v versus V/S) for the formation of R6G by HLM in the absence of alamethicin at pH 7.4 (triangle), pH 8.4 (square) and pH 9.4 (diamond). Points are experimentally determined values and curves are fitted using the substrate inhibition model at all pHs. (C) Rate of R6G formation in recUGT when raloxifene was incubated at 0.5 μM in 0.05 mg/mL recUGT at 37 °C for 7.5 min. *In vitro* conditions were controlled to reflect incubation at pH 5.4 (solid gray bars), pH 7.4 (solid black bars) or pH 9.4 (open bars). The results represent the mean of $N = 6$ with standard error bars. For all graphs, one star (*) represents $p < 0.01$ and two stars (**) represent $p < 0.001$, compared against physiological pH.

optimal activity of recUGT1A9 was observed at pH 9.4 for raloxifene, this pH resulted in the poorest activity with MPA.

Discussion

The ER houses many important drug metabolizing enzymes such as CYPs and UGTs. It comprises a semipermeable membrane that is selective against large and/or charged molecules,[18,19] and is distinct from the content of the cytosol such as with calcium and glutathione levels.[32,33] The ER membrane does not seem to be a physical impediment to CYP function, but because the UGT catalytic site faces the ER lumen, latency in glucuronidation has been observed with microsomal glucuronidation. The present work investigates the role of substrate membrane permeability on microsomal glucuronidation. It is hypothesized that improving membrane permeability would allow substrate−UGT interactions to increase, yielding higher turnover. Ezetimibe, MPA and raloxifene are neutral, acidic and basic compounds, respectively. They were employed as model substrates because they are extensively glucuronidated to form ether glucuronide metabolites.

Glucuronidation of ezetimibe and raloxifene displayed substrate inhibition kinetics. The estimated K_{si} values were greater than their corresponding $K_{m,u}$, suggesting that the substrates have higher affinity to the UGT active site. Such atypical kinetics have been previously reported for raloxifene,[29] but this is the first evidence for ezetimibe. The mechanism of UGT-mediated substrate inhibition remains largely unknown, and possible modes of action include allosteric interactions, multiple active sites and noncompetitive substrate inhibition.[34] The kinetic analyses were not able

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Table 1. Summary of MPA Glucuronidation Kinetics in HLM at pH Ranging from 5.4 to 9.4[a]

			incubation pH				
	activating agents	parameters[b]	5.4[c]	6.4	7.4	8.4	9.4
MPAG	none (control)	$K_{m,u}$	40.6 (3.7)**	55.1 (2.3)**	186 (9)	N/A	N/A
		V_{max}	5.90 (0.24)**	3.33 (0.04)**	2.81 (0.05)		
		$CL_{int,u}$	145 (14)**	60.4 (2.6)**	15.1 (0.8)		
	alamethicin	$K_{m,u}$	35.6 (4.8)**	48.2 (1.1)**	161 (10)	N/A	N/A
		V_{max}	6.27 (0.35)**	3.84 (0.02)**	4.29 (0.09)		
		$CL_{int,u}$	176 (25)**	79.7 (1.9)**	26.6 (1.7)		

[a] Values are reported as mean (SD). The kinetic parameters were obtained with GraFit as outlined in *Materials and Methods*. Unbound Michaelis constant ($K_{m,u}$) and unbound intrinsic clearance ($CL_{int,u}$) values were reported ($f_{u,inc}$ approached 1 at all pHs examined). N/A represents data that was not obtainable because saturation was not observed at the highest substrate concentrations examined. [b] Units for $K_{m,u}$ and K_{si} are μM, V_{max} is nmol/min/mg prot and $CL_{int,u}$ is μL/min/mg microsomal protein. [c] Kinetics at pH 5.4 followed substrate inhibition kinetics. K_{si} was 1860 $\pm$ 330 μM and 2180 $\pm$ 620 μM in the absence or presence of alamethicin, respectively. One star (*) represents $p < 0.01$, and two stars (**) represent $p < 0.001$, compared against physiological pH.

Table 2. Summary of raloxifene glucuronidation kinetics in HLM at pH ranging from 5.4 to 9.4[a]

			incubation pH				
	activating agents	parameters[b]	5.4	6.4	7.4	8.4	9.4
R4G	none (control)	$K_{m,u}$	N/A	N/A	1.72 (0.11)	0.22 (0.01)**	0.22 (0.02)**
		V_{max}			192 (15)	222 (14)	269 (23)*
		K_{si}			45.1 (7)	304 (71)*	507 (56)**
		$CL_{int,u}$			112 (11)	1020 (80)**	1220 (160)**
	alamethicin	$K_{m,u}$	N/A	N/A	4.53 (0.34)	0.22 (0.02)**	0.26 (0.02)**
		V_{max}			796 (24)	389 (32)**	449 (27)**
		K_{si}			7.18 (1.62)	114 (21.2)**	170 (31.2)**
		$CL_{int,u}$			176 (14)	1800 (220)**	1720 (170)**
R6G	none (control)	$K_{m,u}$	N/A	N/A	2.56 (0.21)	0.24 (0.02)**	0.20 (0.02)**
		V_{max}			80.7 (7.2)	71.2 (3.2)	78.7 (3.0)
		K_{si}			454 (49)	1420 (380)	1480 (350)*
		$CL_{int,u}$			31.5 (3.8)	301 (29)**	392 (38)**
	alamethicin	$K_{m,u}$	N/A	N/A	2.16 (0.34)	0.17 (0.02)**	0.18 (0.02)**
		V_{max}			137 (5)	116 (5)*	118 (9)
		K_{si}			414 (74)	370 (61)	544 (72)
		CL_{int}			63.3 (10.1)	670 (31)**	653 (75)**

[a] Values are reported as mean (SD). The kinetic parameters were obtained with GraFit as outlined in *Materials and Methods*. Unbound Michaelis constant ($K_{m,u}$) and unbound intrinsic clearance ($CL_{int,u}$) values were reported by incorporating $f_{u,inc}$ data that were obtained experimentally. $f_{u,inc}$ was determined to be 0.674 $\pm$ 0.089, 0.335 $\pm$ 0.038 and 0.369 $\pm$ 0.047 at pH 7.4, 8.4 and 9.4, respectively. N/A represents data that was not obtainable because saturation was not observed at the highest substrate concentrations examined. [b] Units for $K_{m,u}$ and K_{si} are μM, V_{max} is pmol/min/mg prot and $CL_{int,u}$ is μL/min/mg microsomal protein. One star (*) represents $p < 0.01$, and two stars (**) represent $p < 0.001$, compared against physiological pH.

to distinguish between the various possible mechanisms. However, one possible reason may be due to the generation of multiple glucuronide metabolites from these substrates which may impede each other's glucuronidation. Another reason may be due to the limited mechanistic knowledge of UGTs resulting in lack of appropriate modeling to describe such kinetics.[35] Interestingly, although MPAG followed typical Michaelis−Menten kinetics at pH 7.4 and pH 6.4, substrate inhibition kinetics was observed at pH 5.4. K_{si} was more than 40-fold greater than $K_{m,u}$. These data imply that the affinity to various binding sites may be modulated by pH, which is in agreement with previous literature reports.[23]

Altering the incubation pH had no effect on the kinetic parameters for EZG formation. On the contrary, compared to $CL_{int,u}$ observed at pH 7.4, MPA glucuronidation increased 10-fold as the pH was reduced to 5.4; whereas the formation of R4G and R6G increased 11- and 12-fold at pH 9.4, respectively. In fact, the magnitude of heightened activity was such that glucuronidation at these "optimal" pHs produced higher $CL_{int,u}$ than when alamethicin was present at pH 7.4, suggesting that alamethicin is only partially able to remove latency and does not fully activate the microsomes to attain maximum activity. Kinetic analysis revealed that there was less than 2-fold change associated with V_{max} and that the enhanced activity was mainly driven by the reduction

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Table 3. Summary of Ezetimibe Glucuronidation Kinetics in HLM at pH Ranging from 5.4 to 8.4[a]

			incubation pH			
	activating agents	parameters[b]	5.4	6.4	7.4	8.4
EZG	none (control)	$K_{m,u}$	N/A	2.90 (0.43)	1.51 (0.32)	1.24 (0.11)
		V_{max}		2.74 (0.19)	2.86 (0.09)	2.93 (0.09)
		K_{si}		64.3 (13.2)**	276 (78)	162 (30)**
		$CL_{int,u}$		0.94 (0.16)	1.89 (0.17)	2.36 (0.22)
	alamethicin	$K_{m,u}$	N/A	1.91 (0.12)	1.58 (0.14)	1.60 (0.13)
		V_{max}		7.86 (0.72)	11.2 (1.0)	10.4 (0.31)
		K_{si}		109 (41)	178 (35)	263 (64)
		$CL_{int,u}$		4.12 (0.97)	7.08 (0.65)	6.49 (0.55)

[a] Values are reported as mean (SD). The kinetic parameters were obtained with GraFit as outlined in *Materials and Methods*. Unbound Michaelis constant ($K_{m,u}$) and unbound intrinsic clearance ($CL_{int,u}$) values were reported by incorporating $f_{u,inc}$ data that were obtained experimentally. $f_{u,inc}$ was determined to be 0.734 ± 0.097 and was independent of pH. N/A represent data that were not obtainable because saturation was not observed at the highest substrate concentrations examined. No kinetic data was obtained at pH 9.4 because ezetimibe was unstable at this pH. [b] Units for $K_{m,u}$ and K_{si} are μM, V_{max} is nmol/min/mg prot and $CL_{int,u}$ is mL/min/mg microsomal protein. One star (*) represents $p < 0.01$ and two stars (**) represent $p < 0.001$, compared against physiological pH.

of $K_{m,u}$, indicating that the modified incubation pH improved the affinity of the substrate to the enzyme. This is in contrast to the mechanism reported for activating agents such as alamethicin where it has been shown that higher $CL_{int,u}$ is primarily a V_{max} effect.[36]

One possible explanation for the lower $K_{m,u}$ may be that the ionization of key amino acids that make up the catalytic site is sensitive to shifting incubation pH to affect substrate affinity for UGTs. It has been previously shown that UGT1A1 and UGT1A9 exhibit optimal activity at pH 6.4 and pH 7.6, respectively.[23] Consequently, in the present study, highest activity for MPA glucuronidation with recUGT1A9 was observed at pH 7.4 (Figure 3C). Interestingly however, high rate of glucuronidation was also observed at pH 5.4. Conversely, highest rate of raloxifene glucuronidation for both recUGT1A1 and recUGT1A9 was found at a different pH of 9.4 (Figure 4C and 5C). If one optimal pH exists for a particular isoform, its activity should peak at one specific pH, regardless of the substrate. Instead, these data show that the effect of pH shift on the rate of glucuronidation in HLM was similar to recUGT in a substrate-specific manner. Discrepancies in experimental artifacts have been reported between microsomes and recombinant enzymes, such as the lack of effect of alamethicin on recUGT activities.[37,38] Among many things, differences between the two matrices may be due to protein−protein interactions that are absent in single expression systems, as it has been shown that hetero-dimerization of UGTs affects glucuronidation activity and substrate selectivity.[39,40] This may be contributing to the disparity observed between HLM and recUGT1A9 data for MPA. Despite such difference, both microsome and recombinant data indicate that even though optimum pH exists for various UGT isoforms, it only partially addresses the pH dependence observed with MPA and raloxifene glucuronidation.

In addition to better enzyme affinity, improved membrane permeability may also contribute to decreases in $K_{m,u}$. It is assumed that the biological barrier established by the ER membrane allows only a fraction of the substrate to enter the ER lumen to interact with the UGT active site. Therefore, as the permeability is enhanced, the concentration of the substrate within the microsomal lumen would increase, thereby pressing the reaction forward by increasing the formation rate of the substrate−UGT complex. Meanwhile, the dissociation rate of the substrate−UGT complex reverting back to the substrate could diminish, to again favor the forward direction of generating more glucuronide metabolites. There are at least two ways in which altering the incubation pH can affect membrane permeability. First, adjusting the incubation pH can neutralize the charge on the acidic and basic compounds. This would increase membrane permeability because although the movement of charged compounds is poor, neutral compounds are more readily able to move across biological membranes. The pK_a values for the carboxylic functional group on MPA and piperidine nitrogen on raloxifene are projected to be 4.8 and 8.5, respectively. Theoretical calculations predict that the ratio of charged species versus noncharged species at physiological pH is 400:1 and 12:1 for MPA and raloxifene, respectively. In the case with MPA, the acidic pH neutralizes the molecule

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and at pH 5.4, the ratio is reduced to 4:1. As a result, consistent with the proposed hypothesis, MPA glucuronidation is increased. Similarly, the ratio for raloxifene becomes 1:8 at pH 9.4 as the ions dissociate so that the majority of compounds are noncharged. The result was an increase of raloxifene glucuronidation.

Second, shifting the incubation pH may enhance membrane permeability by changing the substrate binding to membrane channels. It has been proposed that membrane channels may facilitate the passage of charged compounds into the ER lumen.[20] Also, it has been shown that the mechanism behind activating microsomes with alamethicin is to form channels within the microsomal membrane.[16,17] Channels typically express ion selectivity which is propelled by electrostatic interactions mediated by the charge on key residues. Studies have shown that modifications of such residues have resulted in either loss or switch of ion selectivity. A pertinent example is with alamethicin where the substitution of the key glutamine residue at position 18 with lysine resulted in a switch of selectivity from cationic to anionic substrates.[16] Further studies with this lysine 18 mutant demonstrated that selectivity can be controlled by altering the buffer pH, where pH $\leq$ 7.4 preferred anions and pH > 11 preferred cations.[41] In addition to the ionization of key membrane residues, it has been proposed that the charge on or near the surface of the channels may also contribute to selectivity by affecting the chemical environment to favor the accumulation of a particular ion.[42] Thus, changes in surface charge may also affect the potential gradient across the channel. Since the surface charge on the membrane is exposed to the surrounding matrix, its ionization state may be sensitive to the incubation pH. Consequently, it has been demonstrated that the binding affinity is enhanced at optimal pH for capsaicin and succinate channels.[43,44] It is not yet clear if the pH modulates the charge of the channel itself or on the surface membrane or both. However, these studies are evidence which demonstrates that binding affinity to channels can be enhanced by pH.

There are various *in vitro* tools available to study drug metabolism. Among them, microsomes offer advantages of convenience, ease of use and lower cost. In addition, since microsomes can be produced in bulk and stored at length, it can provide a fixed matrix for the long term whose enzymatic expressions and activities are set. Despite its benefits, latency observed with UGT enzymes has limited the use of microsomes to characterize compounds that undergo glucuronidation. There have been many methods proposed to remove latency. One common practice has been to add alamethicin to the incubation mixture.[14,15] However, although alamethicin was able to enhance glucuronidation, it still considerably underpredicted *in vivo* glucuronidation for many compounds.[45] Recently, addition of albumin has been put forward to enhance glucuronidation.[46,47] While it has been demonstrated that albumin improves *in vivo* glucuronidation prediction for few compounds, a detailed study has suggested that its effect may be selective, as only UGT1A9 activity, but not UGT1A1 nor UGT1A6 activities, was increased when using 4-methylumbelliferone as a substrate.[47] It remains to be seen what the scope of the albumin effect would be and its impact across many substrates. In the present work, microsomal glucuronidation was activated by modifying the incubation pH to tailor to the physicochemical properties of the model substrates. As a result, at least 7-fold increases in MPA and raloxifene glucuronidation were observed. In fact, the pH shift had a larger impact than when microsomes were activated by alamethicin at physiological pH, suggesting that alamethicin was not sufficient to completely release UGT activity. Since microsomal glucuronidation oftentimes underpredicts *in vivo* glucuronidation, the higher rate of glucuronidation may give better *in vivo* correlation. Studies are in progress to further examine this pH effect with more compounds whose *in vivo* data are available.

In summary, the effect of incubation pH on the glucuronidation of three compounds that exist in distinct charged state at physiological pH was investigated. By altering the pH of the *in vitro* incubations, elevated levels of MPA and raloxifene glucuronidation were observed; whereas ezetimibe glucuronidation was not changed. The present data suggested that the higher activity may be attributed not only to the optimization of the UGT catalytic site but also to improvements with substrate membrane permeability. The effect of pH on membrane permeability seems to be multifaceted. First, optimizing the pH can neutralize the charge on the molecule to enhance its membrane permeability. Second, pH may increase the binding affinity of ion channels that already show selectivity for a particular charge. Third, pH may allow for the ion selectivity to broaden or to switch for microsomal membrane channels that previously showed selectivity for the opposite charge. For example, at acidic incubation conditions, the charge on MPA would be neutralized. At the same time, the ionization of the positively charged channel that favors anions would increase to yield higher activity, while the ionization of

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the negatively charged channel would neutralize to broaden its ion selectivity. It is not yet known the extent of impact these various parameters have on the overall glucuronidation activity. Nonetheless, data presented here show that the impact of substrate membrane permeability may be as important as pH optimization of the UGT catalytic site.

Abbreviations Used

UGT, UDP-glucuronosyltransferase; recUGT, recombinant UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; CYP, cytochrome P450; ER, endoplasmic reticulum; R4G, raloxifene 4′-glucuronide; R6G, raloxifene 6-glucuronide; EZG, ezetimibe glucuronide; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide; $f_{u,inc}$, fraction unbound in the *in vitro* incubation; HLM, human liver microsomes; $K_{m,u}$, unbound Michaelis constant; V_{max}, maximum velocity; K_{si}, substrate inhibition constant; $CL_{int,u}$, unbound intrinsic clearance; LOQ, lower limit of quantitation.

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