Absorption, distribution, metabolism and excretion of 2,2-bis(bromomethyl)-1,3-propanediol in male Fischer-344 rats

SIMONE I. HOEHLE, GABRIEL A. KNUDSEN, J. MICHAEL SANDERS, AND I. GLENN SIPES*

Department of Pharmacology, College of Medicine, The University of Arizona, Tucson, AZ, USA: SIH, GAK, IGS

Laboratory of Pharmacology and Chemistry, National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA: JMS
Running Title: Disposition of 2,2-bis(bromomethyl)-1,3-propanediol in rats

Corresponding author:

I. Glenn Sipes, Ph.D.
Department of Pharmacology
College of Medicine
The University of Arizona
P.O. Box 245050
Tucson, AZ 85724-5050
Telephone: 520-626-7123
Fax: 520-626-2466
email: sipes@email.arizona.edu

Number of text pages: 19
Number of tables: 1
Number of figures: 6
Number of references: 24
Number of words:

Abstract: 246
Introduction: 396
Discussion: 1274

Nonstandard Abbreviations used:

AUC area under the blood concentration-time curve from time zero to infinity
BDC bile duct cannula
BMP 2,2-bis(bromomethyl)-1,3-propanediol
C_{max} calculated maximum concentration in blood
HPLC high performance liquid chromatography
JVC jugular vein cannula
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>lethal dose for 50 percent of the population</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>limit of quantification</td>
</tr>
<tr>
<td>LSC</td>
<td>liquid scintillation counting</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>R&lt;sub&gt;t&lt;/sub&gt;</td>
<td>retention time</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2α&lt;/sub&gt;</td>
<td>half-life of distribution</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2β&lt;/sub&gt;</td>
<td>terminal half-life of elimination</td>
</tr>
</tbody>
</table>
Abstract

2,2-Bis(bromomethyl)-1,3-propanediol (BMP) is a brominated flame retardant, previously shown to be a multi-site carcinogen in experimental animals. Studies were conducted to characterize the dispositional and metabolic fate of BMP following oral or intravenous (IV) administration to male Fischer-344 rats. Following a single oral administration of [14C] BMP (10 or 100 mg/kg) greater than 80% of the low dose and 48% of the high dose was excreted by 12 h in the urine predominantly as a glucuronide metabolite. Following repeated daily oral doses for 5 or 10 days, route and rate of elimination were similar to those obtained after single administrations of BMP. In all studies the radioactivity recovered in feces was low (<15%). The total amount of radioactivity remaining in tissues at 72 h after a single oral administration of BMP (100 mg/kg) was less than 1% of the dose and repeated daily dosing did not lead to retention in tissues. Following IV administration the radiolabel found in blood decreased rapidly. Excretion profiles were similar to those following oral administration. Parent BMP and BMP glucuronide were present in blood plasma following oral or IV dosing. After an IV dose of BMP (15 mg/kg) the hepatic BMP glucuronide was primarily exported into the bile (>50% within 6 h), but underwent enterohepatic recycling with subsequent elimination in the urine. These data indicate that the extensive extraction and rapid glucuronidation by the liver limits exposure of internal tissues to BMP by greatly reducing its systemic bioavailability following oral exposure.
INTRODUCTION

2,2-Bis(bromomethyl)-1,3-propanediol (BMP) is a brominated flame retardant (BFR) found in unsaturated polyester resins, molded products, and rigid polyurethane foam. It is also used as an additive during manufacture of plastic polymers and as a chemical intermediate for other flame retardants (Larsen, 1969; Larsen and Weaver, 1973). Between 1986 and 2002 the estimated annual aggregate production of BMP in the US was 1-10 million pounds, and it is classified as a high volume chemical (NTP, 1996; USEPA, 2002a).

The compound has a unique aliphatic neopentyl structure containing no hydrogen atoms adjacent to the carbon bonded to the bromine (Figure 1) and is slightly soluble in water (2 g/L at 25°C). BMP may enter the environment through wastewater or dust particles and is expected to be environmentally persistent (NTP, 1996; USEPA 2002b). According to a study conducted in the Ramat Hovav aquitard, its half-life is estimated to be more than 100 years (Ezra et al., 2006).

The acute oral toxicity of BMP in rats is reported to be low (oral LD\textsubscript{50}: 3.5 g/kg) (Keyes et al., 1979). Studies in experimental animals conducted by the National Toxicology Program (NTP) in the 1980s and 90s indicate that BMP is a multi-site carcinogen in rats and mice. In a 13-week sub-chronic study kidney and urinary bladder lesions were reported in both rats and mice receiving BMP either in feed (0-3000 mg/kg) or by oral gavage (0-800 mg/kg). Mostly lesions were observed at the two highest doses of BMP for each the type of administration and test animal (Elwell et al., 1989). Further, in a 2-year dietary toxicity study in Fischer-344 (F-344) rats neoplasms of the skin, subcutaneous tissue, mammary gland, Zymbal's gland, oral cavity, esophagus, forestomach, small and large intestine, mesothelium, kidney, urinary bladder, lung,
thyroid gland, seminal vesicle, hematopoietic system, and pancreas were observed in animals dosed with BMP (Dunnick et al., 1997; NTP, 1996). Based on evidence of carcinogenicity from these studies, BMP is reasonably anticipated to be a human carcinogen (Report on Carcinogens, 2004).

Little information is available on the disposition and metabolism of BMP and their relevance to BMP toxicity. The studies presented here report on the absorption, distribution, metabolism and elimination of BMP, and they describe the pharmacokinetics of BMP following oral or intravenous (IV) administration to male F-344 rats. Additional studies were designed to determine if repeated oral administration of BMP alters its disposition profile.

MATERIALS AND METHODS

Chemicals

[U-\textsuperscript{14}C]-labeled BMP (Lot #10426-17-34), in absolute ethanol (1 mCi/mL) was obtained from Midwest Research Institute (Kansas City, MO). The radiochemical purity of BMP was determined by reversed phase HPLC-UV/Vis-radiometric analysis to be 97.3%. The specific activity was reported to be 65.1 mCi/mmol (247 µCi/mg). Non-radiolabeled BMP (Lot # 04119MD) was obtained from Sigma-Aldrich (St. Louis, MO). Chemical purity of unlabeled BMP was 98%. Soluene-350\textsuperscript{®} and Solvable\textsuperscript{®} tissue solubilization solvents and Pico-Fluor 40 scintillation cocktail solution were received from Perkin-Elmer (Torrance, CA). Hydrogen peroxide (30%) was obtained from VWR (Westchester, PA). Absolute ethanol was from Decon Laboratories, Inc. (King of Prussia, PA). Cremophore\textsuperscript{®} EL, β-glucuronidase (EC 3.2.1.31, Type B-1 from Bovine Liver), sulfatase (EC 3.1.6.1, Type VI from \textit{Aerobacter aerogenes}), D-saccharic acid-1,4-lactone, glycine/HCl, acetonitrile, and all other chemicals and reagents were
obtained from Sigma-Aldrich (St. Louis, MO). All chemicals and reagents were HPLC or analytical grade.

**Animal Studies**

**Animals**

Male F-344 rats of 8-9 weeks of age (182-236 g) without surgical alteration (conventional), or with indwelling jugular vein cannula (JVC) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). All animals, with the exception of rats with surgically implanted bile duct cannula (BDC; study described below), were housed in The University of Arizona Animal Care facility which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The animals were maintained in a temperature controlled (25°C) room under a 12 h light/12 h dark cycle. Conventional animals were acclimated for 5-7 days after receipt. Animals with surgically implanted JVC were acclimated only for 1 day after arrival to ensure the patency of cannula. Animals were allowed food (NTP 2000, Zeigler Brothers, Inc., Gardners, PA) and water *ad libitum* except for a 12 h fasting period prior to single administration of BMP. Food was returned 2 h after dosing. Animals used in the repeated oral administration studies were not fasted. Animals were placed in Nalgene® metabolism cages 24 h prior to administration of BMP. Food was provided as a powder to reduce contamination of fecal matter.

**Dose selection**

The doses used in the *in vivo* studies reported herein, were selected based on the results of toxicity studies published by Elwell et al. (1989). Sub-toxic doses of 10, 100, 150, 300 and 600 mg/kg were chosen to assess the effect of dose on the rate and route of excretion following oral gavage. The doses of 10 mg/kg and 15 mg/kg were...
selected for the IV route of administration. For repeated dose studies 100 mg/kg was administered daily by oral gavage for 1, 5 or 10 days. Doses provided 25-200 µCi/kg \([^{14}\text{C}]\) BMP. In each study, \([^{14}\text{C}]\) BMP was administered to four animals (N=4) in a solution of Cremophore EL: absolute ethanol: water, 3:1:1 (v/v/v), with the exception of the BDC study (N=3).

**Sample collection and preparation**

Following dosing of BMP, the animals were maintained in Nalgene® metabolism cages for collection of urine and feces. In single dose studies, urine was collected at 6, 12, 24, 36, 48, and 72 h; feces were collected at 12, 24, 36, 48, and 72 h. In the repeated dose studies, urine was collected at 6, 12, and 24 h after each dose, while feces were collected at 12 and 24 h after each administration. The metabolism cages were rinsed with methanol following the collection of urine. Radioactivity recovered in cage rinses was added to that determined for urine.

Following collections, triplicate aliquots of urine (10-100 µL) and cage rinse (1 mL) were directly analyzed for \([^{14}\text{C}]\) equivalent content by liquid scintillation counting (LSC). In addition, an aliquot of urine (20-100 µL) was mixed with an equal volume of deionized water, centrifuged (5 min, 1000 x g), and the supernatant subjected to HPLC-radiometric analysis. Samples of urine for LC-MS/MS analysis were from animals that were administered non-radiolabeled BMP. These urine samples were prepared using the same methods as described for HPLC-radiometric analysis. Feces were mixed with water to form a homogenous mixture. To determine total \([^{14}\text{C}]\) radioactivity content, triplicate aliquots of fecal samples (100-200 mg) were solubilized using Soluene-350® according to Thompson and Burns (1996).
At the terminus of each study, animals were euthanized by CO\textsubscript{2} inhalation. Blood was collected from the posterior vena cava into a heparinized syringe and the animals subjected to necropsy. When bladder urine was available at necropsy, samples were prepared as described for urine and analyzed by HPLC with radiometric detection. All collected tissues (adipose, brain, cecum, cecum contents, heart, intestine, intestinal contents, kidneys, liver, lung, muscle, spleen, stomach, stomach contents, skin, and testes) were stored at -20°C until analysis. Blood was processed immediately. Triplicate aliquots from each collected tissue (100-200 mg) and blood (50-100 µL) were solubilized with Solvable\textsuperscript{®} (Thompson and Burns, 1996). Body composition estimates of 11% adipose tissue, 8% blood, 50% muscle, and 16% skin were used to estimate total masses of these tissues (Birnbaum et al., 1980). Following solubilization and addition of 15 mL Pico-Fluor 40, all samples were stored in the dark for 48 h to control for chemiluminescence and were corrected for background. Total [\textsuperscript{14}C] radioactivity in all samples was determined by LSC.

**Biliary excretion study**

This study was conducted at the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, NC, in accordance with institutional guidelines. The animal facilities at NIEHS are AAALAC accredited. F-344 rats, obtained from Charles River (Raleigh, NC) were 3 months old with a weight range of 310-336 g. Each rat was anesthetized with 50 mg/kg pentobarbital intraperitoneal (IP) at a dose volume of 1 mL/kg. Following anesthetization, a midline incision in the abdomen was made, and the bile duct was isolated, distally ligated, and punctured with a 25 gauge needle. A beveled PE10 cannula was inserted through the puncture site into the bile duct for a distance of one centimeter and secured with a suture. Bile flow was confirmed...
and the incision was closed with wound clips. [\(^{14}\text{C}\)] BMP (15 mg/kg; 25 µCi/kg; 1 mL/kg) was administered by tail vein injection and bile was collected on ice at time points from 0.025 to 6 h. The bile samples (10 µl aliquots) were analyzed by LSC for [\(^{14}\text{C}\)] content and stored at -80°C for later analysis by HPLC. Rats were placed on heated pads (40°C), changed hourly to maintain a constant body temperature throughout the procedure. The body temperature of the rats, monitored hourly, ranged from 35-38°C. Each rat remained anesthetized throughout the procedure as a result of hourly IP injections of 0.1 ml pentobarbital. Concurrent IP injections of 300-500 µl of 0.85% saline containing 2.5 mM taurocholic acid maintained hydration of the animals. Euthanasia was by CO₂ inhalation. For HPLC-radiometric analysis, the bile was centrifuged and an aliquot (10 µL) of the supernatant was directly injected into the HPLC system.

**Blood kinetics following intravenous and oral administration of BMP**

For determination of IV blood kinetics [\(^{14}\text{C}\)] BMP (10 or 15 mg/kg; 50 µCi/kg; 2 mL/kg) was administered IV through the JVC (in a solution of Cremophore EL: absolute ethanol: water, 3:1:1 (v/v/v)). Blood (100 µL) was then drawn into the cannula, returned to the circulation and the cannula flushed with normal saline (1 mL/kg). For oral blood kinetic studies, [\(^{14}\text{C}\)] BMP (10 mg/kg; 200 µCi/kg; 4 mL/kg) was administered by oral gavage to JVC F-344 rats. Blood samples (300 µL) were collected via the JVC at 0.083, 0.125, 0.25, 0.5, 1, 1.5, 3, 6, 9, 12, 24, 36, and 48 h into heparinized syringes. The removed aliquots of blood were replaced with an equal volume of saline. The volume of 300 µL blood per time point was chosen based on recommendations made by Diehl et al. (2001) where it was demonstrated that in short term pharmacokinetic studies, the removal of 20% of the blood volume over 24 h produces minimal disturbance of normal
physiological function. Aliquots (2 x 50 µL) from these blood samples were solubilized and [¹⁴C] radioactivity quantified by LSC.

Following IV administration blood samples obtained were centrifuged (750 x g; 15 min) to separate plasma from red blood cells. An aliquot (50 µL) of each plasma sample was mixed with 50 µL acetonitrile, vortexed, and centrifuged to precipitate proteins. The precipitate contained 3% of the radioactivity. To the resulting supernatant 50 µl acetonitrile was added, and the mixture was re-centrifuged. The final supernatant was concentrated under a stream of nitrogen to a volume ≤50 µL and subjected to HPLC analysis with radiometric detection.

Aliquots (150 µL) of each blood sample obtained following oral administration were mixed with 450 µL of ethyl acetate, vortexed for 5 min and centrifuged (5 min; 1000 x g). Extractions were performed 3 times. Supernatants were pooled and an aliquot (20 µL) was analyzed by LSC. The remaining supernatant was evaporated to dryness (MiVac, Genevac, Valley Cottage, NY) under reduced pressure, reconstituted in 150 µL methanol and analyzed by HPLC with radiometric detection.

**Pharmacokinetic analysis**

The blood concentration time data following IV (parent BMP) and oral administration (total [¹⁴C] and parent BMP) were analyzed using a computer modelling program (WinNonlin Professional, Version 5.1, Pharsight Corp., Mountain View, CA). The program was utilized to fit the data to a suitable multi-compartment model using non-linear regression analysis, assuming first-order kinetics for all processes. The model was used to calculate values for the half-life of distribution ($t_{1/2\alpha}$), terminal half-life for elimination ($t_{1/2\beta}$), area under the blood concentration-time curve from zero to infinity (AUC), and maximum oral bioavailability ($F$). Only samples containing quantities of
compound above the limit of quantification (LOQ) were used in pharmacokinetic analyses.

**Identification of phase II metabolites**

To determine the presence of glucuronide conjugates of BMP, an aliquot of urine was mixed with an equal volume of 0.15 M acetate buffer pH 5.0 and incubated with 5000 Fishman U of β-glucuronidase (Type B-1) prior to HPLC-radiometric analysis. For the analysis of sulfate conjugates of BMP, an aliquot of urine was mixed with an equal volume of 0.1 M phosphate buffer pH 7.1 containing 0.1 U of Sulfatase (Type VI) prior to HPLC-radiometric analysis. Control incubations contained an aliquot of identical urine but no enzyme.

**Studies for the identification of $[^{14}C]$ radioactivity in blood and liver**

To characterize the identity of $[^{14}C]$ equivalents in blood by HPLC-radiometric analysis, total blood from rats following IV or oral administration of BMP was used for extraction. Four male F-344 rats were administered $[^{14}C]$ BMP (10 mg/kg; 200 μCi/kg; 2 mL/kg) by oral gavage. Two of these animals were sacrificed at 40 min ($C_{\text{max}}$) post-dose and two at 6 h after dosing. In addition, two rats were administered $[^{14}C]$ BMP by IV via the lateral tail vein (10 mg/kg; 200 μCi/kg; 1 mL/kg). These animals were euthanized at 30 min following dosing. At the terminus of each experiment, the liver and whole blood were collected. The collected blood (5-6 mL/animal) was separated by centrifugation (750 x g; 15 min) into blood plasma and blood cells. The cell pellet was washed with normal saline (2 x 2.5 mL) and the washes were added to the plasma. The plasma/saline mixture (6-7 mL) was acidified with an equal volume of 0.7 M glycine/HCl buffer pH 1.2 and 10 mL of ethyl acetate were added. The mixture was vortexed for 30 min and centrifuged (5 min, 1000 x g) for phase separation. The extraction was
repeated at least 5 times. The combined organic extracts were evaporated to dryness under reduced pressure, reconstituted in 300 µL methanol, and an aliquot (80 µL) was used for HPLC-radiometric analysis. In addition, the extracted aqueous phases were analyzed by HPLC-radiometric analysis after partial evaporation and pH adjustment. An aliquot of the liver was homogenized, extracted under acidic conditions as described above for blood plasma and the reconstituted extracts and aqueous phases were analyzed by HPLC with radiometric detection.

Analytical methods

HPLC analysis

The HPLC system consisted of an Agilent 1100 quaternary pump, thermostated column compartment, thermostated autosampler, and diode array detector (Agilent Technologies, Palo Alto, CA) coupled with 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). Data were acquired and analyzed using HP ChemStation software for LC 3D, (Rev. B.01.01 (164), Agilent Technologies, Palo Alto, CA) and WinFlow software (Version 1.5 (9), LabLogic, Sheffield, UK). Additionally, fractions of the HPLC effluent were collected at 1 min intervals and analyzed with a Beckman LS 3801 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Separation was carried out on 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). The mobile phases consisted of Nanopure water and acetonitrile containing 0.1% formic acid each. The gradient was run from 10% acetonitrile and 90% Nanopure water for the first min, then up to 40% acetonitrile over 12 min, then 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 autosampler temperature was maintained at 10°C. The sample injection volume was 20-100 µL. The limit of detection (LOD) using radiometric detection was 0.72 µg/mL and the LOQ was 2.15 µg/mL. The LOD and LOQ for [14C] equivalents using LSC were 2.9 ng and 11.3 ng, respectively, as determined by the equation described by Zhu et al. (2005).

**LC-MS/MS analysis**

The HPLC system was the same as described above for HPLC analysis but coupled with an Agilent 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 operated in the positive electrospray ionization (ESI) mode over a scan range of m/z = 50 to 500. The flow rate of the nitrogen drying gas was 11 L/min, the nebulizer pressure was set to 60 psi, the drying temperature was maintained at 350 °C, the HV capillary voltage was 3,500 V and the capillary current was 42.7 nA. MS and MS/MS experiments were performed in MRM mode. Two masses were selected for analysis: m/z = 262.9 (BMP) and m/z = 438.9 (BMP mono-glucuronide). The first scan was a full MS scan, and then one or two precursor ions were selected, isolated, and selectively fragmented in the ion trap.

**RESULTS**

**Tissue distribution of BMP**

The distribution of [14C] radioactivity to tissues following single and repeated oral administrations is shown in Table 1. Following a single oral administration of 100 mg/kg [14C] BMP to food-deprived rats, total percent of dose retained in tissues at 72 h was
below 1% (0.13-0.28 nmol/g tissue). When the same dose of BMP was administered orally to non-fasted animals, 4.2% of total radioactivity was recovered from tissues at 24 h post-dose. Adipose tissues, liver, kidneys, muscle and skin contained 0.2, 0.7, 0.1, 0.3, and 0.3% of dose, respectively. The majority of the unexcreted [14C] equivalents (11.9% of dose) were found in the contents of the intestine and cecum. Concentration in nmol/g of tissues and contents ranged from 2.2 to 10.4. Following 5 or 10 days of repeated oral administration (100 mg/kg/day, not fasted) total tissue levels of [14C] equivalents at 24 h after the last dose were in the same range. Also, the tissue distribution was similar, with the majority of the dose remaining in the gastrointestinal (GI) tract. Tissue disposition of BMP was not determined at the lower dose of 10 mg/kg.

Blood levels of [14C] equivalents at 72 h following oral or IV administration of BMP (10 mg/kg) were 0.1% of dose (data not shown). After a single oral administration of 100 mg/kg BMP, 0.3 and 0.2% of dose were recovered in the blood by 24 h and 72 h, respectively. Following repeated oral dosing of BMP (100 mg/kg/day) for 5 or 10 days [14C] blood levels were 0.2% of dose at 24 h after the last dose (Table 1).

**Cumulative elimination of BMP**

The results for the cumulative excretion of BMP-derived [14C] equivalents following IV and oral administration are summarized in Figure 2. When [14C] BMP was administered IV (10 mg/kg) the predominant route of its elimination was urinary. The peak excretion in the urine occurred between 6 and 12 h, and the majority of the dose (>80%) was eliminated by 12 h. By 24 h greater than 89% of the administered radioactivity had been recovered in the urine. Only trace amounts were excreted in the feces by 72 h (<4% of dose). Following a single oral bolus dose (10mg/kg) the excretion rate of BMP-derived [14C] equivalents in urine was similar to that following IV
administration. The fecal elimination accounted for less than 10% of the dose at 72 h. Administration of a 10-fold higher dose of BMP (100 mg/kg) resulted in a similar excretion profile, but urinary excretion was slower; within the first 12 h only 48% of the administered [¹⁴C] equivalents were excreted. The urinary excretion rate at higher oral doses of BMP (150-600 mg/kg) paralleled those of 100 mg/kg (data not shown).

After the rate and route of elimination of BMP following a single oral administration was defined, the effects of repeated daily oral dosing of [¹⁴C] BMP on excretion of BMP-derived [¹⁴C] equivalents were determined (Table 1). After 5 or 10 days of repeated daily dosing of 100 mg/kg [¹⁴C] BMP, the elimination profile of BMP was not notably altered from that of a single dose. The average cumulative amounts of [¹⁴C] equivalents found in urine including cage rinses were 71, 75, and 77% of dose, respectively. The cumulative fecal excretion of BMP from animals receiving 5 or 10 consecutive administrations was slightly increased (14% of dose). In addition, it was noted that the radioactivity recovered in feces at 24 h after administration was increased to 7-8% of dose in non-fasted rats compared to 4% in fasted animals (Figure 2).

Identification of [¹⁴C] radioactivity in urine

Urine collected from rats treated orally with [¹⁴C] BMP was analyzed by HPLC with radiometric detection. A representative radiochromatogram [¹⁴C] radioactivity present in a pooled urine sample is shown in Figure 3. The major peak that eluted earlier than BMP from the reversed phase column with a retention time ($R_t$) of 12.4 min was observed in all samples and at all time points (Figure 3B). Figure 3A shows the elution of the [¹⁴C] BMP standard. In a number of the urine samples a small radioactive peak (1-5% of total [¹⁴C] equivalents) that coeluted with authentic BMP at 14.4 min was detected. The HPLC profiles of urine samples obtained from animals after IV
DMD #23937

administration were similar to those following oral gavage, however, BMP was not detected. Bladder urine contained also exclusively the polar metabolite, and therefore the presence of parent BMP in urine from orally treated rats was suggested to be due to cross contamination from feces. In addition, three minor (≤5% of total [14C] equivalents) and more polar products eluted between 3 and 6 min. The chemical identity of these products was not determined. The product at 5.5 min appears to be a degradation product of the [14C] contaminant originally present in the radiolabeled BMP standard, because the appearance of this peak was accompanied by the loss of the contaminant peak in aging [14C] BMP standards. Incubation of urine with β-glucuronidase prior to HPLC-radiometric analysis resulted in the loss of the polar metabolite with a concurrent increase of parent BMP. Thus, the metabolite was tentatively identified as a glucuronide of BMP.

Based on data obtained from β-glucuronidase assays of urine, animals were administered unlabeled BMP by oral gavage. LC-MS analysis of urine from these studies confirmed the conclusion that the metabolite present in urine was that of BMP monoglucuronide (Figure 4). MS/MS analysis of the metabolite revealed a molecular ion of \( m/z = 438.9 \) ([M+H]⁺) consistent with the mass of a glucuronic acid conjugate of BMP (Figure 4A). In addition, the isotope pattern obtained for this ion was indicative of molecules containing two bromine atoms. The daughter ion spectra obtained by MS/MS analysis from the molecular ion of \( m/z = 438.9 \) was dominated by the loss of one water molecule indicated by \( m/z = 420.9 \) (Figure 4B).

**Identification of \([^{14}C]\) radioactivity in liver and bile**

When liver homogenates from rats treated IV or orally with \([^{14}C]\) BMP (10 mg/kg) were extracted into ethyl acetate in the presence of glycine/HCl buffer at 30 min (IV) and
40 min (oral) post-dose and analyzed by HPLC with radiometric detection, the [\(^{14}\)C] radioactivity in these liver extracts consisted of parent BMP and the glucuronide conjugate identified in urine. Following IV administration of [\(^{14}\)C] BMP (15 mg/kg) to BDC rats, over 50% of the dose was excreted in the bile within 6 h (data not shown). HPLC-radiometric analysis showed that greater than 99% of the BMP-derived [\(^{14}\)C] equivalents excreted in bile over time consisted of a single peak, identified by co-chromatography as the glucuronide conjugate of BMP detected in urine (data not shown).

**Pharmacokinetics of BMP and identification of [\(^{14}\)C] radioactivity in blood**

Blood kinetics following a single IV administration of [\(^{14}\)C] BMP (15 mg/kg) indicated that parent BMP rapidly disappeared from the systemic circulation. The concentration-time profile of [\(^{14}\)C] BMP in blood plasma following IV administration was best described by a biexponential equation that is consistent with a two-compartment model with first order elimination (Figure 5, Insert). After a rapid initial distribution of [\(^{14}\)C] BMP, indicated by a short theoretical half-life of distribution (\(t_{1/2\alpha}: 3.4\) min), a significantly slower elimination (\(t_{1/2\beta}: 2\) h) occurred. Blood plasma concentrations of BMP at times later than 30 min were very low (<1 µg/mL).

When [\(^{14}\)C] BMP (10 mg/kg) was administered orally and extracts obtained from small aliquots of whole blood (150 µl per time point) were analyzed by LSC, the quantities of [\(^{14}\)C] equivalents were close to or below the LOQ. Extracted BMP was quantifiable only at the first four time points (Figure 5). The AUC under these four points, compared to that following IV administration, and adjusted for dose, suggests an oral bioavailability into the systemic circulation of less than 10%. The blood concentration-time profile following oral administration is presented for parent BMP at
the quantifiable time points and for total [\(^{14}\text{C}\)] BMP equivalents (Figure 5). After oral administration absorption was rapid with C\(_{\text{max}}\) reached at 40 min. [\(^{14}\text{C}\)] equivalents were detectable in the blood, although at very low levels, throughout termination at 72 h.

In order to elucidate the identity of the [\(^{14}\text{C}\)] equivalents in blood after oral or IV administration of [\(^{14}\text{C}\)] BMP (10 mg/kg), blood plasma (2-3 mL) from rats was extracted with ethyl acetate in the presence of glycine/HCl buffer and analyzed by HPLC with radiometric detection (Figure 6). Following a single IV administration of [\(^{14}\text{C}\)] BMP (10 mg/kg) both parent BMP and BMP glucuronide were detected in blood plasma (Figure 6A). At 5 min post-IV dose the majority (>90%) of the [\(^{14}\text{C}\)] equivalents was present as parent compound, whereas the amounts of BMP glucuronide (26-57%) were notably increased at all later time points. Following oral administration the [\(^{14}\text{C}\)] radioactivity detected in plasma extracts found both BMP-glucuronide and parent BMP. Because the extraction of the [\(^{14}\text{C}\)] radioactivity in plasma into ethyl acetate was incomplete (33-56% extraction efficiency), the non-extractable [\(^{14}\text{C}\)] equivalents remaining in the aqueous phase were analyzed by HPLC with radiometric detection and were identified as BMP glucuronide. By C\(_{\text{max}}\) (40 min, Figure 6B) the majority of the [\(^{14}\text{C}\)] radioactivity in blood plasma was BMP glucuronide.

**DISCUSSION**

These studies represent the first comprehensive description of the ADME and pharmacokinetic profile of the brominated flame retardant BMP. Following single IV administration to rats, parent BMP disappeared rapidly from the blood. It appears that BMP is efficiently extracted by the liver where it undergoes extensive glucuronidation. The resulting BMP glucuronide is excreted primarily in the bile, but as it is ultimately eliminated predominantly in the urine, some percentage of this glucuronide must also be
exported from liver to blood. Thus, glucuronidation appears to be the key physiological process that governs the clearance of BMP and subsequent exposure to BMP in rats.

Similarly, following oral administration of single or multiple (5 or 10) doses of BMP to rats, the major route of elimination was urinary as a glucuronide. In fact, pharmacokinetic analysis following oral administration showed that $C_{\text{max}}$ in blood was reached quickly, but parent BMP was only detectable at the very early time points after administration. The majority of the [14C] equivalents recovered from blood plasma represents BMP metabolite. Thus, these data demonstrate the critical role of glucuronidation (hepatic and possibly intestinal) in determining the systemic bioavailability of BMP.

Our data show that the hepatic BMP glucuronide was efficiently excreted into the bile but only a low percentage of the dose was eliminated in the feces. These findings suggest that the effectiveness of biliary excretion of BMP glucuronide is greatly limited by enterohepatic recirculation of BMP. After the glucuronide is excreted via the bile into the small intestine it appears to undergo hydrolysis by microbial $\beta$-glucuronidases. The released parent molecule is reabsorbed, subjected to glucuronidation, and is ultimately excreted in the urine. This enterohepatic recycling explains the persistent low blood levels of [14C] BMP equivalents and suggests that the long terminal elimination phases observed in the pharmacokinetic studies most likely result from reabsorption of the aglycone from the intestine rather than retention and slow release from tissues. The effective elimination of BMP glucuronide in bile and enterohepatic recycling results in a low systemic bioavailability of BMP in rats.

The major route of elimination was urinary, regardless of BMP dose level, route of administration or number of doses. However, a slight increase in the rate of fecal
excretion was observed after repeated daily dosing (Table 1). At oral doses of 100 mg/kg BMP and higher (150-600 mg/kg, data not shown), the urinary excretion rate, when expressed as percent of dose, was slower than that of a dose of 10 mg/kg. The radioactivity in urine was excreted predominately as a glucuronide. Formation of glucuronide conjugates is generally regarded as a high-capacity pathway for xenobiotic metabolism which is not readily saturable (Hjelle and Klaassen, 1984). However, studies on the glucuronidation of acetaminophen have demonstrated that certain UGT enzymes with high affinity and low capacity involved in acetaminophen glucuronidation demonstrate concentration-dependent saturation (Court et al., 2001). The decreased elimination rate of BMP glucuronide observed at higher doses may also result from a dose dependent saturation of relevant UGTs. Alternatively, the UGT enzyme(s) responsible for the glucuronidation of BMP may be expressed at lower levels. In addition, glucuronides are transported from the hepatocyte into the plasma and the bile, which have been shown to be saturable as well (Sakamoto et al., 2008).

Tissues were collected at 72 h after oral administration of the high dose (100 mg/kg) and at 24 h after the last dose in repeated dose studies. There was no selective retention of $[^{14}\text{C}]$ radioactivity in tissues reported to develop neoplasms (Dunnick et al., 1997; NTP, 1996) compared to non target tissues. Internal tissues such as liver, kidney and adipose depots contained minor levels of $[^{14}\text{C}]$ BMP equivalents. Radioactivity that had not been excreted within the first 24 h was mainly associated with tissues and contents of the intestine and cecum. Unpublished data (not shown) of a previous study of BMP indicated no preferential distribution to tissues within 24 h after IV administration (15 mg/kg) to F-344 rats. The results of these studies demonstrate that BMP does not accumulate in tissues of rats following oral exposure to low and high doses of BMP.
The aliphatic BMP, with two free hydroxyl groups, behaves somewhat similarly to tetrabromobisphenol A (TBBPA), an aromatic BFR that possesses two phenolic hydroxyl groups. TBBPA is readily absorbed from the GI tract, extensively conjugated in the liver, and the glucuronide metabolite is then eliminated in the bile (Kuester et al., 2007; Schauer et al., 2006). The molecular weights of the glucuronides of both BMP (MW 438) and TBBPA (MW 718) exceed 325, the threshold for biliary elimination in rats (Riviere, 1999). This explains the extensive excretion of these conjugates into bile. However, in contrast to the bulkier TBBPA glucuronide, which is predominantly eliminated via the fecal route (Kuester et al., 2007); the ultimate route of elimination of the BMP glucuronide is urinary. It appears that these two glucuronides are dramatically different with respect to serving as substrates for microbial β-glucuronidases. Interestingly, the molecular weight of the BMP glucuronide is below the threshold for biliary elimination in humans (MW >450), and therefore BMP glucuronide, if formed in human liver, would most likely be released into blood for excretion in the urine without undergoing enterohepatic recirculation.

The disposition of two other higher molecular weight BFRs, TBBPA-2,3-dibromopropyl ether and 1,2-bis(2,4,6-tribromophenoxy)ethane, significantly differs from the ADME data reported here for BMP and for TBBPA. These BFRs do not contain free hydroxyl groups, which enhances their lipophilicity and greatly limits their absorption from the GI tract. Both compounds are minimally absorbed, not metabolized, and therefore excreted in the feces mainly as parent compound (Knudsen et al., 2007; Nomeir et al., 1993).

As stated previously, BMP has been reported to cause malignant tumor formation at multiple tissue sites in rats and mice (NTP, 1996). Another known multi-site
carcinogen in rodents is the aliphatic BFR 2,3-dibromo-1-propanol (DBP) (Eustis et al., 1995). It is a direct acting mutagen and also is bioactivated to reactive intermediates that bind to nucleophilic sites of biological macromolecules. In contrast to DBP, there was no indication that BMP formed reactive metabolite(s) in vivo, only an aliphatic glucuronide conjugate was generated. Preliminary results of in vitro studies with isolated rat hepatocytes demonstrate essentially stoichiometric conversion of BMP to BMP glucuronide. Similarly in other in vitro studies, no evidence for cytochrome P450-mediated monooxygenation or/and glutathione conjugation of BMP was obtained (unpublished data). Most likely the glucuronide of BMP, an ether glucuronide, can be considered as an inactive metabolite because it is lacking the electrophilic character of acyl glucuronides (Zia-Amirhosseini et al., 1994). In addition, there was no evidence for bioaccumulation in tissues, even after repeated daily administration of BMP. In conclusion, the ADME data described here do not provide clues as to the mechanism(s) by which BMP-induced carcinogenesis is initiated. Additionally, it has to be noted that the BMP preparation used in the 2-year feed study conducted by the NTP contained 21.2% impurities (NTP 1996), whereas in the studies reported here the minimum purity of BMP was 97.3%. Whether or not these additional components may contribute to the carcinogenic effects assigned to BMP is not known.

In summary, the data presented here indicate that BMP is rapidly absorbed from the GI tract into the portal circulation, efficiently metabolized in the liver to a glucuronide conjugate that is primarily excreted in the urine of rats. Because of the extensive metabolism the likelihood of systemic exposure of BMP following ingestion of BMP is low. The studies clearly describe the fate of BMP in rats but do not establish
mechanism(s) for the carcinogenic potential of BMP. Thus, how BMP acts as a multi-site carcinogen remains to be determined in further studies.
5 ACKNOWLEDGEMENTS

The authors would like to thank Dr. Michael Cunningham (National Toxicology Program & National Center for Toxicogenomics, NIEHS) and Dr. Robert Kuester for their advice and support as well as Leigh Jacobs and Golriz Rad for their assistance in various aspects of this project.
REFERENCES


Nomeir AA, Markham PM, Ghanayem BI and Chadwick M (1993) Disposition of the flame retardant 1,2-bis(2,4,6-tribromophenoxy)ethane in rats following administration in the diet. Drug Metab Dispos. 21:209-214.


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.

FOOTNOTES

This research was supported by The National Toxicology Program/National Institute Environmental Health Science (NIEHS) contract N01-ES-45529 and by the Intramural Research Program of the NIH and NIEHS. The authors acknowledge the support of the Analytical Core of the NIEHS funded Southwest Environmental Health Science Center (P30-ES 06694).

To whom reprint requests should be addressed:

I. Glenn Sipes, Ph. D.
Department of Pharmacology
College of Medicine
The University of Arizona
P.O. Box 245050
Tucson, AZ 85724-5050

Telephone: 520-626-7123
Fax: 520-626-2466
Email: sipes@email.arizona.edu
Legends for Figures

**Figure 1.** Chemical structure and molecular weight of BMP.

**Figure 2.** Cumulative excretion of radioactivity following IV (10 mg/kg, [○/●]) and oral (10 mg/kg, [◇/◆] or 100 mg/kg, [□/■]) administration of [^{14}C] BMP to male F-344 rats (IV: N=4; oral: N=4; mean ± S.D.; urine: open symbols; feces: closed symbols).

**Figure 3.** Representative HPLC-radio chromatograms of (A) [^{14}C] BMP standard and (B) a pooled urine sample collected 6 h after oral administration of [^{14}C] BMP (100 mg/kg) to male F-344 rats.

**Figure 4.** Representative (A) ESI-LC/MS and (B) MS/MS spectra of the BMP glucuronide (HPLC: \(R_T = 12.4\) min).

**Figure 5.** Time course of total BMP [^{14}C] equivalents (●) and extracted parent BMP (○) in blood following oral administration of [^{14}C] BMP (10 mg/kg) to male F-344 rats (N=4, mean ± S.D.). Insert: Time course of parent [^{14}C] BMP (■) in blood following IV administration of [^{14}C] BMP (15 mg/kg) to male F-344 rats (N=4, mean ± S.D.)

**Figure 6.** Representative HPLC-radio chromatograms of blood plasma following (A) IV (30 min post-dose) and (B) oral (40 min post-dose) administration of [^{14}C] BMP (10 mg/kg) to male F-344 rats (Solid line: Extractable [^{14}C] radioactivity; Dashed line: Non-extractable [^{14}C] radioactivity). Minor shifts in retention time of the ‘non-extractable’ moiety were due to matrix effects.
Table 1. Percent of dose recovered from tissues and excreta following oral administration of [\(^{14}\)C] BMP (100 mg/kg) for 1, 5 or 10 daily administrations to male F-344 rats (mean ± S.D.). \(a\): Time after administration; n.d.: not determined.
Figure 1

\[ \text{C}_5\text{H}_{10}\text{Br}_2\text{O}_2 \]

MW 261.94
Figure 2
Figure 3
Figure 4
Figure 5

[\textsuperscript{14}C] BMP Equivalents (\(\mu\text{g/mL}\))

Time (min)

BMP (\(\mu\text{g/mL}\))

Time (min)
Figure 6