Butylbenzyl Phthalate Hydrolysis in Liver Microsomes of Humans, Monkeys, Dogs, Rats and Mice

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Butylbenzyl phthalate (BBzP) is used as a plasticizer to import flexibility to polyvinylchloride plastics. In this study, hydrolysis of BBzP to monobutyl phthalate (MBP) and monobenzyl phthalate (MBzP) in liver microsomes of humans, monkeys, dogs, rats and mice was examined. The kinetics for MBP formation by human, dog and mouse liver microsomes followed the Michaelis–Menten model, whereas the kinetics by monkey and rat liver microsomes fitted the Hill model. The kinetics for MBzP formation fitted the Hill model for all liver microsomes. The Vmax and in vitro clearance (CLin or CLmax) ratios of MBP/MBzP formation varied among animal species, although the Km for MBP and MBzP formation in each liver microsomes were generally comparable. The hydrolysis of BBzP to monoester phthalates in mammalian liver microsomes could be classified into two types: MBzP>MBP type for humans and dogs, and MBP>MBzP type for monkeys, rats and mice. These findings suggest that the formation profile of MBzP and MBP from BBzP by liver microsomes differs extensively among animal species.

Key words butylbenzyl phthalate (BBzP); hydrolysis; monobutyl phthalate (MBP); monobenzyl phthalate (MBzP); liver microsomes

Butylbenzyl phthalate (BBzP), an aryl alkyl ester of 1,2-benzene dicarboxylic acid, is extensively used as a plasticizer to soften and increase flexibility in polyvinyl chloride plastics. Since BBzP is not covalently bound to plastics, it can be released during the use or disposal of the product. BBzP has been known to be released during the use or disposal of the product. BBzP has been found in meat, fish, milk products and other foods with a high fat content, and the major route of human exposure to BBzP has been reported to be through the ingestion of contaminated food and water. A large number of in vivo studies have found that BBzP causes reproductive and developmental toxicity in rodents. In addition, several in vitro studies have revealed that BBzP is capable of binding to the estrogen receptor, increasing the proliferation of MCF-7 human breast cancer cells. These reports have suggested that BBzP may cause adverse effects in humans as an endocrine-disrupting chemical.

BBzP has been reported to be metabolized in at least two steps in mammals. In the first step, BBzP is hydrolyzed to monobutyl phthalate (MBP) and monobenzyl phthalate (MBzP) by lipases and esterases in the intestines and pancreas (Fig. 1). These monoester phthalates have been reported to be further metabolized by hepatic and/or intestinal uridine diphosphate glucuronosyltransferases to form the hydrophilic glucuronide conjugate. The conjugates are easily excreted into urine. We have recently shown that the rate of MBzP formation from BBzP in human liver microsomes is markedly higher than that of MBP.

Several in vivo and in vitro studies have demonstrated that the estrogenic activities of MBP and MBzP are weaker than that of BBzP; however, the estrogenic activities of monoester phthalates are relatively different between MBP and MBzP. Thus, the toxicity of BBzP is closely associated with the metabolism. Furthermore, the examination of BBzP hydrolysis in experimental animals is an important aspect of toxicological research. The purpose of this study was to clarify the species differences in BBzP hydrolysis by liver microsomes among humans, monkeys, dogs, rats and mice.

Materials and Methods

Materials BBzP, MBP and MBzP were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pooled liver microsomes of humans (race, Caucasian, African American, Hispanic and Asian; gender, male and female; age, 25–75 years old), monkeys (strain, cynomolgus, sex, male, age, 2–7 years old), dogs (strain, beagle; sex, male; age, 8–25 months old), rats (strain, Wistar, sex, male; age, 8–10 weeks old) and mice (strain, CD1, sex, male; age, 8–11 weeks old) were purchased from XenoTech (Lenexa, KS, U.S.A.). All other chemicals and reagents used were of the highest quality commercially available.

Assay for BBzP Hydrolysis Activity BBzP hydrolysis activities in liver microsomes of humans, monkeys, dogs, rats and mice were determined by measuring the formation of MBP and MBzP according to the method reported previously. The incubation mixture contained BBzP (5–1000 μM) and liver microsomes (20 μg protein/μL) in a final volume of 300 μL of 50 mM potassium phosphate buffer (pH 7.4). BBzP was dissolved in methanol (final concentrations in the incubation medium, 1%, v/v). After preincubation for 2 min at 37°C, the reaction was initiated by adding BBzP. Incubation was performed for 20 min at 37°C and terminated by adding 20 μL of 10% phosphoric acid and vortexing. The samples were centrifuged at 12000 × g for 10 min at 4°C. The supernatant was filtered with a polytetrafluoroethylene membrane filter (0.45 μm), and 50 μL of the filtrate was subjected to high-
performance liquid chromatography with an Inertsil ODS-SP column (4.6 mm i.d. × 150 mm; GL Sciences, Tokyo, Japan). The column was maintained at 40°C. The mobile phase for elution of MBP and MBzP was composed of 0.1% phosphoric acid and acetonitrile. Separation was achieved by running the following gradients (percentages of acetonitrile) at a flow rate of 1.2 mL/min: 15% (0–5 min), 15–40% (5–15 min), 40% (15–25 min), 40–85% (25–35 min), 85% (35–45 min), 85–15% (45–50 min) and 15% (50–55 min). UV detection absorbance was recorded at 254 nm. Standard curve samples were prepared in the same manner as incubation samples. Under these conditions, the retention times of MBP, MBzP and BBzP were 19.3, 20.8 and 37.9 min, respectively.

Data Analysis
Kinetic parameters (\(K_m\) or \(S_{50}\), and \(V_{max}\)) and the Hill coefficient (\(n\)) for BBzP hydrolysis were calculated by constructing velocity versus substrate concentration (\(V – [S]\)) plots using SigmaPlot v8.02 software (Systat Software, San Jose, CA, U.S.A.). The kinetic profile was estimated from the respective coefficient of determination and/or Akaike’s information criterion values for Michaelis–Menten, isoenzyme, substrate inhibition and Hill equations. In vitro clearance values were \(CL_{rat} (V_{max}/K_m)\) or \(CL_{max} (V_{max}S_{50}^*(n−1)/(n(n−1)^{1/n})\). All values are expressed as the mean±S.D. of three separate experiments performed in duplicate.

RESULTS AND DISCUSSION

BBzP is first hydrolyzed to MBP and MBzP in mammals,7,8 and the formation of monoester phthalates has been generally regarded to be a detoxification step.1,6,10,11 Mentlein and Butte12 have reported that the hydrolysis of di-n-butyl phthalate and di(2-ethylhexyl) phthalate in rats and humans is catalyzed by hepatic carboxylesterases. Thus, the liver is thought to be an important organ for hydrolysis as the first step of diester phthalate metabolism in rats and humans; however, there is no report concerning the hydrolysis of BBzP in the livers of experimental animals. In this study, the species difference in BBzP hydrolysis by liver microsomes was investigated.

Hydrolysis activities of BBzP in liver microsomes of humans, monkeys, dogs, rats and mice were determined at a substrate concentration of 50 \(\mu M\) (Fig. 2). The activities of BBzP to MBP and MBzP in human liver microsomes were 2.0 and 75 nmol/min/mg protein, respectively, and the pathway for MBzP was suggested to be predominant compared with that for MBP, supporting our previous report.9 The hydrolysis activities of BBzP to MBP in monkey, rat and mouse liver microsomes were 28-, 22- and 44-fold higher than that in human liver microsomes, although the activity of dog liver microsomes was comparable to that in human liver microsomes. In contrast, the hydrolysis activities of BBzP to MBzP in monkey, rat and mouse liver microsomes were 34%, 9.3% and 12% of that in human liver microsomes, respectively, whereas the activity in dog liver microsomes was 1.6-fold higher than that in human liver microsomes.

Kinetic analysis of BBzP hydrolysis by liver microsomes of humans, monkeys, dogs, rats and mice was performed for further detailed information. The calculated kinetic parameters are summarized in Table 1. The kinetics for MBP formation by human liver microsomes was fitted to the Michaelis–Menten model, and the \(K_m\) and \(V_{max}\) values were 37 \(\mu M\) and 3.3 nmol/min/mg protein, respectively. On the hand, the kinetics for MBzP formation by human liver microsomes was fitted to the Hill model with \(n\) of 2.3, and the \(S_{50}\) and \(V_{max}\) values were 16 \(\mu M\) and 72 nmol/min/mg protein, respectively. In the results, \(CL_{max}\) was about 25-fold higher than that of \(CL_{int}\) for MBP formation. The kinetics for MBP formation...
Microsomes could be classified into two types: MBzP formation, the profile of BBzP hydrolysis in mammalian liver MBP, respectively. The species difference in the hydrolysis of BBzP in humans and rats have been reported to be MBzP and MBP type for humans and dogs, and MBP type for monkeys, rats and mice. The urinary major metabolites of monoester phthalate formation was observed between humans and rodents (rats and mice). The kinetics of BBzP hydrolysis in mammalian liver microsomes could be classified into two types: MBzP type for humans and dogs, and MBP type for monkeys, rats and mice. These findings suggest that the formation profile of MBzP and MBP from BBzP by liver microsomes differs extensively among animal species.

**Acknowledgment**

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**REFERENCES**

11. Moore NP. The oestrogenic potential of the phthalate esters. Re-

### Table 1. Kinetic Parameters for BBzP Hydrolysis by Liver Microsomes of Humans, Monkeys, Dogs, Rats and Mice

<table>
<thead>
<tr>
<th>Species</th>
<th>$K_m$ or $S_{50}$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$n$</th>
<th>$CL_{rat}$ or $CL_{max}$ (mL/min/mg protein)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MBP formation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>37.1 ± 2.1</td>
<td>3.25 ± 0.27</td>
<td></td>
<td>0.09 ± 0.01</td>
<td>Michaelis–Menten</td>
</tr>
<tr>
<td>Monkey</td>
<td>56.9 ± 5.0</td>
<td>132 ± 10</td>
<td>1.65 ± 0.11</td>
<td>1.2 ± 0.10</td>
<td>Hill</td>
</tr>
<tr>
<td>Dog</td>
<td>77.2 ± 8.7</td>
<td>3.43 ± 0.36</td>
<td>0.05 ± 0.01</td>
<td>Michaelis–Menten</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>22.9 ± 0.6</td>
<td>54.7 ± 1.9</td>
<td>1.73 ± 0.09</td>
<td>1.21 ± 0.06</td>
<td>Hill</td>
</tr>
<tr>
<td>Mouse</td>
<td>114 ± 21</td>
<td>372 ± 56</td>
<td></td>
<td>3.28 ± 0.39</td>
<td>Michaelis–Menten</td>
</tr>
<tr>
<td><strong>MBzP formation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>16.1 ± 2.4</td>
<td>72.1 ± 5.5</td>
<td>2.29 ± 0.18</td>
<td>2.27 ± 0.2</td>
<td>Hill</td>
</tr>
<tr>
<td>Monkey</td>
<td>41 ± 2.3</td>
<td>52.3 ± 2.4</td>
<td>1.65 ± 0.08</td>
<td>0.65 ± 0.02</td>
<td>Hill</td>
</tr>
<tr>
<td>Dog</td>
<td>28.7 ± 0.7</td>
<td>164 ± 23</td>
<td>2.3 ± 0.23</td>
<td>2.88 ± 0.34</td>
<td>Hill</td>
</tr>
<tr>
<td>Rat</td>
<td>30.6 ± 0.8</td>
<td>9.44 ± 1.47</td>
<td>2.35 ± 0.21</td>
<td>0.16 ± 0.02</td>
<td>Hill</td>
</tr>
<tr>
<td>Mouse</td>
<td>102 ± 17</td>
<td>44.2 ± 8.8</td>
<td>2.17 ± 0.22</td>
<td>0.22 ± 0.03</td>
<td>Hill</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three separate experiments.

by dog and mouse liver microsomes exhibited the Michaelis–Menten model, whereas the kinetics by monkey and rat liver microsomes exhibited the Hill model with $n$ of 1.7. With regard to MBzP formation, the kinetics by liver microsomes of monkeys, dogs, rats and mice as well as humans were fitted to the Hill model with $n$ of 1.7–2.4. Although the $K_m$ for MBP and MBzP formation in each liver microsomes were generally comparable, the $V_{max}$ and in vitro clearance ($CL_{rat}$ or $CL_{max}$) ratios of MBP/MBzP formation varied among animal species.

Species differences in the rate and efficiency of MBP formation in liver microsomes were mice > monkeys > rats > dogs = humans for $V_{max}$, and mice > rats > monkeys > humans > dogs for $CL_{rat}$ or $CL_{max}$. In MBzP formation, the ranking order of $V_{max}$ and $CL_{max}$ was dogs > humans > monkeys > mice > rats. From the in vitro clearance ratios of MBP/MBzP formation, the profile of BBzP hydrolysis in mammalian liver microsomes could be classified into two types: MBzP type for humans and dogs, and MBP > MBzP type for monkeys, rats and mice. In particular, the contracting profile for monoester phthalate formation was observed between humans and rodents (rats and mice). The urinary major metabolites of BBzP in humans and rats have been reported to be MBzP and MBP, respectively. The species difference in the hydrolysis profile of BBzP in this study faithfully reflects the in vivo data reported previously. The structure and function of mammalian carboxylesterases are extensively different among animal species. Surprisingly, the formation profile of MBP and MBzP in liver microsomes of dogs but not monkeys highly paralleled that of human liver microsomes. This phenomenon may mean that the enzymatic properties of carboxylesterase isozyme(s) of dogs involved in BBzP hydrolysis are much more similar to those of humans than other animal species.

In conclusion, we studied the hydrolysis of BBzP to MBP and MBzP in liver microsomes of humans, monkeys, dogs, rats and mice. The kinetics for MBP formation by human, dog and mouse liver microsomes followed the Michaelis–Menten model, whereas the kinetics by monkey and rat liver microsomes were fitted to the Hill model. The kinetics for MBzP formation fitted to the Hill model in all liver microsomes examined. The hydrolysis of BBzP to monoester phthalate in mammalian liver microsomes could be classified into two types: MBzP > MBP type for humans and dogs, and MBP > MBzP type for monkeys, rats and mice. These findings suggest that the formation profile of MBzP and MBP from BBzP by liver microsomes differs extensively among animal species.

