METABOLISM OF 3-BUTENE-1,2-DIOL IN B6C3F1 MICE
Evidence for Involvement of Alcohol Dehydrogenase and Cytochrome P450

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ABSTRACT:
3-Butene-1,2-diol (BDD), a metabolite of 1,3-butadiene, is rapidly metabolized by B6C3F1 mice at doses ranging from 10 to 250 mg/kg. Calculation of plasma clearance suggested that the kinetics of BDD metabolism were dose-dependent. Clearance varied 5-fold in this dose range. Urinary excretion of BDD was also dose-dependent but did not exceed 5% of the administered dose. A small fraction of the dose (<1%) was excreted as glucuronic acid or sulfate conjugates. Benzylimidazole, a cytochrome P450 inhibitor, decreased the clearance of BDD (25 mg/kg) by 44%, whereas 4-methylpyrazole, an alcohol dehydrogenase and cytochrome P450 inhibitor, decreased BDD clearance by 82%. BDD administration (250 mg/kg) resulted in depletion of hepatic and renal nonprotein thiols, by 48 and 22%, respectively. Pretreatment of mice with 4-methylpyrazole provided partial protection against depletion of nonprotein thiols, whereas pretreatment with benzylimida-
butenal and HMVK (fig. 1) was provided (Kemper and Elfarra, 1996). P450 is also capable of metabolizing BDD. Preliminary data from our laboratory indicate that rodent and human liver microsomes oxidize BDD at the C2-position, resulting in formation of HMVK (Kemper et al., 1997). This chemical is highly reactive and is a putative intermediate in the formation of 1,2-dihydroxy-4-(N-acetylcysteinyl)butane, one of the major urinary metabolites of BD in humans (Sabourin et al., 1992; Bechtold et al., 1994). In addition, P450s could catalyze epoxidation (Cheng and Ruth, 1993) or diol cleavage reactions (Clejan and Cederbaum, 1992), which could result in the generation of additional reactive and potentially toxic metabolites (fig. 1). Thus, oxidative metabolism of BDD may result in the formation of several reactive intermediates, which could contribute to the carcinogenic activity of BD.

Because formation of BDD may be a major pathway of BD metabolism in humans, the metabolic fate of this compound warrants examination. Because previous studies have demonstrated that BDD is metabolized by at least two enzyme systems in vitro, the purpose of the studies reported here was to investigate the significance of these pathways in vivo and to characterize the metabolism and urinary excretion of BDD at various doses. An additional goal was to determine whether metabolism of BDD in vivo results in the formation of reactive intermediates, as measured by changes in tissue NPT status and inactivation of PNPH, a marker activity for P450. The data indicate that BDD is eliminated almost entirely by metabolism, and they suggest that both ADH and P450s play significant roles in this process. The results also provide evidence for the formation of reactive BDD metabolites in vivo.

Materials and Methods

Chemicals. (R,S)-BDD (>99%) was obtained from Acros Chemicals (Pittsburgh, PA). GSH and oxidized glutathione, 5,5'-dithiobis(2-nitrobenzoic acid), NaBH₄, 1,4-butanediol, BI, and MP were obtained from Aldrich Chemical Co. (Milwaukee, WI). β-Glucuronidase (type B-1 from bovine liver), cytochrome c (type III from horse heart), sulfatase (type V from Patella vulgata), glutathione peroxidase (from bovine erythrocytes), catalase (from bovine liver), superoxide dismutase (from bovine liver), defereroxamine mesylate, and manitol were obtained from Sigma Chemical Co. (St. Louis, MO). N,O-Bis(trimethylsilyl)trifluoroacetamide with 10% trimethylchlorosilane was obtained from Pierce Chemicals (Rockford, IL).

Animals. Male B6C3F1 mice (6-8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hr light/dark cycle and allowed food and water ad libitum. For urinary excretion experiments, mice were housed individually in polycarbonate metabolism cages (Nalgene, Rochester, NY). Mouse liver microsomes were produced by differential centrifugation, as described previously (Elfarra et al., 1991).

Plasma Clearance Study. Groups of four mice each were given BDD, at doses of 10, 25, 50, and 100, and 250 mg/kg of body weight, by ip injection. BDD solutions were prepared in 0.9% NaCl and administered in a volume of 10 ml/kg of body weight. Blood samples were collected from the orbital sinus at selected time points, using heparinized 25-μl volumetric microcapillaries. Blood samples were collected for 4–8 hr (depending on the dose administered) and analyzed by GC/MS.

Urinary Excretion Study. Groups of four mice each were given BDD at the same doses used for the plasma clearance study described above. Mice were then housed individually in metabolism cages, and urine was collected for 24 hr. Urine was stored at 4°C until analysis by GC/MS. BDD was stable in urine for at least 1 week under these conditions.

Effects of Enzyme Inhibitors on BDD Clearance. For BI experiments, groups of four mice were given 5 mg/kg BI or an equivalent volume of saline by ip injection. 30 min before administration of 25 mg/kg BDD. For MP experiments, groups of four mice were given 10 mg/kg MP with 25 mg/kg BDD, or BDD alone, by ip injection. Inhibitor doses were selected based on literature reports (Benz et al., 1995; Cederbaum and Berl, 1982; Chow et al., 1992). Plasma samples were collected as described above and were analyzed by GC/MS.

Sample Preparation. Blood samples were transferred to 0.8-ml microcentrifuge tubes and centrifuged at 16,000 rpm for 2 min to precipitate cells. Plasma (10 μl) and internal standard (10 μl of 10 μg/ml 1,4-butanediol in water) were applied to 0.3-ml capacity Chem Elut solid-phase extraction columns (Varian, Sunnyvale, CA) and allowed to stand for 5 min. Samples were then eluted with 2 × 3 ml of ethyl acetate, and the eluate was evaporated to a volume of ~1 ml under a stream of N₂ at room temperature. Samples were transferred to conical reaction vials, evaporated to a volume of ~25 μl, and derivatized with 25 μl of N,O-bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane for 20 min at 80°C. Samples were allowed to cool to room temperature before analysis by GC/MS. Recovery of BDD from plasma was ~99%, and silylation of both BDD and the internal standard was quantitative for the conditions used. For determination of free urinary BDD levels, 50 μl of mouse urine and 50 μl of internal standard solution were applied to the extraction columns. Samples were extracted and derivatized as described for plasma samples. Recovery of BDD from urine was >99%. Analysis for conjugated BDD was carried out by a modification of the method of Elfarra and Hwang (1990). For glucuronide and sulfate conjugate analysis, 50 μl of urine was incubated with 20 units of sulfatase and 5000 units of β-glucuronidase in sodium acetate buffer (pH 5) at 37°C for 30 min. Reactions were terminated by rapid cooling in an ice bath and transfer to extraction columns. Samples were extracted and derivatized as described above, except that 3-ml capacity extraction columns were used.

GC/MS Analysis. TMS derivatives of BDD and 1,4-butanediol were detected by GC/MS using selected-ion monitoring mode. Analyses were accomplished using an HP 5890 Series II GC interfaced with an HP 5970 mass-selective detector (Hewlett Packard, Palo Alto, CA). Analytes were separated using a 12-m × 0.20-mm (i.d.), DB-1, capillary GC column (J & W Scientific, Folsom, CA). The injector port and detector temperatures were 235°C and 280°C, respectively. The column temperature was maintained at 70°C for 5 min and was then increased to a temperature of 2°C per min. The final temperature was maintained for 5 min. Injections (2–3 μl) were made in splitless mode, at a column head pressure of 6.5 psi. Under these conditions, BDD and 1,4-butanediol eluted at 4.8 and 6.5 min, respectively. Quantitation of BDD was accomplished using ions at m/z 129 [(M–CH₃OTMS)⁺] and m/z 116 as target ions for BDD and the internal standard, respectively. Compound identity was confirmed by the presence of m/z 103 [(CH₃OTMS)⁺] and m/z 101 ions as qualifier ions for BDD and the internal standard, respectively (fig. 2). The high-abundance fragment at m/z 116 in the internal standard spectrum may represent cleavage between C2 and C3 of bis-TMS-1,4-butanediol, followed by a proton transfer reaction. BDD concentrations were determined by comparison of samples with standard curves produced from control plasma and urine spiked with authentic BDD and extracted as described above. The limit of detection for BDD was approximately 1.5 ng.

Effects of BDD on NPT Status. Groups of four mice were given 250 mg/kg BDD in 0.9% saline solution, or saline alone, by ip injection. After 60 min,
animals were sacrificed by cervical dislocation, and selected tissues were excised for reduced and oxidized NPT determination. NPT status was determined spectrophotometrically using Ellman’s reagent (5,5'-dithiobis(2-nitrobenzoic acid)), as described previously (Sausen et al., 1992). In some experiments, animals were pretreated with BI (5 or 50 mg/kg), MP (10 or 100 mg/kg), or saline (10 ml/kg) 15 min before BDD administration.

Inactivation of Microsomal PNPH Activity In Vivo and In Vitro. Mouse liver microsomes (1 mg/ml) were incubated with BDD (20 mM) in the presence or absence of NADPH (1 mM), or with NADPH alone, in 0.1 M potassium phosphate buffer (pH 7.4) for 0–20 min at 37°C, with shaking. After 20 min, a 25-μl aliquot of the reaction mixture was removed and diluted into 0.675 ml of ice-cold buffer containing 15 mM KCN. Cytochrome c (0.5 ml of a 1.55 mg/ml solution, 0.125 mM final concentration) was added, and the mixtures were incubated for 3 min at 37°C in semimicrocuvettes. Reactions were initiated by addition of NADPH (or buffer, for blanks), and changes in absorbance at 550 nm were measured for 5 min. The slope of the linear portion of the absorbance curve (0.33–2 min) was used to calculate the rate of cytochrome c reduction, using an extinction coefficient of 21 mM⁻¹ cm⁻¹.

Data Analysis. All data are presented as mean values ± SD. AUC values were calculated using the trapezoidal approximation function of the PHARMPC software package (Microcomputer Specialists, Philadelphia, PA). Statistical analyses were carried out using the SigmaStat software package (Jandel Scientific, San Raphael, CA). One-way analysis of variance, followed by the Tukey test, was used for comparisons of multiple group means. The criterion for statistical significance was set at α = 0.05.

Results

Plasma Clearance of BDD. At all doses examined, elimination of BDD from the plasma was rapid (fig. 3A). Even at the highest dose used, plasma BDD levels fell below the limit of detection of the assay within 4 hr. Although the plasma concentration vs. time curves for BDD suggested monoexponential kinetics, the slopes of the curves appeared to decrease with increasing dose. However, marked departure from linearity was not evident, as would be expected if the elimination mechanism was approaching saturation. To test for saturation of the elimination mechanism(s), plasma concentrations were normalized to the administered dose and plotted vs. time (fig. 3B). For a first-order process, the data plotted in this manner should be superimposable. The lack of superimposition of these curves suggests that elimination of BDD in mice cannot be described as a single first-order process. Plasma clearance values (dose/AUC) were also calculated (fig. 4). For calculation of the plasma clearance, the assumption was made that BDD was completely absorbed after ip administration. Plasma clearance of BDD decreased significantly with increasing dose between 10 and 100 mg/kg but did not change significantly between 100 and 250 mg/kg. Values for plasma clearance of BDD ranged from 5658 ± 695 ml/hr/kg at the 10 mg/kg dose to 1089 ± 141 ml/hr/kg at the 250 mg/kg dose.

Urinary Excretion of BDD. The 24-hr urinary elimination profile for BDD is illustrated in fig. 5. Excretion of free BDD increased in a dose-dependent manner but did not exceed 5% of the administered dose. When urine was incubated with β-glucuronidase and sulfatase, modest increases in BDD were seen at the highest doses, whereas formation of conjugates at lower doses was negligible. Because BDD was completely cleared from the plasma within the first 6 hr at all doses, further excretion of BDD beyond 24 hr seems unlikely.

Effects of Enzyme Inhibitors on BDD Clearance. Because urinary excretion of free BDD and glucuronide/sulfate conjugates could account for only a small fraction of the total BDD dose, the effects of ADH and P450 inhibitors on plasma clearance of BDD were examined (fig. 6). When the P450 inhibitor BI was administered at a dose of 5 mg/kg, the clearance of 25 mg/kg BDD was decreased by 44%.
Much more marked inhibition was observed with MP, a classic inhibitor of ADH that can also inhibit certain P450s (Chow et al., 1992). BDD clearance in MP-treated mice was decreased by 82%, compared with untreated controls.

Effect of BDD on NPT Status. Treatment of mice with BDD at a dose of 250 mg/kg resulted in significant depletion of reduced NPTs in liver and kidney (fig. 7). Hepatic and renal reduced NPTs were decreased by 67 and 25%, respectively. No significant depletion of lung NPTs was observed (data not shown). BDD treatment had no effect on oxidized NPT status in liver, kidney, or lung (data not shown). In liver, pretreatment of mice with MP (10 mg/kg) provided partial protection against NPT loss (45% decrease, compared with a 67% decrease in saline-treated controls), whereas BI (5 mg/kg) did not provide any significant protection. A similar pattern of inhibitor effects was observed in kidney. In MP-treated mice, kidney NPT levels were not significantly different from those in saline-treated controls. Ten-fold increases in the inhibitor doses had no effect on protection against NPT depletion, suggesting that maximal effects were achieved at the lower doses.

Effect of BDD Metabolism on PNPH Activity. Preincubation of mouse liver microsomes with BDD (20 mM) resulted in time-dependent inactivation of PNPH activity up to at least 20 min (fig. 8A). The PNPH assay was chosen because it has been used as a marker activity for P4502E1 (Koop et al., 1989), which appears to play a significant role in microsomal BDD oxidation (Kemper et al., 1997). The maximal inactivation observed was approximately 55% of control (without NADPH). When microsomes were preincubated with NADPH alone (i.e. no BDD), residual PNPH activity was approximately 1.5-2-fold higher than in BDD-only reactions and remained constant over time, suggesting that NADPH stabilized P450 to some extent (data not shown). When mice were treated in vivo with 250 mg/kg BDD and sacrificed after 60 min, microsomal PNPH activity was decreased by 51%, compared with saline-treated controls (fig. 8B).

To investigate the mechanism of PNPH inactivation by BDD, the effects of coincubation of microsomes with BDD, NADPH, and a series of antioxidants were examined (table 1). Preincubation with BDD and NADPH alone decreased residual PNPH activity by 42%, compared with controls (without BDD). Addition of the iron chelator deferoxamine, catalase, superoxide dismutase, or mannitol, at the concentrations indicated, provided moderate protection against inactivation of PNPH activity, whereas GSH, with or without glutathione...
peroxidase, was without significant effect. Addition of these agents in the absence of BDD had no effect on PNPH activity (table 1). Preincubation of microsomes with BDD and NADPH for 20 min did not inhibit cytochrome c reduction, a surrogate for NADPH-P450 reductase activity. Cytochrome c reductase activity in microsomes preincubated with BDD and NADPH was 185 ± 6.6 nmol/min/mg, compared with reactions in which either BDD (179 ± 16.7 nmol/min/mg) or NADPH (209 ± 6.3 nmol/min/mg) was omitted.

Discussion

BDD was rapidly cleared from plasma at all dose levels after ip administration. The observation that only a small fraction of the administered dose was recovered in the urine as unchanged BDD or as conjugates of BDD indicates that metabolism of BDD is the primary mechanism for plasma clearance of this compound. Clearance of BDD was dose-dependent, decreasing markedly between 10 and 50 mg/kg and then reaching a plateau between 50 and 250 mg/kg. The rapid metabolism of BDD may partly explain why only small amounts of this compound are found in the urine of animals treated with BD or BMO.

Dose-dependent elimination may result from saturation of a number of processes, including renal excretion, distribution, protein binding, and metabolism. Because only a small fraction of the administered dose of BDD is recovered in the urine, saturation of renal excretion seems an unlikely explanation for the dose-dependent clearance. Dose-dependent distributive processes should be accompanied by dose-related changes in the apparent volume of distribution, which were not observed in our experiments (fig. 3B). Although plasma protein binding was not directly evaluated in these experiments, the apparent volume of distribution for BDD (0.87 ± 0.19 liters/kg) suggests that this compound is not extensively bound to plasma proteins. Therefore, dose-dependent metabolic processes appear to be the most likely cause of the dose-dependent clearance observed in our experiments. Although dose-dependent metabolic processes appear to be the most likely cause of the dose-dependent clearance observed in our experiments. Although dose-dependent clearance of a chemical can occur as a result of saturable metabolism, the pattern of dose dependence observed in our experiments is not consistent with a single saturable process. In the latter case, clearance would be expected to be dose-independent at lower doses (plasma concentrations < K_M) and to become dose-dependent at higher doses, because of enzyme saturation (Shargel and Yu, 1993).

An alternative explanation for the dose-dependent clearance observed in the present study is the existence of parallel supply-limited (first-order) and capacity-limited (Michaelis-Menten) metabolic pathways for BDD. In this model, both the first-order and saturable pathways of BDD metabolism would contribute to plasma clearance at lower BDD doses, whereas the fractional contribution of the saturable pathway(s) would become negligible at higher doses. This situation is analogous to that of a chemical that is cleared entirely by renal filtration (supply-limited) and active renal secretion (capacity-limited). Chemicals of this type produce dose-clearance curves similar to those observed in our experiments. A similar metabolic model has been proposed to account for the dose-dependent clearance of the bronchodilator theophylline, which is metabolized by parallel Michaelis-Menten and first-order processes (Lesko, 1984).
The inhibition data support the existence of at least two pathways of BDD metabolism in vitro. BI is a potent nonspecific inhibitor of P450s (Ortiz de Montellano and Correia, 1995). Pretreatment with this compound caused a significant decrease in the clearance of BDD, consistent with a role for P450 enzymes in BDD metabolism. The involvement of P450s is also supported by our preliminary in vitro studies, which demonstrated oxidation of BDD to HMVK by liver microsomes and recombinant human P450 isoforms (Kemper et al., 1997). MP is a potent inhibitor of ADH used in the treatment of acute ethylene glycol and methanol poisoning (Tolf et al., 1997). MP is also capable of inhibiting ADH from a variety of species (Kemper and Elfarra, 1996; Matos et al., 1985). The profound inhibition of BDD clearance produced by coadministration of MP suggests that ADH plays a major role in BDD metabolism in mice. This is consistent with in vitro studies that demonstrated oxidation of BDD by ADH from a variety of species (Kemper and Elfarra, 1996; Matos et al., 1985). MP is also capable of inhibiting some P450s, notably P4502E1. The dose of MP used in our experiments was intended to inhibit ADH without having significant effects on P450s and was 5–10-fold lower than doses typically used in vivo to inhibit P450-mediated metabolism (Chow et al., 1992). However, follow-up experiments in vitro indicated that MP markedly inhibited PNPH in mouse liver microsomes at concentrations below those expected in our in vivo experiments. Therefore, it is probable that the inhibition of BDD clearance observed in our experiments after treatment with MP was the result of inhibition of both P450 and ADH. On the other hand, BI was found to be a much more potent and complete inhibitor of PNPH (data not shown). These data suggest that the majority of the inhibition of BDD clearance seen after treatment with MP was the result of the effect of MP on ADH.

Dose-dependent inactivation of enzymes underlying BDD clearance could also partially explain our clearance data. HMVK, a microsomal metabolite of BDD in vitro (Kemper et al., 1997), is a highly reactive compound and was shown to react covalently with P450 enzymes, causing inactivation and decreasing BDD clearance. Furthermore, oxidation of BDD by ADH results in formation of 2-hydroxy-3-butenal and HMVK (Kemper and Elfarra, 1996). The results of our in vitro experiments indicated a time-dependent loss of PNPH (fig. 8A), whereas no effect on ADH activity was observed (data not shown). The similar loss of PNPH in vivo suggests that dose-dependent clearance of BDD may be the result, in part, of P450 inactivation.

Numerous studies have demonstrated that ROS participate in microsomal oxidation of alcohols and glycols (Winston and Cederbaum, 1983; Clejan and Cederbaum, 1992). Therefore, experiments were carried out to investigate the possible role of ROS in BDD-mediated inactivation of PNPH (table 1). The finding that various antioxidants provided partial protection against loss of PNPH activity in vitro suggests that ROS may be partly responsible for the observed effect of BDD on P450 activity. However, antioxidants provided only 20–25% protection against loss of P450 activity, suggesting that a large part of the observed inactivation was not mediated by ROS. Furthermore, the lack of protection by GSH suggests that BDD also inactivates PNPH by a suicide-type mechanism, in which the reactive metabolite is not released from the active site. A possible candidate for the metabolite responsible for suicide inhibition of PNPH activity is HMVK, a highly reactive microsomal metabolite of BDD in vitro (Kemper et al., 1997).

In vitro studies of BDD metabolism have demonstrated oxidation of BDD to electrophilic intermediates capable of reacting with GSH and other thiol compounds (Kemper and Elfarra, 1996; Kemper et al., 1997). It was therefore of interest to determine whether conversion of BDD to thiol-reactive compounds could also occur in vivo. At a BDD dose of 250 mg/kg, significant decreases in hepatic and renal NPT levels were observed. These results are consistent with metabolism of BDD to reactive intermediates. The decrease in renal NPTs may indicate that BDD is metabolized to a significant extent in the kidney or that the reactive metabolites formed in the liver possess sufficient...
stability to exert effects on distant target tissues. To determine whether ADH and/or P450 plays a role in depletion of NPTs, the effects of pretreatment with BI and MP on NPT status were examined. The finding that BI provided no significant protection against the loss of NPTs suggests that P450 oxidation products are not responsible. This is consistent with the relatively small effect of BI on BDD clearance. The finding that MP provided partial protection against depletion of NPTs suggests that oxidation of BDD by ADH results in the formation of thiol-reactive metabolites, which is consistent with previous in vitro results (Kemper and Elfarra, 1996). However, the finding that MP pretreatment prevented only approximately 50% of the NPT depletion seen with BDD alone, even when the MP dose was increased 10-fold, suggests the involvement of additional pathways in the mechanism of NPT depletion by BDD. Direct reaction between BDD and GSH was ruled out, because incubation of BDD (2 mM) with 1 mM GSH for 2 hr did not result in any loss of GSH (data not shown). At the high dose of BDD used in this experiment, numerous enzyme systems could potentially oxidize BDD to thiol-reactive species. One possible participant is an MP-insensitive dehydrogenase, which catalyzes reversible ethanol oxidation in extrahepatic tissues of ADH-negative deer mice (Ekström et al., 1993) and humans (Moreno and Parés, 1991).

In summary, the data presented indicate that BDD, a metabolite of BI, is rapidly eliminated in mice almost exclusively (95%) by metabolism. At least two factors may contribute to the dose-dependent metabolism observed, i.e. the activity of multiple pathways and the inactivation of P450. Clearance of BDD is mediated by both P450 and ADH and results in the generation of thiol-reactive intermediates. Partial protection by MP against NPT depletion at high BDD doses suggests the involvement of additional pathways in this effect. Although the effects of BDD on PNPH activity and NPT status at BDD doses suggests the involvement of additional pathways in this effect. Although the effects of BDD on PNPH activity and NPT status might be mediated. Partial protection by MP against NPT depletion at high BDD doses suggests the involvement of additional pathways in this effect. Although the effects of BDD on PNPH activity and NPT status might be mediated.

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