Differences between Human and Rat Intestinal and Hepatic Bisphenol A Glucuronidation and the Influence of Alamethicin on In Vitro Kinetic Measurements

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ABSTRACT:

The extent to which membrane-disrupting agents, such as alamethicin, may alter cofactor transport and influence in vitro kinetic measurements of glucuronidation is a major concern regarding the characterization and extrapolation of inter- and intraspecies pharmacokinetics of bisphenol A (BPA). An additional concern is the omission of a BPA intestinal metabolism component in current pharmacokinetic models used to assess oral exposure. In this study, BPA glucuronidation in native hepatic microsomes from female rat and female human liver displayed higher V_max values than that in males. In the presence of alamethicin, all hepatic V_max values increased; however, this increase was disproportionately greater in males and gender differences were no longer observed. Female rats exhibited a much higher K_m than all other species and genders; the addition of alamethicin had little influence on K_m values for any of the test systems. The dissimilar K_m measured for female rat suggests that different UDP-glucuronosyltransferase (UGT) enzyme(s) are involved in BPA glucuronidation. The presence of different UGTs in female rat was confirmed using Hill coefficients measured from diclofenac-mediated chemical inhibition assays within hepatic microsomes and purified human UGT2B7 and UGT2B15. Mixed-gender human intestinal microsomes showed little BPA glucuronidation reactivity compared with those from male rat intestine. Male rat intestinal microsomes in the presence of alamethicin exhibited a V_max that was nearly 30-fold higher than that for mixed human microsomes. The species and gender metabolic differences we observed between rat and human liver and intestine provide key information for delineating BPA pharmacokinetics needed for human health risk assessment.

Introduction

Bisphenol A (4,4’-isopropylidene-2-diphenol, BPA) is a high production volume chemical used in manufacturing of polycarbonate plastic and epoxy resins for numerous consumer products (Völkel et al., 2005; Patisaul, 2010). Widespread inclusion of BPA in materials for general protective coatings of food cans and polyvinyl chloride pipes and its incorporation into plastic bottles and other amenities has greatly enhanced the potential for human exposure (Pritchett et al., 2002). BPA is classified as a potential endocrine disrupter that disrupts normal hormone function and causes unwanted reproductive and developmental effects in laboratory rodents, which has raised safety concerns about low-dose human exposure (Völkel et al., 2002; Takeuchi et al., 2004; Hess-Wilson, 2009). Accurate characterization of BPA pharmacokinetics (e.g., metabolism) is critical to determining BPA target dose and human susceptibility to low-dose exposure. Equally important is defining the inter- and intraspecies differences in metabolism of BPA to reduce uncertainties associated with data extrapolation from animal studies to humans.

The liver is considered the primary organ responsible for the metabolism of foreign chemicals, and the hepatic transformation of BPA to the monoglucuronide through UDP-glucuronosyltransferase (UGT) catalysis has been well documented in both rodents and humans (Yokota et al., 1999; Pritchett et al., 2002; Kuester and Sipes, 2007; Hanioka et al., 2008). In general, UGT-glucuronidation involves the transfer of a UDP-glucuronic acid (UDPGA) moiety to a foreign chemical (Lin and Wong, 2002). On the cellular level, UGT enzymes are membrane-bound to the endoplasmic reticulum, with the active site residing inside the lumen (i.e., intraluminal). Thus, the susceptibility of BPA and other chemicals to UGT-mediated glucuronidation in vivo depends on the transport of the cofactor, UDPGA, across the membrane bilayer (Killford et al., 2009).

In vitro microsomal assays are routinely used to provide kinetic and metabolite identification data for human health risk assessment and drug discovery (Obach, 1997). Microsomes are the metabolically active subcellular tissue fraction of the endoplasmic reticulum membrane, existing primarily as vesicles that maintain the UGT active site on the intraluminal side as observed in vivo. A major issue regarding

ABBRévIATIONS: BPA, bisphenol A; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; ACN, acetonitrile; HPLC, high-performance liquid chromatography; MSP, microsomal protein.
rates of glucuronidation measured in vitro is the extent to which microsomal incubation conditions may alter UDPGA cofactor transport and, thus, have an impact on UGT reactivity (Mazur et al., 2009). A variety of microsomal incubation procedures including the use of detergents, the addition of bovine serum albumin, and the pore-forming peptide alamethicin have been used to circumvent the native, latent characteristics of UDPGA transport in microsomes (Fisher et al., 2000; Kilford et al., 2009). In most cases, however, the impact of these additives on both the accuracy and validity of measured rates of glucuronidation relative to native conditions has not been addressed adequately.

Pharmacokinetic modeling applications based on in vitro microsomal data generally tend to underpredict rates of in vivo glucuronidation (Cubitt et al., 2009; Kilford et al., 2009). Although many factors may contribute to this occurrence, a primary source of error may be the degree to which UDPGA can access the intraluminally located UGT active site. Microsomes used under native (untreated) incubation conditions may assess the dependence of UGT reactivity on UDPGA cofactor transport, whereas microsomal assays using agents that disrupt the vesicular membrane may display reactivity correlated directly to UGT enzyme content (Lin and Wong, 2002). This dichotomy in experimental approaches has led to high variability in rates of BPA glucuronidation reported by different laboratories and may contribute to inaccurate inter- and intraspecies extrapolation of rodent data to humans (Elsbey et al., 2001; Takeuchi and Tsutsumi, 2002; Völk et al., 2002; Takeuchi et al., 2004; Kuester and Sipes, 2007).

Current pharmacokinetic models for BPA in humans have been based exclusively on rat liver data (Teeguarden et al., 2005). Although the liver has long been recognized as the major organ responsible for xenobiotic glucuronidation, omission of intestinal glucuronidation from an in vitro-in vivo scaling strategy has been shown, in some cases, to underpredict metabolic clearance (Kaminsky and Zhang, 2003; Cubitt et al., 2009). In cases of oral exposure, such as with BPA, it has been proposed that the metabolic capacity of the small intestine should be considered for improving modeling efforts (Lennernäs, 2003; Cubitt et al., 2009; van de Kerkhof et al., 2007). Extrapolation of a rat liver compartment model to humans presents a challenge, given that biliary excretion and enterohepatic recirculation into the intestinal tract occurs in rats but not in humans (Völk et al., 2002; Mielke and Gundert-Remy, 2009). Interspecies differences that may exist in the small intestine of rodents and humans potentially could alter first-pass metabolism and systemic uptake. The purpose of this study was to compare and contrast the kinetics of in vitro BPA glucuronidation in rat and human hepatic and intestinal microsomes under native incubation conditions and in the presence of alamethicin and then to assess the UGT isoform(s) responsible for BPA glucuronidation.

**Materials and Methods**

**Reagents.** BPA (>99%), uridine diphosphoglucuronic acid, saccharic acid-1,4-lactone, alamethicin, magnesium chloride (MgCl2), β-glucuronidase, diclofenac, sodium acetate, and phosphate buffer (pH 7.4) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were analytical grade or better and were used as received.

**Microsomal Incubation Procedure.** Frozen male and female Sprague-Dawley rat and human hepatic microsomes were purchased from Invitro Technologies (Baltimore, MD). Only male Sprague-Dawley rat and mixed-gender human intestinal microsomes were commercially available from Xenotech, LLC (Lexena, KS); all microsomes were stored at −80°C until use. BPA standards were prepared in acetoni triate (ACN) and stored in amber vials at 4°C. Incubations with BPA were conducted in microcentrifuge tubes placed in a heating block at 37°C. Hepatic and intestinal microsome suspensions (0.01–0.05 mg of protein) were dispensed into 180 μl of phosphate buffer (100 mM, pH 7.4, and 1.25 mM MgCl2 final concentration). A pH profile of intestinal metabolism was obtained using sodium acetate and phosphate buffer (100 mM) with pH values ranging from 4.5 to 8.5. All alamethicin assays were conducted at 20 μg of alamethicin/mg microsomal protein and contained saccharalactone (5 mM final concentration). Various concentrations of BPA stock solutions (2.5 μl) were added to the microsomal assays to achieve a final substrate concentration of 0 to 60 μM. The suspension was vortexed and allowed to stand for 10 min in a heating block to ensure temperature equilibration before initiation of the metabolism assay. Phosphate buffer (62.5 μl) containing UDPGA (5 mM final concentration) preincubated to 37°C was used to initiate the reaction. All assays were incubated for a maximum of 30 min, terminated with an equal volume (250 μl) of chilled methanol, and immediately placed on ice. The samples were then centrifuged at 4°C for 10 min at 10,600 × g, and the supernatant was transferred to seal-cap HPLC vials for analysis. Abiotic controls were conducted by adding BPA and omitting UDPGA.

**Chemical Inhibition of Purified UGTs and Microsomes.** Purified human UGT2B7 and UGT2B15 expressed in insect cells were purchased from BD Biosciences (San Jose, CA). Diclofenac inhibitor stock solutions were prepared in methanol and stored in amber vials at 4°C. The inhibitor stock (2.5 μl; 0–500 μM final concentration) was added to the intestinal and hepatic microsomal matrix or purified UGT (1000 μg/ml final) in phosphate buffer and allowed to stand for 10 min at 37°C. BPA was then added at a concentration near the Km and allowed to stand for an additional 10 min in the presence of alamethicin. The reaction was initiated, and the sample was treated as described for the microsomal incubation procedure.

**HPLC Analysis.** Analysis of BPA metabolism samples was performed on an Agilent Series 1100 HPLC quaternary pump system (Agilent Technologies, Santa Clara, CA) equipped with a photodiode array detector (λ = 228 nm). Injections (100 μl) were made onto a Syngery Hydro-RP column (4.6 × 100 mm, 4-μm particle diameter; Phenomenex, Torrance, CA). A gradient elution at 0.4 ml/min was used with ACN and water as follows: 5% ACN for 0 to 4.5 min, 5 to 40% ACN from 4.5 to 5.0 min, 40% ACN for 5.0 to 20.0 min, 5% ACN from 20.0 to 21.0 min, and 5% ACN for 21 to 25 min. BPA calibration curves were prepared in 250 μl of 0.1 M phosphate buffer using 250 μl of methanol and 10 μl of standard stock solution. A minimum of eight standards were used for initial instrument calibration. Instrument calibration was verified by analyzing a standard check sample near the concentration midpoint of the calibration curve before sample analysis and after every 10th sample. Analyte quantitation was considered valid if the standard check sample varied by less than 5% from initial instrument calibration.

**BPA Glucuronide Identification Using β-Glucuronidase.** Because of the unavailability of a BPA glucuronide standard, metabolite confirmation was conducted via a hydrolysis assay using β-glucuronidase. Triplicate active microsomal samples were evaporated in vacuo and then reconstituted in 1.0 ml of 0.05 M sodium acetate buffer (pH 5.0). Next, the samples were added either in the absence (control) or presence of 100 units of β-glucuronidase at 37°C for 1 h. The reaction was then quenched with methanol and centrifuged for HPLC analysis as described above.

**Data Analysis.** Initial zero-order rate constants (kobs; picomoles per minute) for metabolite formation were determined by linear least-squares regression of concentration versus time data. To be valid the regression had to meet the following criteria: less than 20% BPA depletion, ±3 time points, and r² > 0.7. Jackknife residuals and leave-one-out analyses detected outliers. Initial zero-order reaction velocities (picomoles per minute per milligram) were calculated by normalizing kobs to the amount of microsomal protein. The Michaelis-Menten constants Vmax and Km were determined, based on nonlinear regression of product formation velocity at the varying substrate concentrations. CLint values, expressed as Vmax/Km, were adjusted for the unbound fraction of BPA using methods established previously by Austin et al. (2002). A four-parameter nonlinear curve fit was used to determine Hill coefficients and IC50 values based on sigmoidal inhibition plots with the chemical inhibitor diclofenac.

**In Vitro-In Vivo Extrapolation for Hepatic Clearance.** To better understand potential species- and sex-dependent differences in bisphenol A pharmacokinetics of the liver, the kinetic constants for hepatic microsomal protein were incorporated into the well-stirred model of Ito and Houston (2004), as shown in eq. 1:

\[
\text{CL}_{\text{inh}} = \left( Q_s \cdot f_{\text{act}} \cdot 0.05 \text{ mg MSP/pg} / (Q_S + f_{\text{act}} \cdot 0.05 \text{ mg MSP/pg}) \right) \cdot (k_{\text{int}}) (1)
\]
TABLE 1

Kinetics of bisphenol A glucuronidation by intestinal and hepatic microsomes

<table>
<thead>
<tr>
<th>Species</th>
<th>Gender</th>
<th>Microsome Matrix</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>CL&lt;sub&gt;uint&lt;/sub&gt;</th>
<th>CL&lt;sub&gt;n&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Male</td>
<td>Native</td>
<td>7552 ± 553</td>
<td>4.3 ± 1.2</td>
<td>1888</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Native</td>
<td>22,169 ± 1025</td>
<td>6.4 ± 1.0</td>
<td>3695</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Alamethic</td>
<td>20,392 ± 1425</td>
<td>6.2 ± 2.9</td>
<td>1342</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Alamethic</td>
<td>28,607 ± 2258</td>
<td>21.9 ± 4.1</td>
<td>1389</td>
<td>2.0</td>
</tr>
<tr>
<td>Human</td>
<td>Male</td>
<td>Native</td>
<td>2077 ± 186</td>
<td>3.6 ± 1.2</td>
<td>649</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Alamethic</td>
<td>9409 ± 605</td>
<td>5.6 ± 1.3</td>
<td>1882</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Alamethic</td>
<td>4255 ± 375</td>
<td>4.9 ± 1.5</td>
<td>967</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Native</td>
<td>Alamethic</td>
<td>9389 ± 119</td>
<td>7.3 ± 3.2</td>
<td>1444</td>
<td>0.9</td>
</tr>
<tr>
<td>Intestine</td>
<td>Male</td>
<td>Native</td>
<td>6599 ± 306</td>
<td>10.7 ± 1.5</td>
<td>815</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>Alamethic</td>
<td>18,795 ± 1830</td>
<td>37.0 ± 7.7</td>
<td>666</td>
<td>—</td>
</tr>
<tr>
<td>Human</td>
<td>Male</td>
<td>Native</td>
<td>487 ± 119</td>
<td>80.1 ± 35.9</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>Native</td>
<td>660 ± 88</td>
<td>41.9 ± 12.6</td>
<td>26</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Unbound fraction of BPA calculated using logP = 3.4 (Austin et al., 2002).

<sup>b</sup> —, not applicable.

Results

BPA Glucuronide Formation within Microsomes and Purified UG Ts. HPLC analysis of BPA metabolism samples from hepatic and intestinal microsomes and purified UGT assays resulted in the appearance of a single product peak; control assays conducted without UDPGA demonstrated no product formation over time. The formation of BPA glucuronide was confirmed through the β-glucuronidase hydrolysis assay. After active metabolism samples were incubated in the presence of β-glucuronidase, the decrease in concentration of the proposed BPA glucuronide was equivalent to the increase in BPA; no change was observed in control samples (no glucuronidase) over the same time period and conditions. The UV spectra and λ<sub>max</sub> measured for BPA glucuronide were nearly identical to those for BPA. Based on these results and the lack of a BPA glucuronide standard, the concentration of BPA glucuronide present in microsome and UGT assay samples was quantified using the BPA calibration curve.

Species and Gender Comparison of BPA Glucuronidation Kinetics in Hepatic Microsomes. Comparison of species and gender BPA glucuronidation kinetics was performed using rat and human hepatic microsomes. A linear rate of BPA glucuronide formation was determined for each substrate concentration in the absence (i.e., native) and presence of alamethicin. Saturation of BPA metabolism occurred in all hepatic microsome assays and Michaelis-Menten data analysis was applied to BPA glucuronide formation (Fig. 1) to determine V<sub>max</sub> and K<sub>m</sub> (Table 1). For both rat and human native microsomes, females displayed higher V<sub>max</sub> values than males. In the presence of alamethicin, V<sub>max</sub> increased in all cases; however, the increase was disproportionately greater for the males to the point that gender differences were no longer significant for rat or human. The K<sub>m</sub> values in all hepatic microsomal assays increased in the presence of alamethicin but were not statistically different in comparison with those for native microsomes. Male rat, male human, and female human K<sub>m</sub> values were similar and ranged from 3.6 to 7.3 μM among native and alamethicin-amended hepatic microsomes. However, female rats displayed much higher K<sub>m</sub> values (16.2–21.9 μM) in both native microsomes and in the presence of alamethicin. Intrinsically clearances (CL<sub>uint</sub>), expressed as V<sub>max</sub>/K<sub>m</sub>, increased from 1.5- to 2.9-fold in the male rat, male human, and female human with addition of alamethicin to the native microsomes; CL<sub>uint</sub> for female rat remained relatively unchanged; native male human liver microsomes displayed a lower CL<sub>uint</sub> compared with female human liver but displayed a higher CL<sub>uint</sub> with addition of alamethicin. CL<sub>n</sub> values were expressed as milliliters per minute per gram of liver, with values for rats similar for males and females and insensitive to the addition of alamethicin, with values approximating 2.1 ml/min/g of liver. The same pattern was demonstrated for humans, with values in all cases (male or female and plus or minus alamethicin) approximating 0.9 ml/min/g of liver. The sensitivity analysis yielded results generally similar for rats and humans. For each species, hepatic clearance was highly sensitive to hepatic blood flows, with relative sensitivity coefficients of 1.0 for all cases. For rats and humans, under these conditions, hepatic clearance was relatively insensitive to values for K<sub>m</sub>, V<sub>max</sub>, and f<sub>ub</sub>, with sensitivity coefficients for each below absolute values of 0.01.

BPA Glucuronidation Kinetics within Male Rat and Mixed-Gender Human Intestinal Microsomes. A pH profile (pH 5–8) for BPA metabolism was conducted with male rat and mixed-gender human intestinal microsomes in the presence of alamethicin and indicated optimal enzyme activity at the physiological pH 7.4 for each system. A linear rate of BPA glucuronide formation was observed for each BPA concentration tested, in both the absence and presence of alamethicin, for male rat and mixed-gender human intestinal microsomes. Saturation of BPA metabolism occurred in all intestinal microsomes assays, and Michaelis-Menten data analysis was applied to BPA glucuronide formation (Fig. 2) to determine V<sub>max</sub> and K<sub>m</sub> (Table 1).

Transformation of BPA in male rat and mixed-gender human intestinal microsomes exhibited significantly different kinetic profiles. Mixed-gender human intestinal microsomes showed vastly lower rates of BPA transformation compared with male rat intestine. Male rat intestine exhibited a nearly 30-fold higher V<sub>max</sub> relative to the mixed-gender human intestine upon alamethicin activation, whereas the V<sub>max</sub> of native male rat intestine was an order of magnitude above...
that of the native mixed-gender human intestine. \( K_m \) values increased in male rat intestine and decreased in the mixed-gender human intestinal assays upon activation of alamethicin. CL\( _{\text{uint}} \) values for native and alamethicin-activated male rat intestinal microsomes were similar and orders of magnitude higher than those for mixed-gender human intestinal microsomes.

**Chemical Inhibition Assays with Intestinal and Hepatic Microsomes and Purified Human UGTs.** Inhibition of BPA glucuronide formation in intestinal and hepatic microsomes and purified human UGTs was determined in the presence of the UGT inhibitor, diclofenac. UGT isoforms 2B7 and 2B15 were selected, based on previous studies demonstrating their importance in the glucuronidation of BPA (Hanioka et al., 2008; Edginton and Ritter, 2009). Diclofenac is a known inhibitor of purified human UGT2B7 and UGT2B15, and studies were initially conducted with each purified UGT to calculate a Hill coefficient and \( IC_{50} \) value to compare with inhibition results from microsome assays (Ghosal et al., 2004; Zhu et al., 2008). The Hill coefficient, which is determined from the slope of an inhibition sigmoidal dose-response curve, approximates the number of inhibition binding sites outside the active site that can take part in substrate transformation (Wsol et al., 2003). The Hill coefficient for UGT2B7 inhibition with diclofenac was 1.3 and exhibited an \( IC_{50} \) of 19.3 M; UGT2B15 demonstrated a slightly higher Hill coefficient of 1.6 and an \( IC_{50} \) of 29.6 M (Fig. 3).

In the presence of diclofenac, assays with male rat and male and female human hepatic microsomes resulted in similar \( IC_{50} \) values and Hill coefficients (1.2–1.4). Comparison of these Hill coefficient values with those observed with the purified human UGTs suggest that either UGT2B7 or UGT2B15 could be responsible for BPA glucuronidation. However, female rat liver microsomes, which demonstrated a higher \( K_m \) value for BPA glucuronide formation, showed a higher Hill coefficient of 3 (Fig. 4). The different Hill coefficients indicate either the involvement of multiple UGT enzymes, which may include rat isozymes for human UGT2B7 and UGT2B15, or the possibility of a single enzyme with multiple inhibition sites. The male rat intestinal microsomes exhibited a Hill coefficient of approximately 2 and an \( IC_{50} \) value that was more similar to that for the purified human UGT2B15 than to that for UGT2B7. Because of the slow reaction kinetics of the mixed-gender human intestine microsomes, neither a Hill coefficient nor an \( IC_{50} \) was determined.

**Discussion**

Human susceptibility to low-dose BPA exposure continues to be controversial. Because of the intraluminal location of UGTs, microsomal assays are routinely conducted with native incubation matrices and/or with membrane-disrupting agents, including the commonly used pore-forming peptide, alamethicin (Fisher et al., 2000; Kilford et al., 2009). Our investigation of BPA glucuronidation kinetics that compares native rat and human hepatic microsome assays with those amended with alamethicin provided interesting results. In native mi-
Microsomes, both female rat and human liver displayed higher $V_{\text{max}}$ values than males (Fig. 1). In the presence of alamethicin, $V_{\text{max}}$ values increased for all species and genders tested; however, the observed increase was disproportionate to the point that gender differences were no longer apparent as male rat and male human activities increased to near female levels. Although several hypotheses currently exist about the mechanism of UDPGA transport into the lumen of liver microsomes, our results indicate that UDPGA cofactor transport is the rate-limiting step for BPA glucuronidation (Lin and Wong, 2002). It is noteworthy that the rate of UDPGA transport appears to be gender-specific in native rat and human liver microsomes, as evidenced by females exhibiting greater $V_{\text{max}}$ values than males; this gender difference, however, is nearly nonexistent in the presence of alamethicin, suggesting that the UGT enzyme content is similar among male and female liver microsomes within the same species. The application of alamethicin provided only a slight increase in hepatic $K_{m}$ values in all liver microsome assays. A similar $K_{m}$ range (3.6–7.3 µM) was determined among male rat, male human, and female human livers, regardless of assay conditions; however, the female rat consistently exhibited higher $K_{m}$ values (16.2–21.9 µM) in either the absence or presence of alamethicin (Table 1). The large difference in female rat liver $K_{m}$ values relative to those of the other test species suggests that differing UGT enzyme(s) are involved in BPA glucuronidation. Overall, $K_{m}$ values remained nearly constant for each test species regardless of the incubation procedure. Thus, the disproportionate increase in $V_{\text{max}}$ values upon alamethicin activation was the primary kinetic parameter influencing changes in $\text{CL}_{\text{int}}$. 

![Fig. 3. Percent activity of diclofenac chemical inhibition profiles for BPA glucuronide product formation in rat (top) and human (bottom) hepatic microsomes.](image1)

![Fig. 4. Comparison of IC$_{50}$ (top) and Hill coefficient (bottom) values of purified human UGT2B7 and UGT2B15 with intestinal and hepatic microsomes in the presence of the chemical inhibitor diclofenac.](image2)
It has been reported previously that UGT2B1 is the predominant enzyme responsible for BPA glucuronidation in rat liver; findings based on cDNA libraries and substrate overlap suggest that UGT2B7 is the equivalent isozyme in human (Yokota et al., 1999; Takeuchi et al., 2004; Edginton and Ritter, 2009). However, a recent study of BPA glucuronidation using a variety of recombinant human UGT isoforms indicated that UGT2B15 exhibited greater reactivity than UGT2B7 (Hanioka et al., 2008). Based on these findings, we conducted chemical inhibition studies using purified human UGT2B7 (rat UGT2B1 equivalent) and UGT2B15 in the presence of diclofenac, a known inhibitor of these UGT isoforms, to compare the Hill coefficients with liver microsome assays (Ghosal et al., 2004; Zhi et al., 2008). The range of Hill coefficient values measured with purified UGT2B7 and UGT2B15 (1.3–1.6) were similar, which indicates a sole inhibition binding site for both enzymes (Fig. 4). Results of a similar Hill coefficient value measured within a microsome system suggests that either UGT2B7 or UGT2B15 could be involved in BPA glucuronidation, whereas a higher Hill coefficient value indicates that multiple enzymes (including UGT2B7 and UGT2B15) or a single enzyme with multiple inhibition sites is responsible for reactivity.

Gender comparison of diclofenac inhibition profiles for male rat liver, male human liver, and female human liver displaying similar \( K_m \) values for BPA glucuronidation showed similar Hill coefficients (1.2–1.4), suggesting the presence of a sole inhibition site (Fig. 3). Of interest, diclofenac inhibition for female rat liver microsomes, which exhibited a significantly higher \( K_m \) value for BPA glucuronide formation, yielded a Hill coefficient of 3.1. The different Hill coefficient observed in female rat liver indicates the involvement of either multiple UGT enzymes, including the potential rat isozymes for human UGT2B7 and UGT2B15, or the possibility that a single enzyme with multiple inhibition sites is involved during BPA glucuronidation. Previous results from a Northern blot study of male rat liver indicated that rat UGT2B1 (human UGT2B7 equivalent) was the predominant isoform responsible for BPA glucuronidation, whereas other unidentified UGT isoform(s) were believed to be responsible for BPA conjugation in female rat (Yokota et al., 1999). In contrast, another study indicated that higher UGT2B1 mRNA levels were observed in female rats than in male rats (Takeuchi et al., 2004). Although the Hill coefficient cannot confirm enzyme identification, similar inhibition profiles observed for purified human UGT2B7 and UGT2B15 identifies human UGT2B15 and an equivalent rat isozyme as strong candidates for future blotting analysis in both rat and human liver microsomes.

Further studies are needed to identify the specific UGT enzyme(s) responsible for BPA glucuronidation in human liver. We have shown that male and female human liver microsomes exhibited \( K_m \) values and Hill coefficients similar to those for male rat, which suggests the presence of a common UGT enzyme. A previous study reported the correlation of \( K_m \) values determined with purified human UGT2B15 with those observed in pooled human liver microsomes; however, comparable studies were not conducted using UGT2B7 (Hanioka et al., 2008). The metabolic differences we observed between rat and human are a key concern in assessing current BPA human health risks. The current pharmacokinetic approach for BPA consists of a rat oral route model extrapolated to humans (Teeguarden et al., 2005). The model design shows significant sensitivity to \( V_{max} \), which influences BPA elimination in both rats and humans. In developing the model, values of \( V_{max} \) for male and female rat were estimated from radioactive BPA concentration time course data, determined in blood and plasma and elimination kinetics from urine and feces; \( K_m \) values were selected from a second, unrelated in vitro investigation (Pottinger et al., 2000).

Estimating individual kinetic parameters from in vivo studies is difficult, although in vitro assays allow for compartmentalized analysis. Currently, great variability exists for in vivo and in vitro measurements of BPA glucuronidation regarding gender and species (human and rodent) differences (Elsby et al., 2001; Takeuchi and Tutsumi, 2002; Völkel et al., 2002; Takeuchi et al., 2004; Kuester and Sipes, 2007). Addressing these differences in an in vitro to in vivo scaling application for physiologically based pharmacokinetic models reduces uncertainty in key parameters and improves the accuracy of species-specific models. The toxicology of BPA depends on the interaction between unbound agent and target tissues; conjugation with glucuronic acid represents a major detoxication process. This investigation has demonstrated appreciable differences in the kinetic constants governing hepatic glucuronidation of BPA in rats and humans. To make these findings more pertinent to estimating biological longevity, we have extrapolated them to measures of hepatic clearance by incorporating them in a model for the well-stirred liver compartment (Ito and Houston, 2004). Under these conditions, hepatic clearance values in rats are nearly twice those in humans, when expressed as milligrams per minute per gram of liver. In addition, hepatic clearance values seem to be most dependent on the rate of blood flow to the liver. These findings demonstrate that additional studies on the clearance of BPA may be focused on measures and variability of hepatic blood flow; changes in \( V_{max} \) and \( K_m \) values and in binding had little impact on the rate of BPA removal from blood, under the conditions herein. These models may provide more precise estimation of internal target tissue dose in human and will assist in defining the oral exposure of BPA that may result in human target tissue susceptibility.

Although the liver plays a major role in xenobiotic metabolism, the small intestine, as the first site of oral exposure, has recently been recognized as a major organ in the metabolism of many foreign chemicals (Fish et al., 2001; Kaminsky and Zhang, 2003; van de Kerkhof et al., 2007). Intestinal metabolism is complicated by interspecies differences among rodents and humans during biliary elimination because rats exhibit enterohepatic recirculation. Sensitivity analysis of current BPA pharmacokinetic models indicates that terminal elimination rates are strongly influenced by rat enterohepatic recirculation (Teeguarden et al., 2005). Our results from the male rat and mixed-gender human intestinal microsome comparison showed vast differences in BPA glucuronidation kinetics. Enzyme kinetic measurements, using mixed-gender human intestinal microsomes in either the absence or presence of alamethicin, showed little transformation of BPA compared with that in male rat intestine (Fig. 2). Both the native \( V_{max} \) and \( K_m \) values determined in male rat intestine increased nearly 3-fold in the presence of alamethicin. Whereas intestinal microsome preparation is subject to artifacts due to inherent physiology, our results indicate that male rat displayed dramatically enhanced intestinal Cl_{int} compared with mixed-gender human intestinal microsomes (Table 1). Although the intestinal tract is subject to pH fluctuation, our results indicate that physiological pH 7.4 displayed the highest UGT reactivity for intestinal glucuronidation of BPA in both rats and humans (van de Kerkhof et al., 2007).

A limited number of studies have examined BPA metabolism within the intestine. One study conducted using everted rat intestine reported that BPA was glucuronidated primarily during its passage through the intestinal wall, whereas another study focused on hydrolysis of the conjugated BPA via natural rat intestinal glucuronidase activity (Sakamoto et al., 2002; Inoue et al., 2003). Northern blot analysis of rat intestine indicated that the UGT2B1 isozyme was not responsible for BPA glucuronidation and suggested that other UGT isoform(s) may be involved (Yokota et al., 1999). Based on this
evidence, we conducted a chemical inhibition study with male rat intestine in the presence of diclofenac. Male rat intestine displayed a Hill coefficient of 2, which is a value closer to the purified human UGT2B15 value than to the UGT2B7 value. Although the range of Hill coefficient values is too similar to differentiate among the purified UGTs, UGT2B15 also displayed a higher IC₅₀ than UGT2B7. A similar trend was observed with male rat intestine microsomes exhibiting a higher IC₅₀ compared with the liver microsome assays. These results suggest that the rat intestine may possess an isozyme equivalent to human UGT2B15 and could serve as a useful candidate for future blotting analysis to identify the UGT responsible for BPA glucuronidation within rat intestine.

In conclusion, the metabolic differences we observed among rat and human liver and intestine are novel and provide key information for delineating the pharmacokinetics of BPA and determining its systemic availability for human health risk assessment. The species- and gender-specific in vitro data can be scaled to in vivo kinetic parameters to refine and improve BPA physiologically based pharmacokinetic models that, among other things, are focused on understanding low-dose BPA bioavailability. In future metabolic studies, species-specific parameters should be used for both liver and intestinal metabolism of BPA; gender differences should also be considered and incorporated into these models. Combining these data may help assess the susceptibility of humans to oral exposure of BPA by refining the metabolism information with more accurate, species-specific data.

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References


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