Effect of Intestinal Glucuronidation in Limiting Hepatic Exposure and Bioactivation of Raloxifene in Humans and Rats

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Raloxifene (Evista) is a second generation selective estrogen receptor modulator used in the treatment of osteoporosis and for chemoprevention of breast cancer. It is bioactivated to reactive intermediates, which covalently bind to proteins and form GSH conjugates upon incubation with NADPH and GSH-supplemented human and rat liver microsomes. Despite these in vitro findings, no major raloxifene-related toxic events have been reported upon its oral administration to humans. This disconnect between safety of raloxifene and its in vitro bioactivation is attributed to its presystemic metabolism via glucuronidation. Current studies investigated the effect of hepatic and intestinal glucuronidation in modulating hepatic availability of raloxifene and its subsequent bioactivation, in vitro. The study design involved preincubation of raloxifene with intestinal microsomes followed by a sequential incubation with liver microsomes. The degree of bioactivation of raloxifene was assessed from the percentage of GSH conjugate formed in liver microsomal incubations or the amount of covalent binding of raloxifene-related material to liver microsomal proteins. The results indicated that human intestinal glucuronidation limited the hepatic exposure of raloxifene that underwent bioactivation in the liver. Similar experiments with rat microsomal preparations showed very little effect of intestinal glucuronidation. This effect of intestinal glucuronidation and the observed species difference were explained by comparing the efficiency (Cl_{int}) of glucuronidation and oxidation in the two species. These findings suggested that even though the rate of bioactivation in the two species was similar, the Cl_{int} of glucuronidation was 7.5-fold higher in the human intestine as compared to rats. These results support the hypothesis that intestinal glucuronidation modulates the amount of raloxifene undergoing bioactivation by liver and corroborate the importance of assessing other competitive metabolic pathways and species differences in metabolism prior to extrapolation of bioactivation results from rats to humans.

Introduction

Raloxifene (Evista) is a second generation SERM approved by the Food and Drug Administration for the prevention and treatment of osteoporosis in postmenopausal women (*1*−*3*). It also exhibits strong antiestrogenic effects on breast tissue and the endometrium and is currently prescribed for the chemoprevention of breast cancer (*4*).

Earlier in vitro studies on the bioactivation of raloxifene using NADPH-supplemented human (HLMs)[1] or rat liver microsomes (RLMs) have demonstrated the propensity of this drug to undergo CYP3A4-mediated metabolic activation, leading to several reactive intermediates. These intermediates have been trapped by nucleophiles such as glutathione (GSH) or *N*-acetylcysteine (NAC), and the corresponding GSH and NAC adducts have been identified (*5, 6*). The studies have also demonstrated that raloxifene is a suicide inhibitor of CYP3A4. This mechanism-based inactivation is probably mediated through a reactive metabolite formed by metabolic activation of raloxifene since addition of GSH attenuates the degree of inactivation. Several studies demonstrating the modification of amino acids of the CYP3A4 active site have also been published recently (*7, 8*).

Despite these observations of in vitro bioactivation, raloxifene is very well-tolerated in patients (*9*). The only adverse effects seen with raloxifene were hot flashes, vaginal dryness, and leg cramps. More serious but less frequent adverse effects include thromboembolic events such as deep venous thrombosis, pulmonary emboli, and retinal vein thombosis. These side effects are probably due to target pharmacology of the drug since all drugs belonging to this class, for example, tamoxifen, toremifene, and arzoxifene, exhibit this side effect profile (*10*). Also, no significant drug−drug interactions with CYP3A4 have been reported in the clinic following oral administration of raloxifene despite the inactivation of the enzyme in vitro.

The human pharmacokinetics of raloxifene is characterized by extensive metabolism, mainly via glucuronidation, resulting in 2% bioavailability (*9*−*11*). This low bioavailability is due to first pass metabolism of raloxifene. Two glucuronides, the 4′-β-glucuronide (4-R-G) and 6-β-glucuronide (6-R-G), have been detected in humans of which the 4-R-G is present in higher concentrations (8-fold higher) than 6-R-G (*12*). No oxidative metabolites or its GSH or NAC adducts, which were detected

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[1] Abbreviations. GSH, reduced glutathione; NAC, *N*-acetylcysteine; NADPH, UDPGA, uridine-5′-diphosphoglucuronic acid; UGT, uridine 5′-diphosphoglucuronosyl transferase; G1, glutathione conjugate of raloxifene; M1 and M2, glutathione conjugate of raloxifene; HIM, human intestinal microsome; RIM, rat intestinal microsome; HLM, human liver microsome; RLM, rat liver microsome.

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in the in vitro studies, have been observed in circulation or excreta of the human subjects (*11, 12*).

The disparity between the in vitro bioactivation liability of raloxifene and its good safety record in humans is possibly attributed its rapid detoxication via intestinal glucuronidation in vivo. Even though published reports on metabolism and disposition of raloxifene suggest that presystemic glucuronidation of raloxifene serves as a protective mechanism against its hepatic bioactivation, this has never been explored. Thus, the primary objective of the current study was to investigate the effect of hepatic and intestinal glucuronidation in modulating hepatic availability of raloxifene and its subsequent bioactivation using microsomal preparations. The second objective of the study was to gain insight on the differences in the protective effect of glucuronidation between rats and humans. Although pharmacokinetic and metabolism studies of raloxifene in rats suggest that it is primarily cleared via glucuronidation in this species like humans (*13*), its thioether conjugates (GSH and NAC adducts) have been observed in the bile and urine of rats dosed with 5 mg/kg raloxifene (*5*). Because these in vivo studies have demonstrated the bioactivation potential of raloxifene in rats, the current study was aimed at comparing the differences in glucuronidation and bioactivation pathways between the two species.

Experimental Procedures

Caution: *Because of its potent pharmacological activity, raloxifene is hazardous and should be handled carefully*.

Materials. Raloxifene, GSH, magnesium chloride ($MgCl_2$, 1 M solution), alamethicin, uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), and β-nicotinamide adenine dinucleotide 2′-phosphate, reduced (NADPH), were obtained from Sigma/Aldrich (St. Louis, MO). [^{14}C]Raloxifene was prepared by custom synthesis by Nerviano Medical Sciences (Nerviano, Italy). The specific activity was 52 mCi/mmol, and the radiochemical purity was 99%. Pooled human intestinal microsomes (HIMs) (protein concentration, 10 mg/mL) were purchased from Celsis In Vitro Technologies (Baltimore, MD). Pooled male rat intestinal microsomes (RIMs) (protein concentration, 10 mg/mL) and pooled male RLMs (protein concentration, 20 mg/mL) were obtained from XenoTech Laboratories (Lenexa KS). Pooled HLMs were prepared under contract by BD Gentest (Woburn MA). Aliquots from the individual preparations from 56 individual human livers were pooled on the basis of equivalent protein concentrations to yield a representative microsomal pool with a protein concentration of 20.4 mg/mL (determined using the Bicinchoninic Acid Assay; Pierce, Rockford, IL). A 3 mM stock solution of raloxifene was prepared using DMSO and acetonitrile as solvents, and this was used for all incubations. Alamethicin was dissolved in ethanol at a concentration of 20 mg/mL. Other chemicals and reagents were of the highest quality available.

Incubations of Raloxifene with Rat or Human Liver, Intestinal, and a Mixture of Intestinal and Liver Microsomes in the Presence of NADPH, GSH, and UDPGA. All microsomal incubations (total volume, 1.0 mL) were conducted at 37 °C for 45 min in 100 mM potassium phosphate buffer (pH 7.4) containing magnesium chloride (10 mM); liver, intestinal, or a mixture of liver and intestinal microsomes, mixed in equal proportions (1.0 mg/mL); alamethicin (10 μg/mg protein); and NADPH (2 mM); GSH (3 mM), with or without (controls) UDPGA (3 mM). Reactions were initiated by the addition of raloxifene (10 μM) after preincubation of the incubation mixture for 5 min. The final concentration of DMSO in the incubation media was <0.1%, and the final concentration of all organic solvents in the incubation mixture was <1%. Incubations that lacked NADPH, GSH, or microsomes served as negative controls. Reactions were terminated with 1 mL of acetonitrile and centrifuged at 3000*g* for 10 min.

For metabolite profiling, the resulting supernatant was removed and evaporated to dryness under a steady stream of nitrogen. The residue was reconstituted in 30% acetonitrile in 0.1% formic acid (200 μL), vortex mixed, and centrifuged. Aliquots (30 μL) of the final supernatant were analyzed as described in the HPLC/MS analysis section.

For the determination of relative amounts of GSH conjugate (G1, glutathione conjugate of raloxifene) formed and the amount of raloxifene remaining, the incubation mixture was quenched with acetonitrile (1 mL) containing buspirone (10 μM), used as an internal standard (IS). An aliquot (10 μL) was then analyzed using the method described in the HPLC/MS analysis section. The amount of G1 formed was determined by estimating the G1/IS peak area ratio, and the value obtained in incubations containing UDPGA was normalized to the value obtained in the incubations that lacked UDPGA. The percent of raloxifene remaining in the incubation mixture was also determined in a similar manner. All experiments were conducted in duplicate. The percent change in G1 formed or raloxifene remaining was determined using eq 1:

$$\%\ \text{decrease in G1} = \%\ \text{of G1 formed in the absence of UDPGA} - \%\ \text{of G1 formed in the presence of UDPGA} \quad (1)$$

Studies on Effect of Human and Rat Intestinal Glucuronidation on the Metabolic Activation of Raloxifene by HLMs and RLMs. The effect of intestinal glucuronidation on bioactivation of raloxifene by liver microsomes was determined by using an experimental design depicted in Scheme 1. Raloxifene (10 μM) was preincubated with a mixture of potassium phosphate buffer (100 mM, pH 7.4), magnesium chloride (10 mM), HIMs or RIMs (0.3 mg/mL), alamethicin (10 μg/mg protein), NADPH (2.0 mM), GSH (3 mM), and UDPGA (3 mM). The reaction was initiated by the addition of raloxifene to the incubation mixture, which was prewarmed at 37 °C for 5 min. At selected preincubation times of 0, 5, 15, and 30 min, a 100 μL aliquot of this preincubate was added to an incubation mixture (final volume, 1 mL) containing potassium phosphate buffer (100 mM, pH 7.4), magnesium chloride (10 mM), HLMs or RLMs (1.0 mg/mL), alamethicin (10 μg/mg protein), NADPH (2.0 mM), GSH (3 mM), and UDPGA (3 mM) that was prewarmed at 37 °C for 5 min. After 45 min, the mixture was quenched with acetonitrile containing 10 μM buspirone (IS, 1 mL) and centrifuged at 3000*g* for 10 min. A 10 μL aliquot of the supernatant was analyzed for G1 formation and unchanged raloxifene, as described in the HPLC/MS analysis section. The percent of G1 formed or raloxifene remaining in the incubations at 0, 5, 15, and 30 min was estimated from peak area ratios of the respective analytes to IS and normalizing the values at 5, 15, and 30 min to those obtained at 0 min.

Control experiments were performed by preincubating raloxifene (10 μM) with HIMs and RIMs in the absence of UDPGA. The conditions and the final concentrations of all other reagents were similar to that described above. A 100 μL aliquot of this preincubation mixture was then added to rat or human liver microsomal incubation mixture containing all reagents described in the liver microsomal incubation above.

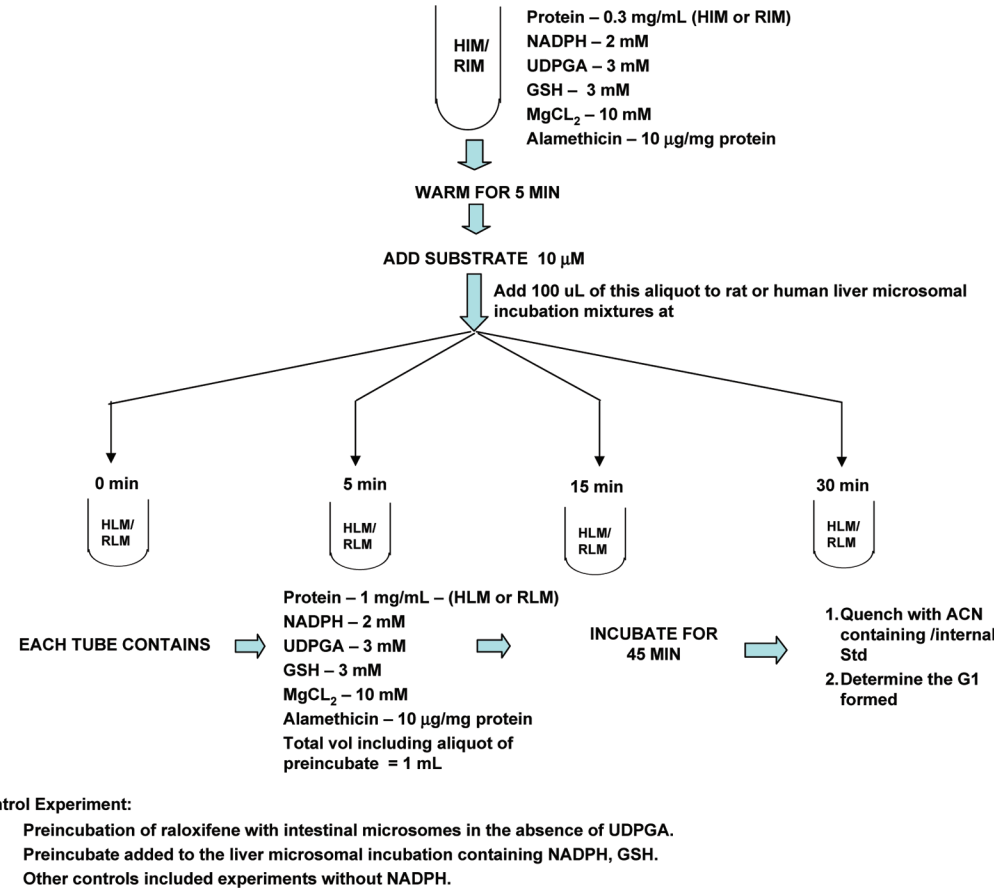
The percentage decrease in G1 formation was determined using Equation 2

$$\%\ \text{decrease in G1} = \frac{[\text{amount of G1}]_{\text{control}} - [\text{amount of G1}]_{\text{UDPGA}}}{[\text{amount of G1}]_{\text{control}}} \times 100 \quad (2)$$

$[\text{Amount of G1}]_{\text{control}}$ is the relative amount of G1 formed at 0, 5, 15, and 30 min in the control (absence of UDPGA in the preincubation with intestinal microsomes), and $[\text{Amount of G1}]_{\text{UDPGA}}$ is the relative amount of G1 formed at 0, 5, 15, and 30 min in incubations containing UDPGA in the preincubation with intestinal microsomes. All experiments were conducted in duplicate.

Effect of Glucuronidation on Covalent Binding of [^{14}C]Raloxifene-Related Material to Liver Microsomes. Covalent binding experiments were done in two parts. Human intestinal or liver

Scheme 1. Experimental Design To Demonstrate the Effect of Intestinal Glucuronidation on Bioactivation of Raloxifene by the Liver Microsomes



microsomes (0.3 mg/mL) were treated with alamethicin (0.015 mg/mL) in 0.1 M KH_2PO_4, pH 7.5, containing 3.3 mM $MgCl_2$ on ice for 15 min. [^{14}C]Raloxifene (9 μM) was added, and the incubations were prewarmed to 37 °C for 5 min. The first incubation was commenced with the addition of either water (control), NADPH (1.0 mM), or a mixture of NADPH and UDPGA (1.0 and 3.0 mM, respectively), and the total incubation volume was 0.1 mL. Incubations were carried out for 15 min, shaking open to the air. To these incubation mixtures was added 1 mL of HLMs (2.0 mg/mL) in 0.1 M KH_2PO_4, pH 7.5, containing 3.3 mM $MgCl_2$ and either water, NADPH (1.0 mM), or a mixture of NADPH and GSH (1.0 and 3.0 mM, respectively). This second incubation was conducted for 20 min at 37 °C shaking open to air.

The incubations were terminated with the addition of 5.5 mL of CH_3CN and vigorously mixed, followed by spinning in a centrifuge at 1700*g* for 5 min. The supernatants were decanted, and the pellet was resuspended in 1 mL of water, followed by the addition of 5 mL of CH_3CN/CH_3OH (4:1), vigorous mixing, and spinning as before. This washing procedure was repeated two more times. Subsequently, the pellets were washed three more times using 5 mL of CH_3CN. The supernatnants after this washing procedure were sampled and shown to possess radioactivity at the level of background. The final pellets were dissolved in 1 mL of NaOH (1 M) at 37 °C overnight. The resulting solutions were subjected to liquid scintillation counting in 18 mL of Ultima Gold scintillation fluid (Packard). All experiments were done in triplicate.

Enzyme Kinetics. Kinetic studies were performed to determine the efficiencies (Cl_{int}) of glucuronidation and bioactivation of raloxifene in the gut and liver microsomes of human and rat. Authentic standards for raloxifene glucuronides and glutathione conjugates were not available, so a two-step approach was used to determine the Cl_{int} for these biotransformation pathways. In the first step, estimates for K_M were made by monitoring the peak area intensities for the protonated molecular ions for raloxifene glucuronides and GSH conjugate G1 on HPLC/MS at varying raloxifene substrate concentrations. In the second step, first-order substrate depletion rates were measured at a concentration of raloxifene below the experimentally estimated K_M values, in liver and intestinal microsomes containing various cofactors (i.e., UDPGA or NADPH) to determine Cl_{int} values.

In Vitro Incubation Conditions. All incubations were conducted in triplicate, in 96 well cluster tubes (1.2 mL; Corning Life Sciences, Acton, MA), by shaking reaction mixtures in air in a heated water bath at 37 °C. Incubations were carried out in 100 mM phosphate buffer (pH 7.4), containing gut or liver microsomes from the rat or humans (final concentration of 0.6 mg of microsomal protein/mL), 3 mM $MgCl_2$, 3 mM GSH, alamethicin (10 μg/mg protein), and raloxifene (dissolved in methanol). The final concentration of organic solvent in the incubation mixture was 1%. All reactions were initiated by adding either NADPH (1 mM) or UDPGA (3 mM).

Estimation of K_M Values. Incubation times and protein concentrations were selected that had provided a linear reaction velocity for the formation of both of the products in preliminary experiments. Incubation mixtures containing 0, 1, 3, 5, 8, 10, 12, 15, and 25 μM raloxifene (final volume of the incubation mixture was 100 μL) were prewarmed for 5 min in a water bath at 37 °C, and reactions were initiated by adding either NADPH to assess G1 formation or UDPGA to assess glucuronide conjugates. All incubations (except those containing HIM and UDPGA) were terminated after 10 min by adding 100 μL of acetonitrile containing 10 μM buspirone. The reaction mixtures containing HIM and UDPGA were quenched after 5 min, as described above. The terminated reaction mixtures were centrifuged 3000*g* for 10 min, and a 10 μL aliquot of the supernatant was analyzed for G1 and the glucuronide conjugate as described in the HPLC/MS section.

The rate of formation of the two metabolites at various concentrations was expressed as the ratio of the peak areas of the

metabolites to IS, which was then normalized to the time and amount of protein used (peak area ratio of analyte to IS/min/mg protein). The K_M values for product formation were determined using nonlinear regression by fitting the reaction velocity vs substrate concentration data to the Michaelis−Menten model (eq 3) using GraphPad Prism version 5.0.

$$v = V_{max} \times [S]/(K_M + [S]) \quad (3)$$

v is the reaction velocity, V_{max} is the maximum velocity, and [S] is the substrate concentration. Even though a good estimate of the K_M of the glucuronide and GSH conjugate formation could be obtained using this approach, the V_{max} for glucuronidation or or GSH formation could not be estimated using this data. Because the analytical method did not separate the two glucuronides M1 and M2 (glutathione conjugates of raloxifene), the peak area of glucuronide conjugate measured in this study gave an aggregate of both of the conjugates and therefore an estimate of an apparent K_M of the overall glucuronidation pathway.

Determination of Cl_{int} Values. The mixtures containing raloxifene (1 μM, final concentration) were preincubated for 5 min, and reactions were initiated by addition of either NADPH or UDPGA. The reactions were terminated by adding 100 μL of the incubation mixture to 100 μL of acetonitrile containing 10 μM buspirone (IS) at 0, 2, 5 10, 15, and 20 min. The sample for the 0 min time point was taken immediately following addition of either NADPH or UDPGA. A 10 μL aliquot of the quenched incubation mixture was analyzed using the method described in HPLC/MS section. The percent substrate remaining was then estimated by dividing the raloxifene/IS peak area ratios at each of the 2, 5, 10, 15, and 20 min time points with the respective ratio at the 0 min time point and multiplying by 100%. The corresponding rate constants of substrate loss, $k_{loss\text{-}glu}$ for the glucuronidation pathway and $k_{loss\text{-}oxi}$ for the oxidation pathway, were estimated by fitting the percent of substrate remaining vs time data to eq 4 in the GraphPad Prism version 5 software package (San Diego, CA).

$$\%\ \text{raloxifene remaining} = 100 \times e^{k_{loss} \cdot t} \quad (4)$$

Intrinsic clearance was then calculated using k_{loss} data as follows (eq 5),

$$Cl_{int} = k_{loss\ (glu\ or\ oxi)} \times \frac{\text{incubation volume}}{\text{mg microsomal protein}} \quad (5)$$

and was expressed as a function of the microsomal protein (μL/min/mg protein).

HPLC/MS Analysis. Metabolite profiling and identification of metabolites in the microsomal incubations were achieved by separating the metabolites and the GSH conjugates on a Kromasil C4 100A column (3.5 μm, 150 mm × 2.0 mm, Phenomenex, Torrance, CA) by reverse phase chromatography at ambient temperature. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) and was delivered at 0.200 mL/min. The initial composition of solvent B was maintained at 1% for 5 min and then increased in a linear manner as follows: 20% at 8 min, 40% at 35 min, and 90% at 42 min. It was maintained at 90% for up to 45 min and then decreased to 1% in the next 5 min. The column was allowed to equilibrate at 1% solvent B for 5 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 5 min after sample injection.

Mass spectrometric analyses were performed on a ThermoFinnigan Deca XP ion trap mass spectrometer, which was interfaced to an Agilent HP-1100 HPLC system (Agilent Technologies, Palo Alto, CA) and equipped with an electrospray ionization source. The values for ESI were as follows: capillary temperature, 270 °C; spray voltage, 4.0 kV; capillary voltage, 4.0 V; sheath gas flow rate, 90; and auxiliary gas flow rate, 30. The mass spectrometer was operated in a positive ion mode with data-dependent scanning. The ions were monitored over a full mass range of *m/z* 125−1000. For a full scan, the automatic gain control was set at 5.0×10^8, the maximum ion time was 100 ms, and the number of microscans was set at 3. For MS^n scanning, the automatic gain control was 1.0×10^8, the maximum ion time was 400 ms, and the number of microscans was set at 2. For data-dependent scanning, the default charge state was 1, the default isolation width was 3.0, and the normalized collision energy was 40 V.

The analysis of raloxifene, G1, and glucuronide conjugates M1 and M2 in all incubations was performed using a Sciex Q-Trap 4000 (Applied Biosystems/MSD Sciex, Concord, ON, Canada) interfaced to an HPLC system consisting of Shimadzu LC-10AD VP binary pumps (Shimadzu, Columbia, MD) and a PAL system (Leap Technologies, Carrboro, NC). All three analytes were separated by injecting 10 μL of the sample on a Synergi Fusion-RP column (4 μm, 100 mm × 2.0 mm, Phenomenex, Torrance, CA). The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B), and the flow rate was 400 μL. A gradient was used for the separation and was initiated with 20% B for 1 min and then increased in a linear manner to 65% at 2.3 min and to 100% at 4.5 min. It then was maintained at 100% B for 5 min and decreased to 20% at 5.5 min. The column was allowed to equilibrate at 20% solvent B for 0.5 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 1 min after sample injection.

The mass spectrometer was fitted with a Turbo IonSpray and operated in a positive ion mode with a capillary voltage of 4.5 kV. The source temperature was set at 450 °C, declustering potential at 65 V, and the entrance potential at 10 V. The collision-activated dissociation of MH^+ ions was performed using a collision energy of 40 V. The analytes were monitored in multiple reaction monitoring mode (MRM) using the transitions of 779 → 650 (or 391 → 253 for a doubly charged ion) for G1 (retention time, 1.88 min), 650 → 473 for glucuronide conjugates (retention time, 1.98 min), 473 → 269 for raloxifene (retention time, 2.08 min), and 386 → 122 for buspirone (IS; retention time, 1.86 min). All data were processed using Analyst software 1.4.1 (Applied Biosystems, Foster City, CA) to obtain the peak area ratios (analyte/IS). The peak area for the glucuronide conjugate at the retention time of 1.98 min was an aggregate of the two conjugates M1 and M2 since these two metabolites coeluted and could not be separated by this method. The raloxifene concentrations were quantified by comparing the peak area ratios (raloxifene/IS to the peak area ratios generated with a standard curve) using linear regression. In some instances, the raloxifene/IS peak area ratios were used. The peak area ratios were then used to determine the % of each analyte remaining or formed as well as the % change, as described in individual sections.

Results

Effect of Glucuronidation on Bioactivation of Raloxifene in Incubations with Human and Rat Microsomal Preparations. Preliminary experiments to evaluate the impact of glucuronidation on the P450-mediated bioactivation of raloxifene were conducted by incubating raloxifene (10 μM) with NADPH and glutathione (GSH)-supplemented liver and intestinal microsomes from human and rat in the presence and absence of UDPGA. Incubations were also conducted with a microsomal system prepared by mixing equal amounts of pooled human or rat intestinal and liver microsomes.

The first step, however, was to assess the metabolic profile of raloxifene in these preparations. Metabolites of raloxifene were identified by analyzing aliquots of the incubation mixtures on an ion-trap mass spectrometer in positive ion mode as described in the Experimental Procedures. Figure 1A−C shows representative total ion chromatograms following in vitro incubation of raloxifene with HLMs, HIMs, and a mixture of human intestinal and liver microsomes (HIM/HLM). All major

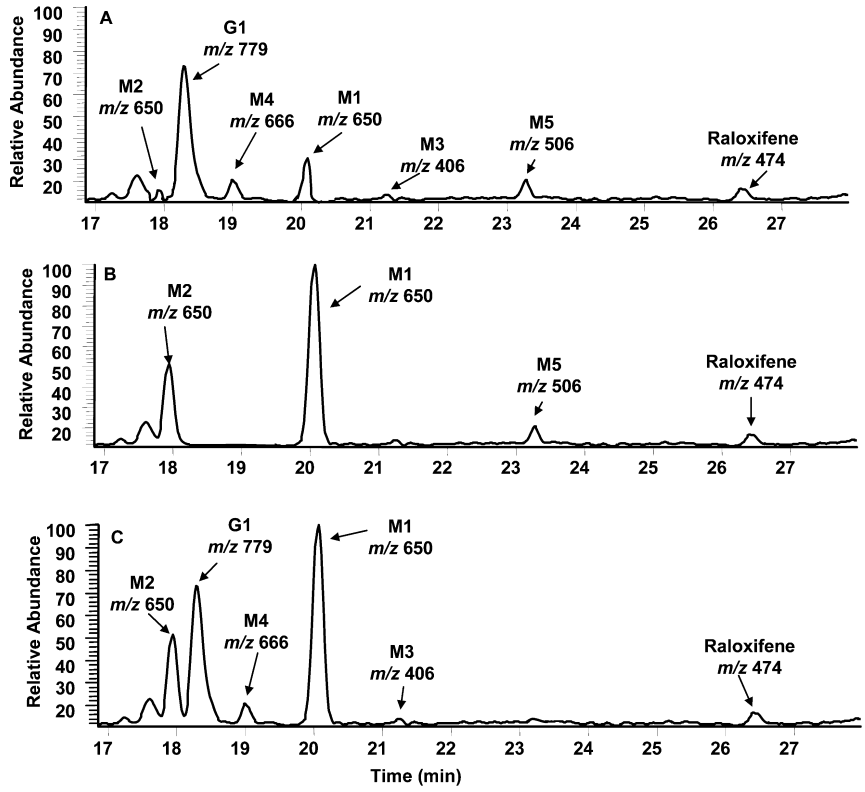
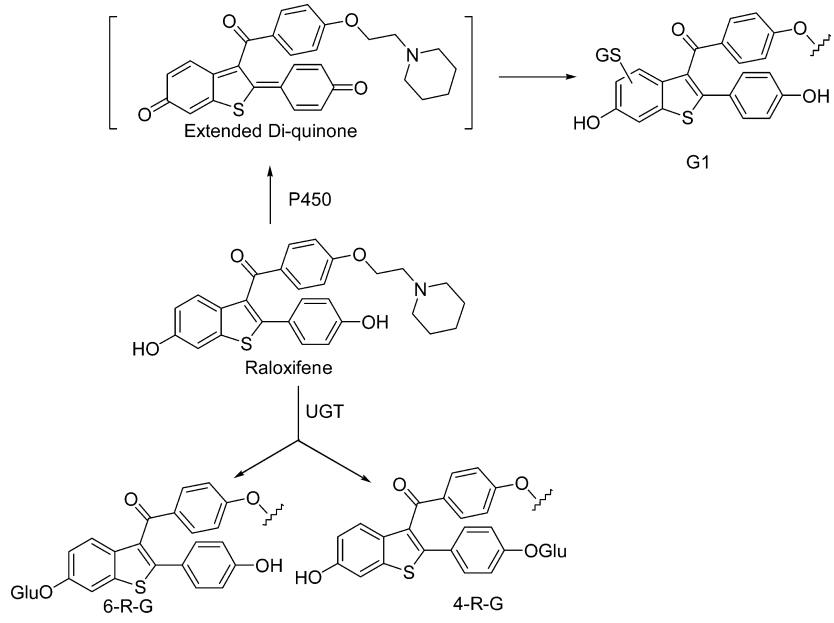


Figure 1. Total ion chromatograms following incubation of raloxifene with (A) HLMs, (B) HIMs, and (C) a mixture of human liver and intestinal microsomes mixed in a 1:1 ratio (HLM/HIM).

Scheme 2. Proposed Metabolic Pathway of Raloxifene Following Its Incubation with Rat or Human Microsomal Preparations (Liver Microsomes, Intestinal Microsomes, or a Mixture of Liver and Intestinal Microsomes) in the Presence of NADPH, GSH, and UDPGA[a]



[a] The metabolic scheme also shows the plausible reactive intermediates that could lead to the formation of the glutathione conjugate G1. All metabolites observed in this study are consistent with those observed in the previous studies (*5, 6, 14*).

metabolites of raloxifene observed in the current study were consistent with those observed in previously reported studies (*5, 6*). Scheme 2 shows the structures of major metabolites formed following an in vitro incubation of raloxifene with rat or human microsomal preparations. Metabolites M1 and M2 were identified as glucuronide conjugates of raloxifene and were absent in control incubations without UDPGA, whereas G1 was identified as the glutathione conjugate and was absent in the control incubations without NADPH or GSH. Comparison of the overall metabolic profiles from HLM and HIM incubations indicated that M1 and M2 were formed in larger amounts in the incubation with HIM than HLM, whereas metabolite G1 was formed in substantially larger amounts in the HLM incubation. Raloxifene incubation with rat liver (RLM), rat intestinal (RIM), and a mixture of rat liver and intestinal microsomes (RLM/RIM mix) also resulted in raloxifene me-

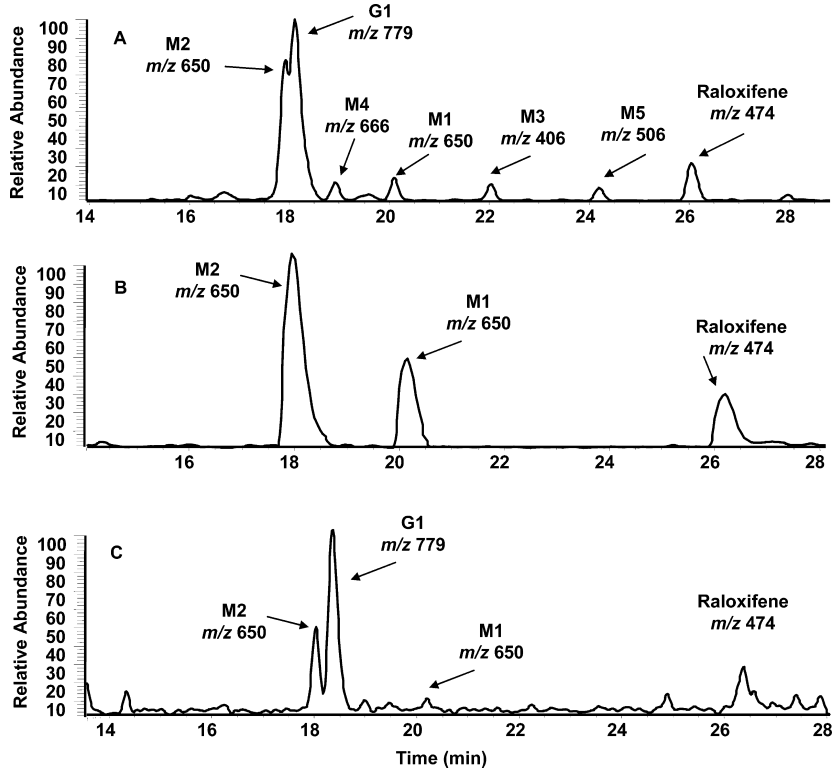


Figure 2. Total ion chromatograms following incubation of raloxifene with (A) RLMs, (B) RIMs, and (C) a mixture of rat liver and intestinal microsomes mixed in a 1:1 ratio (RLM/RIM).

tabolites that were similar to those observed in human microsomal preparations (Figure 2A−C). A comparison of raloxifene metabolic profiles from RLM and RIM incubations indicated that M2 was formed in larger amounts in incubations with both of the preparations. Metabolite M1 was only observed in incubations with RIM, whereas G1 appeared to be a major peak in RLM incubations. The minor metabolites M3, M4, and M5 (Figures 1 and 2) were oxidative (M5), cleaved (M3), or conjugative (M4, glucuronide conjugate of hydroxyraloxifene) metabolites of raloxifene, and no attempt was made to further characterize these metabolites.

In contrast to the previous reports (*5, 6*), which suggested the formation of three glutathione conjugates of raloxifene, only one peak was observed in the current study, in incubations with both species. It is possible that the chromatography conditions used in this study resulted in the coelution of all three peaks. In addition, our studies did not reveal the formation of a reported glutathione conjugate of hydroxyraloxifene. No attempts were made to resolve the isomers of G1 or to modify incubation conditions to detect the glutathione conjugate of hydroxyraloxifene.

The effect of glucuronidation on bioactivation of raloxifene was assessed by comparing the amount of G1 formed in NADPH and GSH-supplemented human and rat microsomal preparations, in the presence or absence of UDPGA. Figure 3 shows the effect of glucuronidation on G1 formation in human and rat microsomal preparations. Incubation of raloxifene with NADPH and GSH-supplemented HLM, HIM/HLM mix, and HIM preparations containing UDPGA for 45 min resulted in about 84, 22, and 21% formation of G1 relative to incubations lacking UDPGA. Similar experiments with the RLM, RLM/RIM mix, and RIM resulted in a ~96, 73, and 72% formation of G1 relative to the incubations lacking UDPGA, respectively (Figure 3). This suggested that addition of UDPGA changed the levels of G1 formed in the microsomal incubations.

The amount of unreacted raloxifene remaining in incubations of the three microsomal systems of human and rat containing UDPGA was also determined and compared to incubations lacking UDPGA. In human preparations, the percentage of raloxifene remaining was 79% in the HLM-mediated preparations and 2.0% in the preparations containing HIM (HLM/HIM mix and HIM alone) when UDPGA was added (Figure 3). Similarly, the determination of percent raloxifene remaining in UDPGA-supplemented RLM, RLM/RIM, and RIM incubations suggested that 88, 40, and 45% of the parent was left over in these reactions after 45 min when compared to incubations lacking UDPGA (Figure 3). The greater consumption of raloxifene in UDPGA-supplemented incubations was probably due to glucuronidation of the substrate relative to bioactivation.

Effect of Intestinal Glucuronidation on Bioactivation of Raloxifene by RLMs or HLMs. A method involving two sequential incubations was used to evaluate the effect of intestinal glucuronidation on bioactivation of raloxifene by liver. In this experiment, raloxifene was preincubated with HIM or RIM containing NADPH, GSH, and UDPGA first. At various times after starting the incubation, a small aliquot was transferred to a tube containing HLM or RLM that was supplemented with NADPH, GSH, and UDPGA (Scheme 1). This allowed only the amount of unreacted raloxifene in the preincubate to undergo metabolic activation by P450 in the liver microsomes and therefore helped to evaluate the impact of intestinal glucuronidation on the bioactivation of raloxifene by liver microsomes. As described before, the degree of bioactivation of raloxifene was assessed by the amount of G1 formed in the liver microsomal incubations. The percentage of raloxifene remaining in the preincubation mixture was also determined to gauge the correlation between the amount of G1 formed and the unchanged parent.

To properly determine the effect of intestinal glucuronidation, it was crucial to ensure that the amount of intestinal microsomes

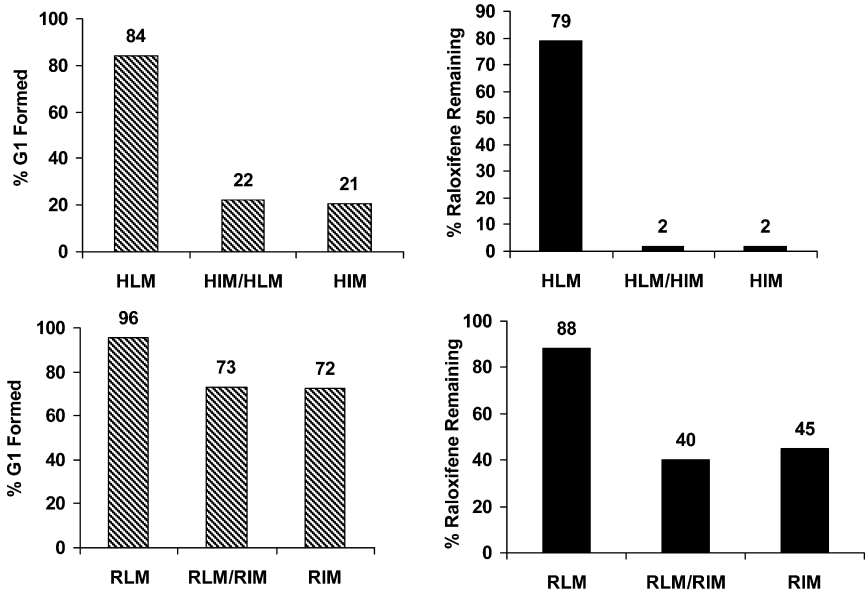


Figure 3. Percentage of glutathione conjugate G1 formed and raloxifene remaining in humans and rats after incubation of raloxifene with human and rat microsomal preparations in the presence UDPGA. The amount of G1 formed and raloxifene remaining in the presence of UDPGA is normalized to amounts in incubations containing NADPH and GSH but without UDPGA. HIM/HLM, a mixture of human intestinal and liver microsomes (mixed in a 1:1 ratio). RLM/RIM, a mixture of rat liver and intestinal microsomes (mixed in a 1:1 ratio). Data presented here are an average of two experiments.

in the aliquot of preincubate would not affect the oxidative metabolism of raloxifene by P450 and GSH conjugation. To achieve this condition, only 10% of the preincubation mixture was transferred to the vial containing the liver microsomal incubation mixture, thus diluting the intestinal microsomes by 10-fold. The control experiment lacked UDPGA in the preincubation step with intestinal microsomes. This eliminated the formation of glucuronide conjugates M1 and M2 in the intestinal microsomal preparations. All of the other controls that lacked either NADPH or GSH in liver microsomes did not produce G1.

Figure 4A shows the effect of preincubation of raloxifene with HIM on hepatic bioactivation in the presence or absence of UDPGA. When 10 μM raloxifene was preincubated with HIM containing UDPGA for 5 or 15 min, about 48 and 10% of G1 was formed relative to the amount of G1 formed without preincubation ($t_{\text{preincubation}} = 0$ min) (Figure 4A). No G1 was detected when raloxifene was allowed to preincubate with HIM for 30 min. Similar experiments that lacked UDPGA while incubating raloxifene with HIM resulted in 89% G1 formation (relative to $t_{\text{preincubation}} = 0$ min) upon preincubation for 5−30 min (Figure 4A). A decrease (after 5 and 15 min of preincubation) in the control experiment was probably due to the residual HIM that was present in the second incubation with HLM. Overall, a 46−89% reduction in G1 formation was observed when raloxifene was preincubated for 5−15 min with HIM containing UDPGA relative to HIM preincubations that lacked UDPGA (Table 1).

With rat preparations, preincubation of raloxifene with RIM containing NADPH, GSH, and UDPGA for 5 and 15 min resulted in about 82 and 58% of the G1 formation as compared to the amount of G1 formed in the absence of preincubation ($t_{\text{preincubation control}} = 0$ min) (Figure 4B). The control experiments that lacked UDPGA (but contained the remaining cofactors) resulted in 95 and 93% formation of G1, respectively. This resulted in a 14−38% decrease in G1 formation relative to the control (Table 1).

Assessment of the corresponding raloxifene concentrations in these experiments indicated that about 61 and 13% of the

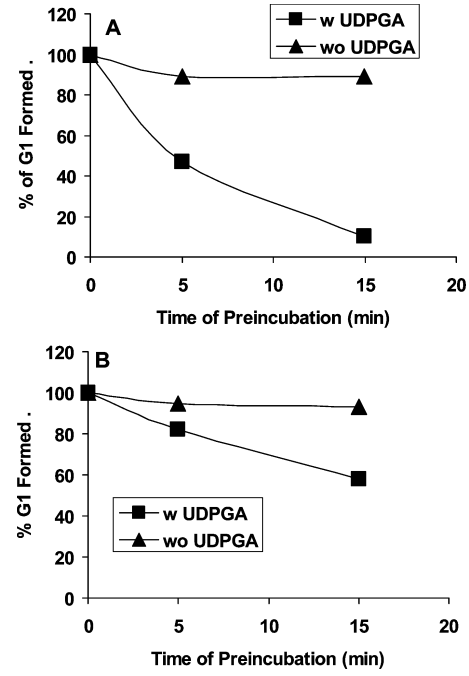


Figure 4. Effect of intestinal glucuronidation on the formation of the glutathione conjugate (G1) by liver microsomes. (A) Preincubation of raloxifene with HIMs with and without UDPGA, followed by a second incubation of the preincubate with HLMs. (B) Preincubation of raloxifene with RIMs followed by a second incubation of the preincubate with RLMs. Experiments were conducted as depicted in Scheme 1. Data presented here are an average of two experiments. The amount of G1 formed was determined as described in the Experimental Procedures. The values of G1 formed in the liver microsomal incubation following preincubation with intestinal microsomes containing UDPGA were normalized to the values of G1 formed in the liver microsomal incubation following preincubation of raloxifene with intestinal microsomes in the absence of UDPGA.

substrate remained at 5 and 15 min of incubation with HIM in the presence of UDPGA, while 80 and 70% of unchanged raloxifene was left over after preincubation with RIM for 5 and 15 min, respectively. On the other hand, in incubations lacking UDPGA, ~98% of unreacted raloxifene was present after 15

Table 1. Percentage Decrease in G1 Formation upon Preincubation with HIMs and RIMs Containing UDPGA[a]

preincubation with HIM containing UDPGA at time (min)	% decrease in G1 formation relative to control in human preparations	% decrease in G1 formation relative to control in rat preparations
5	46	14
15	89	38

[a] The percentage decrease was calculated as described in the Experimental Procedures.

min. This indicated that the amount of raloxifene consumed in the intestinal microsomal incubations correlated inversely with the amount of G1 formed following subsequent incubation with liver microsomes.

Effect of Intestinal and Hepatic Glucuronidation on Covalent Binding of [^{14}C]Raloxifene-Related Material to Pooled HLMs. The effect of intestinal glucuronidation on P450-mediated bioactivation of raloxifene by liver microsomes was also assessed by determining the amount of raloxifene-related material that was covalently bound to the proteins. For ease of handling radioactivity, the preincubation method used in determining the amount of G1 formed was slightly modified as described in the Experimental Procedures. Figure 5A shows the effect of intestinal glucuronidation on covalent binding of [^{14}C]raloxifene-related material to microsomal proteins. Preincubation of [^{14}C]raloxifene with HIM in the absence of UDPGA resulted in covalent incorporation of 26 pmol of raloxifene-related material into the microsomal protein after sequential incubation of preincubate with NADPH-supplemented HLMs (Figure 5A, bar B). Irreversible binding observed was due to P450-mediated metabolism of raloxifene (as shown previously) since there was minimal covalent binding (5 pmol of raloxifene-related material) of radioactivity in the absence of cofactors (Figure 5A, bar A). The amount of covalent binding decreased to 13 pmol when GSH was added along with NADPH and HLM, indicating a 50% reduction in covalent binding (Figure 5A, bar C). However, the addition of UDPGA along with NADPH (with or without GSH) to the preincubation of [^{14}C]raloxifene with HIM resulted in an 87−90% reduction of unextractable raloxifene-related material (~2.5−3.4 pmol) (Figure 5A, bars E and F) and was almost equivalent to background levels (Figure 5A, bar D).

Similar incubations were conducted using only HLM to assess the impact of liver glucuronidation on P450-mediated liver bioactivation. Incubation of [^{14}C]raloxifene with pooled HLM resulted in covalent binding of 44 pmol of raloxifene-related material into the protein after precipitation and multiple washing steps (Figure 5B). Inclusion of GSH reduced this to 30 pmol (32% reduction), consistent with the observation of GSH adduct formation. In contrast to the results following preincubation with HIM, addition of UDPGA to HLM resulted in binding of 22−32 pmol of raloxifene-related material to proteins in the presence or absence of GSH, indicating only a 28−50% decrease in covalent binding (Figure 5B). These results indicate that intestinal glucuronidation prevents the covalent binding of raloxifene-related material, while hepatic glucuronidation has only a partial effect.

Determination of Efficiency (Cl_{int}) of the Glucuronidation and Bioactivation Pathways of Raloxifene in Intestinal and Liver Microsomes of Humans and Rat. Kinetic studies were performed to gain some more insight in the observed effect of intestinal glucuronidation on bioactivation of raloxifene. The efficiency (Cl_{int}) of glucuronidation ($Cl_{int\text{-}glu}$) and oxidation ($Cl_{int\text{-}oxi}$) of raloxifene in the gut and liver microsomes of human and rat was estimated by incubating raloxifene with intestinal and hepatic microsomes with either UDPGA or a combination of NADPH/GSH, and the contribution of these two pathways in the two tissues was determined. Because the metabolic profile of raloxifene following incubation with liver or intestinal microsomes in the presence of NADPH and GSH mostly resulted in G1 as a primary product, it was fair to assume that bioactivation of raloxifene was the primary pathway of oxidative metabolism. Hence, $Cl_{int\text{-}oxi}$ values obtained following incubation of raloxifene with NADPH/GSH supplemented liver and gut microsomes were essentially an estimation of the bioactivation pathway of raloxifene.

A substrate depletion approach (also called as the half-life method) was used to estimate Cl_{int} values for both pathways. Traditionally, the metabolite formation method has been used for the determination of in vitro Cl_{int} (μL/min/mg protein) where the initial rate of metabolite production following a microsomal incubation of a substrate is measured over a range of concentrations to obtain the kinetic parameters (K_M and V_{max}) and the

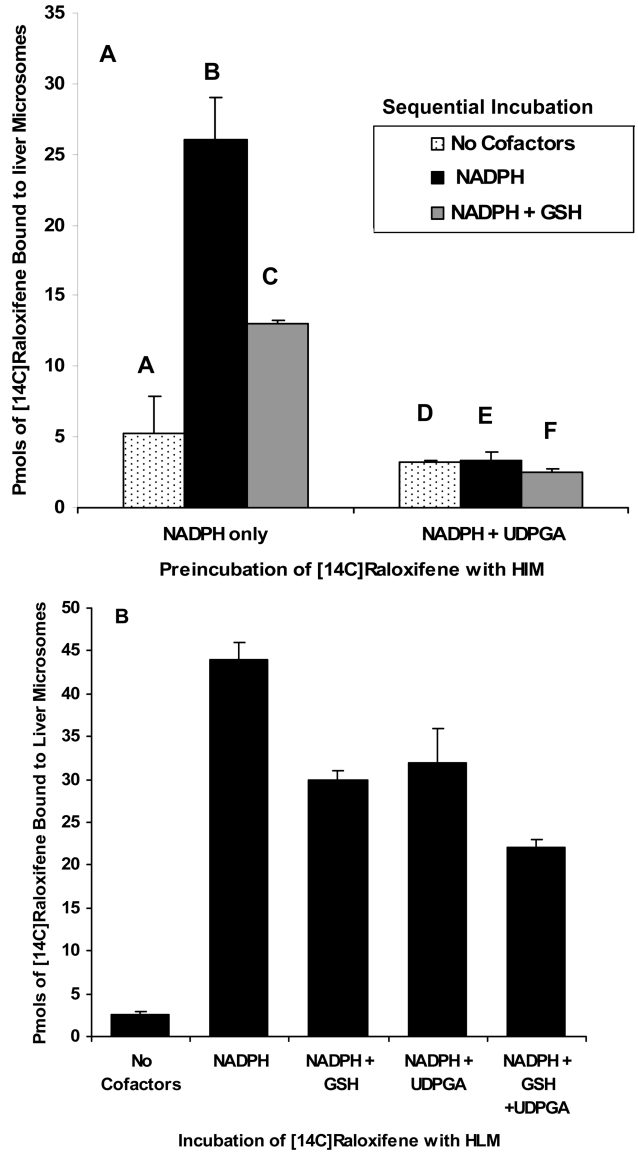


Figure 5. Effect of glucuronidation on the covalent binding of raloxifene-related material to liver microsomal proteins following (A) preincubation of [^{14}C]raloxifene with HIMs followed by a sequential incubation of the incubate with HLMs and (B) incubation of [^{14}C]raloxifene with HLMs. The data presented here are means of three experiments.

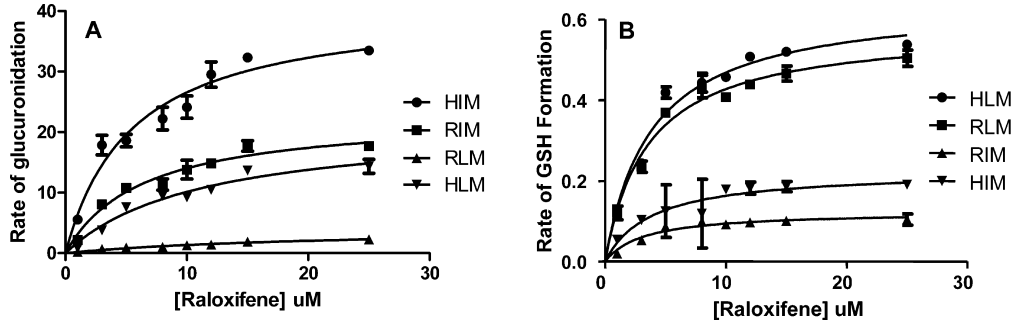


Figure 6. Substrate saturation profiles for the formation of (A) glucuronide and (B) GSH conjugate (G1) in the gut and liver microsomes of humans and rats. The rates are expressed in peak area ratiod of metabolite to IS/min/mg protein. The synthetic standards of the metabolites were not available; therefore, the V_{max} could not be determined, and only apparent K_M values were estimated in these experiments. For the glucuronide plot (A), the K_M value represents an apparent K_M of the overall glucuronidation process and is a conglomeration of K_M values of the two individual glucuronides M1 and M2. The data presented here are means of three experiments.

Cl_{int} is estimated using the formula $Cl_{int} = V_{max}/K_m$. However, this method requires authentic standards of metabolites as well as methods for measurement of metabolite concentrations. Although one can use radiolabeled material to achieve this, the substrate depletion approach, where the consumption of the parent drug is monitored over time in a defined metabolizing system, is a much simpler way for estimating Cl_{int} values, especially in the absence of metabolite standards (*14*−*16*). More recently, this method has also been used to predict in vivo clearance of compounds that are primarily cleared via glucuronidation (*17*). Both the V_{max}/K_M and the substrate depletion methods have been shown to provide similar and accurate estimations of Cl_{int} values and have been used to predict in vivo clearances of drugs (*17, 18*). Because the authentic standards of the glucuronide and glutathione conjugates of raloxifene were not available, the substrate depletion approach seemed to be the approriate method to estimate the $Cl_{int\text{-}glu}$ and $Cl_{int\text{-}oxi}$ for the glucuronidation and oxidation processes in gut and liver microsomes.

The Cl_{int} measurement using substrate depletion method should be performed at substrate concentrations that are well below the K_M of each relevant enzyme involved in the metabolism. This way, the consumption of the substrate follows a first-order decay and prevents the underestimation of Cl_{int} values (*14, 15, 19*). Therefore, the first step in this exercise was to determine the apparent K_M of glucuronidation and G1 formation in the gut and liver microsomes. The enzyme kinetics for the glucuronidation and bioactivation processes were performed using the standard approach described in the Experimental Procedures. However, in the absence of authentic standards, the rates of glucuronide and GSH conjugate formed metabolically at various substrate concentrations had to be expressed as the peak area ratio (metabolite to IS)/min/mg protein. Although such an approach gave a good estimation of K_M, it precluded an accurate estimation of V_{max} since exact concentrations of the two metabolites could not be determined (due to lack of synthetic standards). Because incubation and mass spectrometry conditions as well as the analytes measured at all raloxifene concentrations were identical, it was presumed that the response factor for each analyte was linear over the substrate concentration selected. Figure 6A,B shows the substrate saturation plots from the glucuronide and GSH conjugate formation data following incubation of raloxifene with gut and liver microsomes of humans and rat in the presence of either UDPGA or NADPH and GSH. The K_M values for each of these reactions are shown in Table 2. The K_M for glucuronide formation was estimated to be 5.5 and 6.4 μM in the gut microsomes and 11 and 21 μM in liver microsomes of the human and rat, respectively. Despite the fact that the kinetic values for glucuronides M1 and M2 have been previously reported earlier (*20, 21*), a direct comparison of these values could be made with those in the current study. This was because the kinetic parameters are affected by the conditions and the source of the enzymes used, and second the K_M values obtained in our study were a conglomeration of the K_M values of M1 and M2 (as mentioned before). For G1 formation, the estimated K_M values ranged from 3.3 to 3.9 μM following incubation of raloxifene with NADPH- and GSH-supplemented gut and liver microsomes of the two species (Table 2).

Table 2. Apparent Kinetic Parameters for the Glucuronidation and Oxidation Pathways of Raloxifene in Intestinal and Hepatic Microsomes of Human and Rat[a]

	glucuronidation		oxidation	
	K_M (μM)	$Cl_{int\ glu}$ (μL/min/mg protein)	K_M (μM)	$Cl_{int\ oxi}$ (μL/min/mg protein)
HIM	5.5 ± 0.83	397 ± 14	3.9 + 1.5	26 + 3.1
HLM	11.2 ± 0.88	37 ± 1.6	3.8 + 0.43	142 + 5.8
RIM	6.4 ± 1.9	53 ± 2.9	3.3 + 0.63	31 + 1.9
RLM	21.1 ± 5.2	9.3 ± 1.3	3.3 + 0.38	164 + 20

[a] Both Cl_{int} and K_M values are means ± SD (n = 3). $Cl_{int\ oxi}$ is the Cl_{int} for bioactivation of raloxifene since all or most P450-mediated oxidation of raloxifene resulted in bioactivation of raloxifene and could be trapped by GSH. The apparent K_M for glucuronidation is conglomeration of the K_M values of the two glucuronide conjugates M1 and M2, whereas the apparent K_M value of oxidation is that of the GSH conjugate (G1) formation following bioactivation of raloxifene. The Cl_{int} values were estimated using the substrate depletion approach, whereas K_M estimates were obtained using the substrate saturation method as described in the Experimental Procedures.

On the basis of the K_M values for the glucuronidation and oxidation processes that metabolized raloxifene, an initial substrate concentration of 1.0 μM was selected to estimate the Cl_{int} for the two pathways. This ensured that the experiments were performed under nonsaturating conditions and did not result in the underestimation Cl_{int}. Figure 7A,B shows the percentage of raloxifene remaining over time following incubation of 1 μM raloxifene with HIM, RIM, RLM, and HLM in the presence of UDPGA and NADPH/GSH. As mentioned in the Experimental Procedures, the k_{loss} values for each reaction (glucuronidation and oxidation) were obtained by fitting eq 4 to the % substrate remaining vs time data. The Cl_{int} was then estimated using these k_{loss} values that are depicted in Table 2. In humans, intestinal microsomal incubations of raloxifene with UDPGA and NADPH/GSH as cofactors resulted in $Cl_{int\text{-}glu}$ and $Cl_{-int\text{-}oxi}$ estimates of 397 and 26 μL/min/mg protein, whereas incubations with liver microsomes resulted in estimates of 37 and 142 μL/min/mg protein for glucuronidation and oxidation, respectively (Table 2). The results showed that $Cl_{int\text{-}glu}$ of

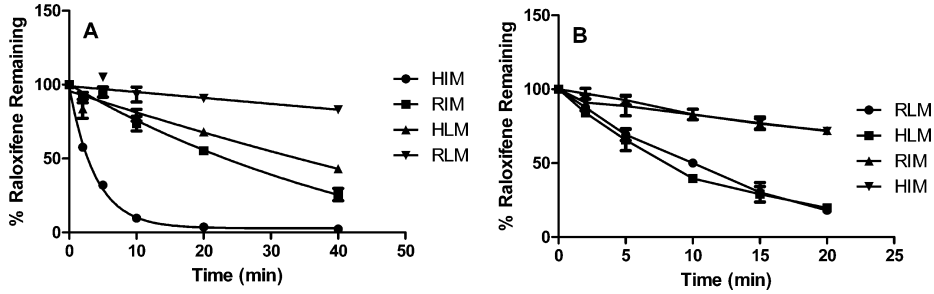


Figure 7. Percent of raloxifene remaining vs time profile after incubation with intestinal and liver microsomes in rats and humans in the presence of UDPGA (A) and NADPH/GSH (B).

raloxifene was ~15-fold greater than $Cl_{int\text{-}oxi}$ in the intestinal microsomes. Similarly, comparison of $Cl_{int\text{-}glu}$ for intestinal and hepatic glucuronidation suggested that the efficiency of intestinal glucuronidation was ~11-fold greater than that in the liver (Table 2). These data were consistent with the observations in the previous studies (*20*), which reported that the turnover of raloxifene was much faster in the intestinal microsomes than hepatic microsomes. In comparing the liver and gut $Cl_{int\text{-}oxi}$ estimates for the bioactivation process, the hepatic oxidation was about 5.5-fold faster than gut bioactivation (Table 2). Thus, the relative contribution of glucuronidation to the overall metabolism in the two tissues of humans ($Cl_{int\ gut} + Cl_{int\ liver}$) was about 72%, whereas the oxidation was only 28% of the total Cl_{int}.

In experiments with rat intestinal and liver preparations, comparison of $Cl_{int\text{-}glu}$ values in the gut and liver microsomes indicated that intestinal glucuronidation of raloxifene was ~6-fold faster to that in the liver ($Cl_{int\text{-}glu}$ = 53 vs 9 μL/min/mg protein), and the $Cl_{int\text{-}oxi}$ in the liver was 5.3-fold faster than oxidation in the gut. However, in contrast to humans, the rate of glucuronidation was only 1.7-fold faster than that of oxidation (53 vs 31 μL/min/mg protein) in the rat intestine (Table 2). Overall, in rats, the contribution of glucuronidation to overall metabolism in the two tissues was only 24% as compared to bioactivation pathway, which was ~76%.

Nonspecific binding of the substrate can also impact the estimated Cl_{int}, and an assessment of the free fraction (f_u) was made. The determined free fractions indicated that the f_u of raloxifene was similar in the gut and liver microsomes of the two species and ranged from 0.092 to 0.11. Similarity in the values across the subcellular fractions tested suggested that the impact of f_u on the Cl_{int} estimates was minimal.

Discussion

The present study investigated the effect of glucuronidation on P450-mediated metabolic activation of raloxifene. Previous in vitro studies have shown that rat and human liver microsomal incubation of raloxifene in the presence of NADPH results in P450-mediated metabolic activation to electrophilic extended diquinone and *ortho*-quinone intermediates that could be trapped by nucleophilic thiols including GSH and NAC (*5, 6*). However, the impact of the glucuronidation pathway on raloxifene bioactivation has never been explored.

In the current study, preliminary incubations of raloxifene with NADPH- and GSH-supplemented human and rat liver, intestinal, and a mixture of liver and intestinal microsomes resulted in a reduction of the amount of G1 formed when UDPGA was added to the incubation mixture. Although reactions of raloxifene with NADPH, GSH, and UDPGA-supplemented HLMs and RLMs decreased G1, the impact of glucuronidation was more noticeable in both species when raloxifene was incubated with liver preparations containing intestinal microsomes (HLM/HIM or RLM/RIM mix) (Figure 3). A similar change in the extent of G1 formed was observed when raloxifene was incubated with NADPH-, GSH-, and UDPGA-supplemented intestinal microsomes alone (Figure 3), suggesting that intestinal glucuronidation modulated the exposure of raloxifene and therefore its bioactivation more than the liver alone. This was further confirmed from the observation that only 2 and 40% raloxifene was present in the incubation mixtures containing HIMs and RIMs, respectively, while 79 and 88% of unchanged drug was present in incubation mixtures containing liver microsomes alone, as shown in the results.

The effect of intestinal glucuronidation on P450-mediated metabolic activation of raloxifene by liver was further explored by a sequential incubation method, which involved preincubation of raloxifene with gut microsomes in the presence of UDPGA followed by a subsequent incubation of the preincubate in liver microsomes. Such an experiment imitated intestinal first pass metabolism observed in humans or rats in vivo, in which the drug given orally is first exposed to the GI tract (intestine) and the absorbed fraction that escapes intestinal metabolism is exposed to liver where it undergoes further metabolism. Because the exact residence time of raloxifene in the enterocytes is unknown, raloxifene was preincubated for various times for up to 30 min in both the RIMs and HIMs prior to determination of G1 in liver microsomes. Analysis of the data clearly showed a decrease in the amount of G1 when UDPGA was present in the incubation mixtures relative to the controls (experiments lacking UDPGA) (Figure 4). A corresponding decrease in the amount of raloxifene remaining in the preincubation mixtures (HIM and RIM) was also apparent, and this correlated quite well with the amount of G1 formed after sequential incubation. The findings of this study further confirmed that intestinal glucuronidation limits the amount of raloxifene that undergoes P450-mediated metabolic activation and hence influences bioactivation, in both the humans and the rats. An assessment of the impact of human intestinal glucuronidation on human hepatic bioactivation of raloxifene was also conducted by measuring covalent binding of raloxifene-related material to human microsomal proteins, using [^{14}C]raloxifene. This was done since all reactive intermediates are not trapped by GSH. A substantial reduction (87−90%) in the covalent binding was observed when HIM and UDPGA were included in the incubation (Figure 5A). This further confirmed that the protective effect of glucuronidation on raloxifene is more associated with glucuronidation activity in the intestine rather than the liver (Figure 5B).

Although the effect of intestinal glucuronidation on the parent drug available for hepatic bioactivation was observed in both of the species, comparsion of the amount of G1 formed or amount of raloxifene left over in the above experiments suggested some difference in the metabolism of raloxifene in

the rat and humans. Previous studies have also indicated that in contrast to poor bioavailability in humans (2%), raloxifene has fairly good bioavailability in rat (39%) (*13*). Upon coincubation of raloxifene with a mixture of gut and intestinal microsomes, there was a considerable decrease in the amount of G1 formed in human tissue preparations as compared to rats (Figure 3). Likewise, preincubation of raloxifene with HIM followed by a sequential incubation with HLM resulted in a substantial decrease in G1 formation (relative to control) when compared to the rat incubations (Figure 4 and Table 1). Determination of unchanged raloxifene in the intestinal microsomal mixtures (preincubation mixtures) of rat and human also suggested that the metabolism in the gut microsomes of the rat was slower than that of humans since only 13% of unchanged raloxifene remained in the human intestinal microsomal mixtures after 15 min. In contrast, 70% raloxifene remained in the gut microsomal incubation mixture of rats after 15 min. These results suggested that differences in the rates of glucuronidation influenced the bioactivation of raloxifene in the two species.

To gain a better understanding of the impact of glucuronidation on hepatic bioactivation of raloxifene as well as the species differences observed between the rat and the humans, the rates of glucuronidation and bioactivation in the gut and the liver microsomes of these two species were compared. In humans, high $Cl_{int\text{-}glu}$ of glucuronidation in HIM incubations revealed that this route predominated (Table 2). On the other hand, an approximately 4-fold faster rate of oxidation (as compared to glucuronidation) in hepatic microsomes explained the lack of protection against bioactivation in this tissue. Hence, efficient and extensive metabolism of raloxifene in human intestine reduces the availability of raloxifene to be bioactivated by P450 in the liver. A similar comparison of Cl_{int} estimates in rat preparations suggested that Cl_{int} for glucuronidation was only 1.7-fold greater than oxidation of raloxifene in the intestinal microsomes and 3-fold less efficient than the hepatic oxidation (Table 2). This supported the greater degree of bioactivation in the rat experiments by liver. Because the Cl_{int} values for the oxidation pathway between humans and rats were similar (Table 2), the species difference observed in the degree of metabolic activation after sequential incubations could be explained by the highly efficient glucuronidation pathway in HIM vs RIM (the rat was 7.5-fold less efficient than humans) (Table 2). Further analysis of the data suggested that the species difference was primarily driven by V_{max} since the estimated K_M values for glucuronidation were quite similar in human and rat incubations (5.5 and 6.4 μM in human and rat, respectively). (A rough estimation using the observed $Cl_{int\text{-}glu}$ and K_M values suggested that the V_{max} values were 2183 and 339 pmol/min/mg protein for humans and rat, respectively).

The above assessment was in agreement with the previously published results from studies on glucuronidation of raloxifene (*20, 21*). These studies have demonstrated that raloxifene glucuronidation is primarily catalyzed by uridine 5′-diphosphoglucuronosyl transferase (UGT) 1A10 and UGT1A8 isoforms. Both of these enzymes are primarily expressed in the human intestine and are completely absent in human liver (*20, 22, 23*). Kinetic studies with the two isoforms have indicated that UGT1A10 is very efficient in metabolizing raloxifene to the primary circulating metabolite R-4-G, whereas UGT1A8 catalyzes the formation of both R-4-G and R-6-G metabolites (*20, 21*). These studies have also shown that the efficiency of the HIMs to metabolize raloxifene is 2−5-fold higher than that in the hepatic microsomes. In contrast to humans, the efficiency of glucuronidation by RIMs has been reported to be significantly lower than HIMs due to lack of UGT1A10 expression in the rat intestine (*20*). So, although glucuronidation is a primary route of metabolism for raloxifene in rats, the slower rate of glucuronidation in the rat intestine probably results in a greater amount of unchanged raloxifene that can be metabolized by P450 in RLMs to electrophilic intermediates, as compared to humans. An attempt was also made to estimate the intestinal first pass extraction ratio by extrapolation of in vitro Cl_{int} data from the intestinal incubations in the two species using the method published by Chiba and co-workers (*24*). These calculations predict that in human the liver is exposed to only 11% of a dose as unchanged raloxifene in contrast to 68% in rats based on our data. However, no attempt was made to evaluate this in an in vivo experiment.

A 10 μM raloxifene concentration was used in the current study to illustrate the role of glucuronidation and its effect on bioactivation. Theoretically, the amount of drug to which the enterocytes are exposed depends upon its intestinal solubility and intestinal permeability (*25, 26*). Raloxifene falls into low solubility and high permeability class of drugs according to the BCS classification system (*25*). This limits its amount coming into the enterocytes, thereby providing little opportunity to saturate intestinal enzymes including the UGTs (*25*). Recent reports have shown that raloxifene is a good Pgp substrate, which also limits the bioactivation of this drug by reducing its residence time with oxidative metabolism enzymes (*27*). Consequently, one can infer that if the intestinal processes are not saturated, efficient glucuronidation and efflux of raloxifene by the gut coupled with a moderate dose and low solubility probably restrict the amount of raloxifene that can undergo hepatic metabolic activation (*28*). This will decrease the body burden of the reactive intermediates of raloxifene in humans.

Estimation of raloxifene concentrations using the following equation (*29*)

$$[\text{raloxifene}] = \frac{k_{abs} \times F_a \times \text{dose}}{Q_g}$$

suggests that the concentration used in these experiments was proximal to those estimated to be present in enterocytes during absorption after administration of a 60 mg dose. Using values of 0.03/min for the absorption rate constant (k_{abs}), 60% for the fraction of the dose absorbed (*10*), and a value of 3.5 mL/min/kg for villous blood flow. an estimate of 9 μM was obtained.

In conclusion, the findings of this study support the hypothesis that intestinal glucuronidation limits hepatic exposure of raloxifene undergoing bioactivation in the liver. Although raloxifene is readily metabolized by P450 to electrophilic intermediates in the liver of both rat and humans, a species-dependent balance between the oxidation and the glucuronidation of the drug modulates its bioactivation. While other unknown biochemical processes could play a role in the lack of raloxifene toxicity, the current study demonstrates that in the case of raloxifene, extensive first-pass glucuronidation by the intestine could at least partially explain why the drug that is bioactivated by liver P450s does not demonstrate significant hepatotoxicity.

Over the years, in vitro experiments have been used to identify those xenobiotics that undergo metabolism-dependent bioactivation to reactive electrophilic intermediates. These have included observations of covalent incorporation into proteins as well as observation of intermediates trapped with nucleophiles such as GSH and cyanide. Positive results from these experiments have been interpreted as indicative of the potential for various toxicities (e.g., hepatotoxicity, blood dyscrasias, etc.).

However, several recent reviews and commentaries have been published that highlight the caution of overinterpreting metabolism-dependent positive covalent binding and GSH adduct formation when evaluating the bioactivation potential of a new drug (*30*−*33*). These reviews also emphasize the importance of considering the contribution of other metabolic pathways (hepatic or extrahepatic), in light of a positive signal in a reactive metabolite screen and assessing species differences prior to extrapolating bioactivation results (*34, 35*). The results of this study also support this view.

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