In vitro glucuronidation of 2,2-bis(bromomethyl)-1,3-propanediol by microsomes and hepatocytes from rats and humans

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Nonstandard Abbreviations used:

BPA  Bisphenol A
BMP  2,2-bis(bromomethyl)-1,3-propanediol
TFMU  4-(Trifluoromethyl)umbelliferone
HPLC  high performance liquid chromatography
LC  liquid chromatography
DMD #32110

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<th>Acronym</th>
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<td>LOD</td>
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<td>LOQ</td>
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<td>LSC</td>
<td>liquid scintillation counting</td>
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<td>$R_t$</td>
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<td>UDPGA</td>
<td>uridine diphosphate glucuronic acid</td>
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<td>UGT</td>
<td>Uridine Diphosphate Glucuronosyltransferase</td>
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Abstract

2,2-bis(bromomethyl)-1,3-propanediol (BMP) is a brominated flame retardant used in unsaturated polyester resins. In a two-year bioassay BMP was shown to be a multi-site carcinogen in rats and mice. Because glucuronidation is the key metabolic transformation of BMP by rats, this study compared the *in vitro* hepatic glucuronidation of BMP across several species. Also, the glucuronidation activities of human intestinal microsomes and specific human hepatic UGT enzymes for BMP were determined. To explore other possible routes of metabolism for BMP, studies were conducted with rat and human hepatocytes. Incubation of hepatic microsomes with BMP in the presence of UDPGA resulted in the formation of a BMP-monoglucuronide. The order of hepatic microsomal glucuronidation activity of BMP was rats, mice >> hamsters > monkeys >>> humans. The rate of glucuronidation by rat hepatic microsomes was 90-fold greater than that of human hepatic microsomes. Human intestinal microsomes converted BMP to BMP-glucuronide at a rate even lower than that of human hepatic microsomes. Among the tested human UGT enzymes, only UGT2B7 had detectable glucuronidation activity for BMP. BMP-monoglucuronide was the only metabolite formed when BMP was incubated with suspensions of freshly-isolated hepatocytes from male F-344 rats or with cryopreserved human hepatocytes. Glucuronidation of BMP in human hepatocytes was extremely low. Overall, the results support *in vivo* studies in rats in which BMP-glucuronide was the only metabolite found. The poor glucuronidation capacity of humans for BMP suggests that the pharmacokinetic profile of BMP in humans will be dramatically different than that of rodents.
INTRODUCTION

The brominated flame retardant 2,2-bis(bromomethyl)-1,3-propanediol (BMP) is used in the manufacture of unsaturated polyester resins, molded products and rigid polyurethane foam. The estimated annual production of BMP in the US is 3 to 4 million pounds, but current production figures are not available (Elwell et al., 1989). In contrast to other known brominated flame retardants, BMP is a small aliphatic molecule. Its unique structure contains the bromine bonded to the carbon adjacent to the central carbon that has no hydrogen atoms (Figure 1). This provides a compound very resistant to dehydrobromination. It is soluble in organic solvents (acetone, ethanol, ether), and slightly soluble in water (2 g/l at 25°C) (NTP, 1996; USEPA 2002).

No quantitative data were available on human exposure to BMP during the preparation of this manuscript. Occupational exposure as a result of production of flame-retardant resins could occur (NIOSH, 1995). In such situations, the primary routes of human exposure to BMP are expected to be via inhalation and dermal contact (Larsen, 1969; NTP, 1996; USITC, 1994). BMP may also enter the environment as fugitive dust and through wastewater, where it is expected to be persistent.

Based on animal studies conducted by the National Toxicology Program (NTP), BMP is anticipated to be a human carcinogen (Report on Carcinogens 2004). In NTP’s two year feeding study, BMP was found to be a multi-site carcinogen in Fischer-344 (F-344) rats and B6C3F1 mice (Dunnick et al., 1997). Increased incidences of neoplasms were observed in a variety of tissues (i.e., esophagus, lung, kidney, urinary bladder) (NTP, 1996). Because of BMP’s carcinogenic potential, it is critical to know if it is metabolized similarly in rodents and humans to better extrapolate animal toxicity data to humans.
The hydroxyl groups present on BMP (Figure 1) provide functional groups for glucuronidation and sulfation. Indeed, results of a recent in vivo study with male F-344 rats show that BMP is extensively excreted in the urine (80 % in 12 h) solely as a monoglucuronide conjugate (Hoehle et al., 2009). This BMP-glucuronide is most likely formed in the liver as it is secreted into the bile at early time points after administration. However, following enterohepatic recycling it is primarily eliminated in the urine.

The present study investigated the in vitro glucuronidation of BMP using hepatic microsomes obtained from a number of species including humans, as well as six human hepatic recombinant Uridine Diphosphate Glucuronosyltransferase (UGT) enzymes. To better assess its metabolism in a more integrated system, BMP was also incubated with rat and human hepatocytes.
MATERIALS AND METHODS

Chemicals

[U-\(^{14}\)C] BMP (Lot #10426-17-34) in absolute ethanol (1 mCi/mL) with a specific activity of 65.1 mCi/mmol (247 µCi/mg) was obtained from Midwest Research Institute (Kansas City, MO). The radiochemical purity of [\(^{14}\)C] BMP was determined by reversed phase HPLC-UV/Vis-radiometric analysis to be 97.3%. Non-radiolabeled BMP (Lot #04119MD) was obtained from Sigma-Aldrich (St. Louis, MO). Chemical purity of unlabeled BMP was 98%. [Propyl-2-\(^{14}\)C] bisphenol A (BPA) was obtained from Moravek Biochemicals (Brea, California). Liver digest media (Lot #289936) and Williams Medium E (WME) without phenol red (Lot #1282834) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). Flo-Scint III and Pico-Fluor 40 scintillation cocktail solutions were received from PerkinElmer (Torrance, CA). 4-(Trifluoromethyl)umbelliferone (TFMU), UDPGA, D-saccharic acid-1,4-lactone, \(\beta\)-glucuronidase (EC 3.2.1.31, Type B-1 from bovine liver), sulfatase (EC 3.1.6.1, Type VI from *Aerobacter aerogenes*), acetonitrile, alamethicin and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO). All chemicals and reagents were HPLC or analytical grade.

Biological Materials

Animals

Male F-344 rats from Harlan Sprague-Dawley (Indianapolis, IN) weighing 200-325 g were used. Animals were fed standard commercial diet (Harlan Teklad 4% mouse/rat diet, Harlan Teklad, Indianapolis, IN) and were allowed food and water *ad libitum*. All animals were housed in The University of Arizona Animal Care facility which is accredited by the Association for Assessment and Accreditation of Laboratory Animal
Care. The animals were maintained in a temperature controlled (25°C) room under a 12 h light/12 h dark cycle and acclimated for at least 5-7 days after receipt.

**Microsomal Fractions**

For the preparation of microsomal fractions, male rats were euthanized by CO$_2$ inhalation and livers were excised immediately. Pooled F-344 rat liver microsomes were prepared from 8 male F-344 rats according to the procedure described by Guengerich, (1989). Pooled liver microsomes from female F-344 rats (pool of 105 animals), male B6C3F1 mice (pool of 347 animals), male Golden Syrian Hamsters (pool of 101 animals), and male Rhesus monkeys (pool of 12 animals) were purchased from XenoTech (Lenexa, KS).

Pooled human liver microsomes were also purchased from XenoTech. They were prepared from the livers of 50 donors (29 males and 21 females of Caucasian, Hispanic and African American race, with ages ranging from 7 to 76 years). Pooled human intestinal microsomes were purchased from Gentest (Woburn, MA) and contained equal amounts of microsomes prepared from both the duodenum and jejunum section of each of the 10 donors (6 males and 4 females of Caucasian, Hispanic and African American race, with ages ranging from 5 to 62 years). Supersomes, i. e., microsomes from insect Sf-9 cells infected with a baculovirus strain containing the cDNA of human UGT1A1, 1A3, 1A4, 1A6, 1A9, or 2B7, were obtained from Gentest.

All microsomal preparations were stored at -80°C. Protein concentrations were measured using the BCA Protein Assay Kit from Pierce (Rockfort, IL) with bovine serum albumin as standard (Smith et al., 1985).
Rat and Human Hepatocyte Preparations

Primary hepatocytes from male F-344 rats were prepared in-house via a two-stage perfusion method as described by Pritchett et al. (2002). Only those cell preparations with >90% viability (as determined by trypan blue exclusion) were used for incubations.

Pooled cryopreserved human hepatocytes were purchased from CellzDirect (Austin, TX) and were prepared from the livers of 10 donors (5 males and 5 females of Caucasian and African American race, with ages ranging from 20 to 74 years). The post-thaw viability was determined by trypan blue exclusion to be ≥ 75%.

Glucuronidation Assays

To determine UGT activity towards BMP, hepatic microsomes (from male and female F-344 rats, male B6C3F1 mice, male Golden Syrian hamsters, male Rhesus monkeys, and humans), intestinal microsomes (humans) or expressed individual UGTs (human) were incubated with [14C] BMP. To ensure activation, microsomes were pre-incubated on ice with alamethicin (5 µg/mL) for 10 min (Hoehle et al., 2007). Incubations were then carried out in a total volume of 0.2 mL, consisting of microsomal protein (0.1-1.0 mg/mL), 0.1 M phosphate buffer pH 7.4, magnesium chloride (10 mM), the β-glucuronidase inhibitor D-saccharic acid-1,4-lactone (10 mM), UDPGA (4 mM) and [14C] BMP ([50 µM; 1.1 µCi/mL] dissolved in DMSO; final concentration 1%). The samples were incubated at 37°C for 15 to 360 min depending on the source of microsomes. A number of control incubations were also performed. These included the known substrate for a number of UGT enzymes, TFMU, as well as incubations with heat denatured microsomes, or with intact microsomes in the absence of UDPGA. For incubations with recombinant UGTs, the control incubations consisted of supersomes.
lacking UGT enzymes, but in the presence of UDPGA. All reactions were terminated with 0.2 mL ice-cold methanol. Samples were centrifuged (5 min, 14,000 rpm) and the supernatants (50-100 µL) were subjected to HPLC-radiometric or UV/Vis analysis. All incubations were performed in duplicate on at least three different occasions. For concentration-dependent metabolism studies, incubations were conducted with [14C] BMP at final concentrations of 3.5, 7, 15, 25, 50, 100, 250, 500, or 1000 µM (0.2-0.9 µCi/mL) and rat liver microsomes (0.25 mg/mL) as described above. Glucuronidation activities for each substrate concentration were determined in three independent experiments in duplicate.

**Hepatocyte Incubations**

Rat hepatocytes (0.25 - 1 x 10^6 cells/mL) were incubated in suspension with WME and [14C] BMP (2, 25, 50, 75, or 100 µM), (0.2-0.6 µCi/mL, 0.25% DMSO: absolute ethanol, 10:1 (v/v)) at 37°C in a shaking water bath for 0-120 min in a total volume of 1 mL. Human hepatocytes (0.25 x 10^6 cells/mL) were incubated under similar conditions with [14C] BMP (2, 25, or 50 µM) for 0-360 min. Aliquots (150-250 µL) were removed at various times and reactions were terminated by snap freezing in liquid nitrogen. Samples were thawed and then stored at -20°C until analysis. Prior to HPLC-radiometric analysis the samples were centrifuged (5 min, 14,000 rpm) to obtain a supernatant. Incubations with rat hepatocytes were conducted three times in duplicate for each BMP concentration. [14C] BPA (50 µM, 0.2 µCi/mL), was included to verify glucuronidation activity of rat and human hepatocytes (Pritchett et al., 2002; Kuester and Sipes, 2007). Negative controls were conducted using substrate in WME without cells.
**HPLC Separations and Analyses**

To separate BMP from its metabolite(s), an Agilent HP 1100 HPLC system equipped with a quaternary pump, thermostated column compartment, thermostated auto-sampler, and diode array detector (Agilent Technologies, Palo Alto, CA) was used. The HPLC system was coupled to a flow-through β-RAM detector for [14C] radioactivity (IN/US systems, Tampa, FL) and to an in-line fraction collector (Gilson Inc., Middleton, CA). UV/Vis detection was used to detect TFMU and its metabolites. Data were acquired and analyzed using HP ChemStation software for LC 3D, (Rev. B.01.01, Agilent Technologies, Palo Alto, CA) and WinFlow software (Version 1.5, LabLogic, Sheffield, UK). Samples (50 to 100 µL of supernatant) were injected onto a 250 x 4.6 mm i.d., 5 µm, reversed-phase Luna C18 column coupled with a 4.0 x 3.0 mm i.d. SecurityGuard C18 guard cartridge (Phenomenex, Torrance, CA) and eluted with a mobile phase consisting of Nanopure water and acetonitrile, both containing 0.1% formic acid. The gradient was run from 10% acetonitrile and 90% water for the first min, then up to 40% acetonitrile over 12 min, and finally up to 90% acetonitrile in one min and held for 5 min. The column was re-equilibrated to initial conditions for 10 min between injections. The flow rate was 0.9 mL/min at 25°C. The auto-sampler temperature was maintained at 10°C. Due to the low conversion of BMP to BMP-glucuronide by human hepatocytes, and in order to confirm the absence or presence of any additional metabolites, the fractions of the radio-HPLC effluent were subjected to further analysis with a Beckman LS 3801 liquid scintillation counter (LSC) (Beckman Coulter, Inc., Fullerton, CA). Fractions were collected at a rate of 1 min/tube. The LOD
and LOQ for [14C] equivalents for the LSC were 0.22 and 0.83 pmol, respectively, as determined by the equation described by Zhu et al. (2005).

**Identification of phase II metabolites**

For the identification of conjugates of BMP, samples from microsomal and hepatocyte incubations were subjected to enzymatic hydrolysis with β-glucuronidase or sulfatase followed by HPLC analyses. LC-MS and MS/MS analyses of the conjugate peak were conducted using methods previously described by Hoehle et al. (2009). The Agilent HP 1100 HPLC system was coupled with an MSD-Trap-SL ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA) and LC/MSD Trap software (Version 5.3) for data collection and analysis (Bruker Daltonics, Billerica, MA). The mass spectrometer was operated in the positive electrospray ionization (ESI) mode and two masses were selected for analysis: \( m/z = 438.9 \) (BMP-monoglucuronide) and \( m/z = 262.9 \) (BMP). The first scan was a full MS scan, and then precursor ions were selected, isolated, and selectively fragmented in the ion trap as previously described (Hoehle et al., 2009).

**Data analysis**

The amount of BMP-glucuronide formed and the glucuronidation activity were calculated from the area of the HPLC glucuronide peak using the specific activity of the [14C] BMP stock solution. For the kinetic studies, the data were subjected to analysis based on Michaelis-Menten kinetics by OriginPro 6.0 software (OriginLab Corporation, Northampton, MA). This program determines the \( K_m \) and \( V_{max} \) values from enzyme-kinetic data using non-linear regression analysis. The results from the hyperbolic regression were used to determine the in vitro metabolic clearance (\( CL = V_{max} / K_m \)) which is expressed in volume/time. The calculated CL was scaled up to total liver
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microsomal protein per kilogram (CL_{int}) by assuming a rat liver weight of 30 g/kg body weight, and a conversion factor of 45 mg microsomal protein per 1 g liver (Corley et al., 2005). The total hepatic clearance (CL_{H}) was calculated using the following formula from the well-stirred model:

\[ CL_{H} = \frac{Q \times f_u \times f_{u \text{inc}} \times CL_{int}}{Q + (f_u \times f_{u \text{inc}} \times CL_{int})} \]

where \( f_u \) is the unbound fraction of BMP in blood, \( f_{u \text{inc}} \) is the fraction of BMP unbound to microsomal protein, and \( CL_{int} \) is the total \textit{in vitro} intrinsic clearance (Houston, 1994). The rat hepatic blood flow (Q) was assumed to be 45 mL/min/kg (Corley et al., 2005) and \( f_u \) was determined to be 0.6 by a method previously described by Cheng et al. (2009). \( f_{u \text{inc}} \) was estimated to be 0.94 (Hallifax and Houston, 2006). The liver extraction ratio was determined using the CL_{H} to Q ratio.
RESULTS

BMP-glucuronide formation by hepatic microsomes from F-344 rats

In pilot studies $[^{14}\text{C}]$ BMP (or non-radiolabeled BMP for MS analysis) was incubated with various concentrations of hepatic microsomal protein (0.1-0.5 mg/mL) from male F-344 rats in the presence of UDPGA. HPLC analysis of these samples revealed a single product peak ($R_t = 12.4$ min) eluting earlier than BMP ($R_t = 14.4$ min) from the reverse phase column (Figure 2). This product was tentatively identified as a glucuronide of BMP (Figure 1) as incubation with $\beta$-glucuronidase resulted in a loss of this peak with a corresponding increase in a peak that co-eluted with the BMP standard. MS/MS analysis of this metabolite in incubations of rat liver microsomes with BMP revealed a fragmentation pattern identical to that reported previously for the monoglucuronide of BMP (Hoehle et al., 2009). The formation of the BMP-glucuronide was stoichiometric with the loss of BMP, and it increased with an increase in concentration of microsomal protein and with time of incubation. Linear product formation for up to 120 min was achieved using protein concentration of 0.25 mg/mL.

A single contaminant eluting at 16.4 min was observed in radio-HPLC analyses of $[^{14}\text{C}]$ BMP standard (Data not shown). This peak was not observed in any incubated samples. However a new peak eluted at 5.5 min which contained the same amount of radiolabel (< 3%). This peak appears to be a degradation product of the original contaminant as described previously (Hoehle et al., 2009)

Incubation of $[^{14}\text{C}]$ BMP with primary hepatocytes from male F-344 rats

When $[^{14}\text{C}]$ BMP was incubated in suspensions of primary hepatocytes obtained from male F-344 rats, the same glucuronide identified in incubations using rat liver microsomes was formed (Figure 3 A). There was no evidence for the formation of other
metabolites of BMP by rat hepatocytes (sulfatase treatment, HPLC analyses). A time course showing the conversion of BMP into BMP-monoglucuronide is depicted in Figure 4. The formation of the BMP-monoglucuronide was stoichiometric, regardless of the concentration of BMP (2-100 µM) or the concentration of rat hepatocytes (0.25-1.0 x 10^6 cells/mL). Data are only presented for incubations containing 50 µM BMP.

**Kinetics of BMP glucuronidation in hepatic microsomes from F-344 rats**

Kinetic analysis of BMP-glucuronide formation was performed using hepatic microsomes from male and female F-344 rats. The formation of the monoglucuronide of BMP followed Michaelis-Menten kinetics (Figure 5). The V_max values were 6161 ± 173 and 6339 ± 250 pmol/min/mg protein, and K_m values were 95 ± 8 and 153 ± 17 uM, for hepatic microsomes from male and female F-344 rat, respectively (Table 1). The CL (V_max /K_m) was scaled up to CL int and modelled to estimate CL_H (Table 1). The total hepatic clearances (CL_H) were found to be 23.5 and 18.5 mL/min/kg, for male and female rats, respectively.

**Glucuronidation of BMP by human hepatic and intestinal microsomes, and UGTs**

When [14C] BMP (50 µM) was incubated with human liver microsomes and UDPGA under the same conditions used for rat liver microsomes (0.25 mg/mL), no BMP-glucuronide was detected. When higher amounts of human hepatic microsomal protein (0.5 and 1.0 mg/mL) and longer incubation times (30-360 min) were used, the HPLC-radiochromatograms revealed a small radioactive peak that co-eluted with the BMP-glucuronide. In addition, MS/MS analyses confirmed that this peak was the monoglucuronide conjugate. Remarkably, even after 360 min of incubation at the high microsomal protein concentration of 1.0 mg/mL, only 8% of the initial BMP was converted into BMP-glucuronide. The glucuronidation activity of human liver
microsomes was 12 pmol/min/mg protein as compared to 1110 pmol/min/mg protein for F-344 rat liver microsomes (Figure 6). Because the rate of BMP-glucuronide formation by human hepatic microsomes was so low and did not follow Michaelis-Menten kinetics, CL could not be determined. There was no evidence of saturation even at concentrations up to 1000 µM. Results from positive control incubations conducted with TFMU confirm that these human hepatic microsomes possessed glucuronidation activity. After 10 min of incubation under similar conditions as with BMP, greater than 90% of TFMU was converted to TFMU-glucuronide by human hepatic microsomes.

To further investigate the capacity for human enzymes to glucuronidate BMP, studies were conducted with intestinal microsomes and a series of recombinant cDNA-expressed UGT enzymes (Supersomes). Human intestinal microsomes converted BMP to BMP-glucuronide at a slower rate (2.3 pmol/min/mg) than hepatic microsomes. Of the 6 expressed human hepatic UGTs incubated with [14C] BMP, only UGT2B7 was active. It formed BMP-glucuronide at a very low rate (7 ± 2 pmol/min/mg protein).

**Incubations of [14C] BMP with human cryopreserved hepatocytes**

When [14C] BMP (50 µM) was incubated with suspensions of cryopreserved human hepatocytes, the only metabolite detected was the same monoglucuronide identified in human microsomes (Figure 3 B). The BMP-glucuronide was generated at very low rates. Less than 3% of initial BMP concentration was converted to this metabolite over the 6 h incubation at the three BMP concentrations. For example at 50 µM BMP, the rate of BMP glucuronidation in human hepatocytes (10 pmol/min/10^6 cells) was about 150-fold lower than that of F-344 rat hepatocytes (1498 pmol/min/10^6 cells). These human hepatocytes possessed efficient UGT activity as demonstrated by the glucuronidation of [14C] BPA. When the human hepatocytes were incubated with 50 µM
[\textsuperscript{14}C] BPA, by 360 min, 26% of the starting concentration of BPA was converted to BPA-glucuronide. The glucuronidation of BPA by human cryopreserved hepatocytes has been previously reported by Kuester and Sipes (2007).

**Glucuronidation of BMP by hepatic microsomes from different species**

In addition to liver microsomes from male and female F-344 rats and for humans, the glucuronidation of BMP was determined in hepatic microsomes obtained from: male B6C3F1 mice, male Golden Syrian hamsters and male Rhesus monkeys. Microsomes from all sources catalyzed the formation of the BMP-monoglucuronide, although with markedly different activities (Figure 6). Hepatic microsomes from male mice showed similar activities to those from male and female F-344 rats. Activities of hepatic microsomes from male hamsters were 3-fold lower than those of rats and mice. BMP glucuronidation activities of monkey hepatic microsomes were 6-fold lower than those of rodents but 14-fold higher than the activities in human liver microsomes.
DISCUSSION

The present study was conducted to characterize the *in vitro* metabolism of the brominated flame retardant, BMP. Based on previous *in vivo* studies indicating that the formation of a BMP-glucuronide is the major route of BMP metabolism in rats (Hoehle et al., 2009), the studies reported herein focused on interspecies variations in hepatic glucuronidation of BMP. The *in vitro* experiments using hepatic microsomes from rats, mice, hamsters, monkeys and humans as well as in suspensions of hepatocytes from rats and humans revealed the formation of only one metabolite, BMP-monoglucuronide. The extensive glucuronidation of BMP by rat hepatic microsomes and rat hepatocytes is consistent with *in vivo* results obtained in this species which revealed the rapid excretion of BMP as a glucuronide conjugate. The rapid glucuronidation of BMP also explains its low oral bioavailability in rats (Hoehle et al., 2009).

Because the glucuronidation of BMP followed Michaelis-Menten kinetics, it was possible to calculate an *in vitro* intrinsic clearance, CL, and then scale this value to estimate its *in vivo* hepatic clearance (CL_H) via glucuronidation. The mean value for the overall CL_H of BMP by hepatic microsomes from male and female F-344 rats was estimated to be 23.5 and 18.5 mL/min/kg. This value indicates a clearance of BMP in rats that corresponds to approximately 50% of the rat liver blood flow that was reported to be 45 mL/min/kg (Corley et al., 2005). These estimated hepatic CL_H values suggest a liver extraction ratio of 0.4 to 0.5 for rats. Compounds with extraction ratios of greater than 0.7 are considered to be non-restrictively cleared, while those with extraction ratios below 0.3 are restrictively cleared and are considered to have a low hepatic clearance (Rowland and Tozer 1995). The hepatic extraction ratios of rat hepatic microsomes for BMP fall into the intermediate range. As stated previously, CL values could not be
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determined for human hepatic microsomes and thus extraction ratios were not estimated.

The extrapolated CL_H of 18.5 – 23.5 mL/min/kg correlates well with the in vivo plasma clearance of BMP, 22 mL/min/kg, calculated from the data provided by Hoehle et al. (2009). It is somewhat surprising that these values compare so favorably because BMP-glucuronide is known to undergo enterohepatic recycling, an event which can prolong plasma half-life and decrease clearance. As this does not occur in vitro, the extrapolated CL_H could exceed in vivo CL. However, it is primarily BMP-glucuronide that circulates in plasma after enterohepatic recycling, which would not affect the clearance of the parent compound. Also, it is possible that tissues other than the liver contribute to the glucuronidation of BMP in vivo and thus increase its overall rate of clearance.

In contrast to the results obtained with rat hepatic preparations, the results of in vitro studies with human liver microsomes and human hepatocytes indicate that the capacity for glucuronidation of BMP by human liver is very low. Incubations of BMP with hepatocytes, which contain a spectrum of metabolic enzymes and cofactors, did not result in the formation of any additional metabolites. Thus it appears that glucuronidation is the sole route of BMP metabolism by both rat and human hepatocytes. If any additional metabolites are formed, they are formed at levels below the limit of detection. However, recovery of radiolabel from the column as parent and glucuronide was usually greater than 95%.

Because of the minimal metabolism of BMP by human hepatic microsomes and hepatocytes, its glucuronidation by human intestinal microsomes was investigated. Intestinal glucuronidation is known to occur for numerous drugs and chemicals (Cubitt et al., 2009), and may play a more important role in humans than rats (Dalvie et al.,
2008; Wong et al., 2009). Some UGT enzymes (i.e. UGT1A8) are expressed primarily in the intestinal system (Fisher et al., 2001). Although human intestinal microsomes did glucuronidate BMP, the rate was slower than that of hepatic microsomes. Thus, it appears that BMP undergoes minimal glucuronidation by human tissues, and no other significant metabolic pathways are apparent.

The only expressed UGT enzyme to glucuronidate BMP was UGT2B7. This human enzyme glucuronidated BMP at a rate that was similar to that of human hepatic microsomes. Members of the UGT1A family were inactive. Because there is a high homology among members of the UGT2B family (Mackenzie et al., 2005), and UGT2B7 was the only member of the UGT2B family tested, it is not known if other UGT2B enzymes may contribute somewhat to the low level of BMP glucuronidation observed in human liver and intestine. It is likely that UGT2B enzymes are responsible for the glucuronidation of BMP by hepatic microsomes of rats, as well as those of the other species tested. However, the dramatic variation in the extent of BMP glucuronidation between rats and humans suggests that rats may possess a specific UGT2B enzyme that is not, or is only poorly expressed in human hepatic microsomes. Other investigators have reported that human liver microsomes possess dramatically less glucuronidation activity towards aliphatic alcohols than rat liver microsomes. For example, the $V_{\text{max}}/K_m$ ratios for the glucuronidation of n-propanol by rat and human hepatic microsomes were 3.0 and 0.5 μL/min/mg, respectively (Iwersen and Schmoldt, 1997 and Jurowich et al., 2003).

As BMP is a di-alcohol, it may be metabolized by the same UGT as simple alcohols. Iwersen and Schmoldt (1997) reported that a specific hydroxysteroid UGT (UGT2B3) present in rat liver microsomes catalyzed the glucuronidation of aliphatic
alcohols (C1-C7). However, the affinity of this enzyme was low with $K_m$ values ranging from 0.3 – 86 mM ($K_m$ values for glucuronosyl transferase(s) decreased with increasing chain length). The alcohol with the highest affinity (heptanol) yielded a $V_{\text{max}}/K_m$ value of 40 $\mu$L/min/mg, which is similar to the value of 41-65 $\mu$L/min/mg obtained for BMP reported here. In studies with human liver microsomes, Jurowich et al. (2003) reported a similar structure-activity relationship for glucuronidation of these alcohols, but found the affinities of them to be 2 to 6-fold lower for human liver microsomes than for rat liver microsomes. Thus, BMP appears to behave similarly to aliphatic alcohols (C5-C7) with respect to glucuronidation by rat and human liver microsomes. Understanding the molecular aspects of this rodent/human difference in glucuronidation capacity towards chemicals such as BMP has important toxicological ramifications, particularly when metabolic data are used to extrapolate findings in rats to humans.

In conclusion, glucuronidation appears to be the sole route of metabolism for BMP in rodents and humans and most likely for the other species investigated. Among the tested species, rodents displayed the highest glucuronidation capacity. Our data suggest that interspecies differences in UGT enzymes significantly alter the glucuronidation of BMP and result in severely compromised capacity to metabolize BMP in some species. The poor glucuronidation capacity of BMP by humans suggests that its $\text{in vivo}$ pharmacokinetic profile will differ dramatically from that obtained in rodents. How such dramatic differences in pharmacokinetics will affect the potential for BMP toxicity in humans remains to be established.
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REFERENCES


Hallifax D and Houston JB. 2006 Binding of drugs to hepatic microsomes; Comment and assessment of current prediction methodology with recommendation for improvement. Drug Metabolism and Dispos 34: 724-726.


National Toxicology Program (NTP) Toxicology and Carcinogenesis Studies of 2,2-Bis(Bromomethyl)-1,3-Propanediol (FR-1138(R)) (CAS No. 3296-90-0) in F344 Rats and B6C3F1 Mice (Feed Studies). *Natl Toxicol Program Tech Rep Ser* 452:1-465.


U.S. Environmental Protection Agency (USEPA) (2002b). Test plan for 2,2-bis(bromomethyl)-1,3-propanediol CAS No. 3296-90-0, High Production Volume (HPV) Chemical Challenge Program, Washington, DC.


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Figure 2. Representative HPLC-radiochromatograms from incubations of BMP with hepatic microsomes obtained from male F-344 rats. The 30 min incubations contained 0.25 mg microsomal protein per mL, and 50 µM $[^{14}C]$ BMP. (A) Incubation without UDPGA and (B) Incubation in the presence of UDPGA (4 mM). Peak at 5.5 min represents a contaminant (see text).

Figure 3. Representative HPLC-radiochromatograms of the BMP-glucuronide formed after the incubation of 50 µM BMP with (A) freshly-isolated male F-344 rat hepatocytes (30 min, 0.5 x 10$^6$ cells/mL) and (B) human cryopreserved hepatocytes (240 min, 0.25 x 10$^6$ cells/mL). (Note the difference in scale on the y-axis).

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Figure 6. Activities of hepatic microsomes from male and female F-344 rats, male B6C3F1 mice, male Golden Syrian hamsters, male Rhesus monkeys, and humans (mixed gender) for the glucuronidation of BMP. Rates of glucuronidation were
determined at 50 µM BMP and are expressed as pmol/min/mg protein (mean ± SD of at least three independent experiments).
Table 1. Kinetic parameters for the glucuronidation of $[^{14}C]$ BMP by F-344 rat hepatic microsomes. All data are converted to the rate of BMP-glucuronide formed per minute per mg microsomal protein (mean ± SD of three independent experiments). CL$_{\text{int}}$ and CL$_{\text{H}}$ determinations were carried out as described under Materials and Methods.

<table>
<thead>
<tr>
<th>Rat Hepatic Microsomes</th>
<th>$V_{\text{max}}$ (pmol/min/mg protein)</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}/K_m$ (µL/min/mg protein)</th>
<th>Scaled CL$_{\text{int}}$ (mL/min/kg)</th>
<th>Scaled CL$_{\text{H}}$ (mL/min/kg)</th>
<th>Extraction Ratio (ER)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>6161 ± 173</td>
<td>95 ± 8</td>
<td>65</td>
<td>88</td>
<td>23.5</td>
<td>0.54</td>
</tr>
<tr>
<td>Female</td>
<td>6339 ± 250</td>
<td>153 ± 17</td>
<td>42</td>
<td>56</td>
<td>18.5</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.

(A) [14C] equivalents (dpm) vs. Retention Time (min)

(B) [14C] Equivalents (dpm) vs. Retention Time (min)

BMP

BMP glucuronide
Figure 3.
Figure 4.

![Graph showing BMP Glucuronide Formation and BMP Depletion over Incubation Time (min)].

- **BMP Glucuronide Formation (nmol/10^6 cells)**
- **BMP Depletion (nmol/10^6 cells)**

The graph illustrates the decrease in BMP depletion and the increase in BMP glucuronide formation over time.
Figure 5.

BMP Glucuronidation Activity (pmol/min/mg protein) vs. [14C] BMP (µM)
Figure 6. BMP Glucuronidation Activity (pmol/min/mg protein)

- Rat (male)
- Rat (female)
- Mouse (male)
- Hamster (male)
- Monkey (male)
- Human (mixed)