ABSTRACT:
The disposition of carbamazepine (CBZ) was investigated in the SWV mouse. A $^{14}$C-CBZ dose was administered to CBZ pretreated mice, and the distribution of radiolabeled material was determined. Twenty-four hours after the $^{14}$C-CBZ dose, 92.5% of the dose was accounted for in urine (56%), in the visera and carcass (22%), in feces (11%), and expired as $^{14}$CO$_2$ (2%). CBZ metabolites present in hydrolyzed urine were also identified using a combination of spectroscopic techniques. CBZ, CBZ-10,11-epoxide (CBZE), 2- and 3-hydroxy-CBZ, methylsulfonyl-CBZ, and glucuronides of CBZ and CBZE accounted for 64% of total urinary radioactivity (0–24 hr) in CBZ pretreated mice. Minor metabolites of CBZ included novel cysteine and $N$-acetylcysteine conjugates of CBZ, as well as a methylsulfonyl conjugate of CBZE not previously reported. The urinary excretion of these thioether conjugates was increased in CBZ/phenobarbital pretreated mice and decreased in CBZ/stiripentol pretreated mice in comparison with CBZ-only treated mice. Preliminary studies of the effects of phenobarbital and stiripentol on the urinary abundance of these metabolites are consistent with the modulation of teratogenicity in the SWV mouse by the same pretreatments. These data suggest the formation of thioether metabolites of CBZ may be related to CBZ teratogenicity in the SWV mouse.

To reduce the risk of harmful seizures to both the mother and child, pharmacological intervention in the treatment of epilepsy is often continued throughout pregnancy. However, as a therapeutic class, antiepileptic drugs are associated with an enhanced risk of birth defects. Although CBZ$^1$ monotherapy is commonly prescribed to the pregnant epileptic mother (1, 2), CBZ-related teratogenic effects have been observed clinically (3–5). Moreover, the sometimes unavoidable pharmacokinetic interactions that occur between CBZ and other co-administered anticonvulsants with CBZ in some individuals has been associated with an increased incidence of birth defects among neonates exposed in utero (3, 6, 7). Lindhout and coworkers (3) reported that prenatal exposure to the combination of CBZ, PB, and valproate was associated with a 58% incidence of congenital anomalies (3). This high rate of malformation could not be explained by the additive toxicities of these three anticonvulsants or simply by the number of anticonvulsants coadministered, because other combinations of 3–4 drugs had a 7% incidence of malformation. On the basis of these findings, and an understanding of the complexities of pharmacokinetic interactions that occur between CBZ and other co-administered anticonvulsants, several investigators have speculated that the teratogenic effects associated with CBZ exposure are due to a metabolite of CBZ rather than the parent drug itself (3, 4, 8, 9).

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1 Abbreviations used are: CBZ, carbamazepine; PB, phenobarbital; CBZE, carbamazepine-10,11-epoxide; STP, stiripentol; LSC, liquid scintillation counting; ip, intraperitoneal; BaOH, barium hydroxide; LC/MS/MS, liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; CID, collision-induced dissociation; NAC, $N$-acetylcysteine; CYS, cysteine; EI, electron ionization; GSH, glutathione.
changes in the fraction of CBZ dose metabolized to individual urinary metabolites. Given the complexity of CBZ metabolism, metabolites whose formation was modulated in a manner consistent with the results of teratogenicity studies were given high priority for identification, although it was recognized that changes in the qualitative profile of specific CBZ urinary metabolites excreted in urine would not necessarily reflect the true magnitude of cotreatment effects. Thus, this investigation was an initial attempt to identify possible metabolites that could be involved in a mechanism of CBZ teratogenicity.

Materials and Methods

Chemicals. 14C-CBZ, labeled at the carbonyl carbon of the side chain (10 mCi/mmol), was purchased from Sigma Chemical Co. (St. Louis, MO). The chemical and radiochemical purities were >99% by HPLC. 14C-CBZ was diluted with unlabeled CBZ (Abbott Laboratories, Chicago, IL) to a final specific activity of 2.25 mCi/mmol. 2-Hydroxy CBZ and 3-hydroxy CBZ were the gifts of Dr. René Levy (University of Washington, Seattle, WA). CBZ-10,11-trans-dihydrodiol was prepared as described previously (16) from CBZ supplied by Biocodex Research Laboratories (Montrouge, France). CBZ-glucuronide (EC 3.2.1.31) crude solution were obtained from Helix pomatia; hydrogen peroxide was obtained from Sigma; ethanolamine and 2-ethoxethanol were obtained from Aldrich Chemical Co. (Milwaukee, WI); and trifluoroacetic acid was obtained from Pierce Chemical Co. (Rockford, IL). l-Ascorbic acid and Optima grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA); ammonium acetate, barium hydroxide, and hydrochloric acid were purchased from J. T. Baker, Inc. (Phillipsburg, NJ); Ready-Safe liquid scintillation cocktail was purchased from Beckman Instruments, Inc. (Fullerton, CA); and Flo-Scint II LSC cocktail was purchased from Packard (Downers Grove, IL).

Animals. The highly inbred SWV/Fnu mouse strain was used in this study. Breeding stock was obtained from the Comprehensive Teratology Institute (University of Washington, Seattle, WA) and was continually back bred in the Laboratory of Chemically Induced Teratogenesis, University of Virginia. Mice were randomly assigned to three treatment groups: CBZ, 1,500 mg/kg/day; CBZ, 1,500/STP 300 mg/kg/day; and CBZ, 1,000/PB, 45 mg/kg/day. The dose of STP (300 mg/kg/day) was previously determined to be protective against a teratogenic dose of CBZ (1,500 mg/kg/day), whereas the coadministration of PB (45 mg/kg/day) had been found to protect teratogenicity with a nonteratogenic dose of CBZ (1,000 mg/kg/day) in the SWV mouse (13). During the 7-day pretreatment period, mice were housed in polyethylene cages with sawdust bedding. Food and water were provided ad libitum. Urinary and fecal samples were individually filtered (ultraspin centrifuge filters, 10,000 molecular weight cut-off; Alltech Associates, Inc., Deerfield, IL) before HPLC analysis.

CBZ Urinary Metabolites. Mice were randomly assigned to three treatment groups: CBZ, 1,500 mg/kg/day; CBZ, 1,500/STP 300 mg/kg/day; and CBZ, 1,000/PB, 45 mg/kg/day. The dose of STP (300 mg/kg/day) was previously determined to be protective against a teratogenic dose of CBZ (1,500 mg/kg/day), whereas the coadministration of PB (45 mg/kg/day) had been found to protect teratogenicity with a nonteratogenic dose of CBZ (1,000 mg/kg/day) in the SWV mouse (13). During the 7-day pretreatment period, mice were housed in polyethylene cages with sawdust bedding. Food and water were provided ad libitum. Urinary and fecal samples were individually filtered (ultraspin centrifuge filters, 10,000 molecular weight cut-off; Alltech Associates, Inc., Deerfield, IL) before HPLC analysis.

Mass Balance of Radiolabel. Mice (N = 5) received CBZ (20 mg/kg/day) in cyclodextrin intraperitoneally over a 7-day pretreatment period. Food and water were provided ad libitum. On day 7, 14C-CBZ (8 mg/kg, 5 μCi) was administered by ip injection, as described previously. The actual injected dose was determined gravimetrically. After administration of 14C-CBZ, mice were placed into a single sealed glass metabolic cage (Lab Glass, Inc., Vineland, NJ) for collection of excreta and 14CO2 (18, 19). Water was provided ad libitum. Positive air flow through the glass cage was maintained by connection to an aspirator. Air was drawn through a lime trap in series before the cage, and expired air was bubbled through three liquid reservoirs connected in series between the cage and the aspirator. The reservoirs were arranged such that expired air was first bubbled through aqueous HCl (4% w/v) before going to the first CO2 trap, which contained an ethanolamine-ethoxyethanol mixture (1:2). The second CO2 trap contained a saturated BaOH solution (20% w/v). The ethanolamine-ethoxyethanol mixture was replaced at 8 and 16 hr and the BaOH solution at 12 hr. The animals were euthanized under an atmosphere of ether 24 hr after administration of 14C-CBZ. Tissues and excreta were collected at 24 hr for the determination of 14C disposition. Cage parts were rinsed with methanol until counts declined to background levels. The methanol rinse was collected, reduced (<4 ml) by rotary evaporation, and added to the pooled urine sample from which a small aliquot was removed to determine the concentration of radiolabel by LSC. Feces were added to a solution of sodium methoxide (2 M) and repeatedly vortexed to achieve a slurried mixture. Before LSC, an aliquot of the slurry was decontaminated with hydrogen peroxide (30%) and adjusted to a neutral pH with concentrated HCl. The viscera were removed from the carcasses and homogenized mechanically in sodium methoxide (2 M). Carcasses were cut into small pieces after removing the skin and homogenized mechanically in sodium methoxide (2 M). A small aliquot of each homogenate mixture was sampled for the quantitation of radiolabel by LSC. Exhaled 14CO2 contained in the BaOH solutions (0.5 ml diluted with 0.5 ml H2O) and ethanolamine-ethoxyethanol solutions (1 ml) were also determined by LSC.

Chromatographic and Spectroscopic Analysis of CBZ Metabolites. Three HPLC systems are described. The isolation of minor CBZ metabolites for which no synthetic standards exist was necessary before mass spectral analysis. In general, urinary CBZ metabolites could not be resolved using mobile phase conditions optimized for atmospheric pressure ionization.

System 1. Neat and hydrolyzed urine was analyzed using a Hewlett-Packard Series 1050 HPLC with a variable-wavelength UV detector set at 230 nm. For some analyses, an A-100 β-radioactive flow detector (Radiomatic, Tampa, FL) was used. System 1 consisted of solvent A (10 mM ammonium acetate, pH 7) and solvent B (50% methanol, 50% water). Absorbance spectra of specific chromatographic peaks were obtained using a Hewlett-Packard Series II 1090 HPLC with a diode array detector scanning from 200 to 350 nm. These HPLC systems were equipped with a 5 μm Econosil C8 column (25 cm × 4.6 mm; Alltech Associates) protected by a C8 guard column (10 mm × 4.6 mm; Alltech Associates). The mobile phase consisted of solvent A (10 mM ammonium acetate, pH 7) and solvent B (50% methanol and 50% acetonitrile) delivered at a constant flow rate of 1 ml min⁻¹. The gradient mixture was initially held at 10% B (0–10 min), increased linearly to 35% B (10–35 min), maintained at 35% B (35–45 min), and increased linearly to 55% B (45–65 min). Retention times of the synthetic standards were: CBZ-10,11-dihydrodiol, 32 min; 2-hydroxy-CBZ, 41 min; CBZ, 42 min; 3-hydroxy-CBZ, 43 min; and CBZ, 52 min.

System 2. CBZ urinary metabolites were partially purified using a Beckman 112 Solvent Delivery Module and a 5 μm Ultrasphere C8 column (25 cm × 10 mm; Phenomenex, Torrance, CA) protected by a C8 guard column. UV absorbance was monitored using a Hewlett-Packard Series 1050 detector set at 230 nm. Mobile phase mixtures of acetonitrile (10–35%) in 10 mM ammonium acetate were isocratically delivered at a constant flow rate of 3 ml min⁻¹. The proportion of acetonitrile in the mobile phase was adjusted for the relative polarity of the metabolite being isolated. Fractions were collected by hand or using a fraction collector (Fra-100, Pharmacia, Sweden). In some cases, individual fractions were further purified using system 1. Fraction eluents collected using system 1 or system 2 were frozen, lyophilized, and stored at −20°C. Lyophilized samples were reconstituted in ammonium acetate buffer (0.01 M, pH 7) to achieve a 2- to 5-fold concentrated mixture (−100 μl) just before mass spectral analysis.

System 3. On-line LC/MS and LC/MS/MS of partially purified metabolites were conducted with a Shimadzu LC-10AD twin-pump system equipped with a 5 μm Solvent Miser C8 column (25 cm × 2.1 mm; Alltech Associates) and a Shimadzu SPD-10AV variable wavelength detector (220 nm) positioned in series just before the mass spectrometer. Isotopic mixtures of solvent A (0.05% trifluoroacetic acid, v/v) and solvent B (50% methanol:50% acetonitrile) ranging from 35 to 55% B were delivered at a constant rate of 0.2 ml min⁻¹. MS/MS Positive-ion mass spectrometric analyses were performed on a VG
CBZ Mass Balance. The distribution of radiolabeled material (0–24 hr collection) in CBZ/pretreated mice after intraperitoneal administration of $^{14}$C-CBZ (8 mg/kg, 5 μCi) is shown in fig. 1. Nearly 68% of the radioactive dose was excreted in urine and feces, consistent with previous findings in humans and rats (11, 12). A preliminary study determined that ~50% of a single CBZ dose in mice was excreted in urine from 0 to 12 hr, 7% from 12 to 24 hr, and 1% from 24 to 48 hr. The amount of radiolabel remaining in the viscera and carcass at 24 hr accounted for <22% of the dose. Approximately 2% of the dose was exhaled in the form of $^{14}$CO₂. Less than 8% of the dose was unaccounted for. These findings indicate that a significant proportion of a CBZ dose is excreted in urine within 24 hr. Moreover, CBZ metabolism involving loss of the $^{14}$C-containing carbamoyl side chain does not constitute a significant pathway of elimination in the SWV mouse model. On the basis of these findings, we focused on the identification of CBZ metabolites in the urine of pretreated SWV female mice.

Results

Effect of Pretreatments on CBZ Urinary Metabolites. To contrast the effects of PB and STP on CBZ metabolism, pooled urine from treated animals was analyzed by HPLC and MS. Female mice pretreated (7 days) with CBZ in the absence and presence of PB or STP received a single $^{14}$C-CBZ dose (8 mg/kg, 5 μCi) by ip injection. CBZ metabolites were separated by HPLC using chromatographic system I and analyzed by UV and radiometric detection. Urine from each exposure group was incubated alone or with β-glucuronidase added. A comparison of urine samples before and after β-glucuronidase treatment revealed that 25–33% of $^{14}$C-CBZ derived material was hydrolyzed (chromatograms not shown).

Radiochromatograms of hydrolyzed urine samples collected from CBZ/PB, CBZ, and CBZ/STP treated mice are shown in fig. 2 (a–c, respectively). To minimize variability, these chromatograms were obtained by consecutive HPLC analyses on a single day. When $^{14}$C-CBZ was dosed to pretreated mice, only 3–6% of the dose was excreted in urine as unchanged drug. In each sample there were 10 peaks of $^{14}$C-CBZ–derived material, labeled alphabetically in fig. 2b. Peaks A–J of each chromatogram represent ~70% of total urinary radioactivity of the single $^{14}$C-CBZ dose. Recovery of label in this study was less than in the mass balance study, because cage washing was limited to avoid unnecessary dilution of urine.

Peaks A–J were quantified as a percentage of radioactive CBZ dose and as a percentage of total excreted radioactivity (table 1). In our previous teratogenicity study, the incidence of malformation was increased by PB and decreased by STP coadministrations relative to CBZ-only exposure (13). Urinary comparisons between treatment groups in the present study revealed that the relative abundances of peaks A, D, and E were modulated by STP (decreased) and PB (increased) coadministrations in a manner consistent with a contribution to teratogenicity. Conversely, the abundance of peak B was decreased by coadministration of PB and increased by STP. The remaining peak abundances (C, F, G, H, I, and J) were not modulated in a pattern related to teratogenicity (table 1).

Peak A represented 0.9% of the total radioactive dose in urine collected from CBZ pretreated mice. The relative abundance of peak A was diminished to 0.7% by STP, while accounting for 1.2% of the dose in PB cotreated animals. Similarly, as a function of CBZ dose, coadministration of PB was associated with increases in peak D (1.7%) and peak E (1.4%), compared with the CBZ-only treated sample, 0.4% and 0.9% respectively. However, STP cotreatment was not associated with changes in peaks D and E (% dose) in comparison with the CBZ treatment group. When the areas of peaks A–J were coadministered in a manner consistent with a contribution to teratogenicity. Conversely, the abundance of peak B was decreased by coadministration of PB and increased by STP. The remaining peak abundances (C, F, G, H, I, and J) were not modulated in a pattern related to teratogenicity (table 1).

Identification of CBZ Metabolites (Peaks A–J). Synthetic standards cojected individually with hydrolyzed urine confirmed the identity of peaks F, G, H, and J as 2-hydroxy-CBZ, CBZE, 3-hydroxy-CBZ, and CBZ, respectively. The 10,11-dihydrodiol-CBZ standard did not coelupe with any of the 10 radioactive peaks, consistent with the low microsomai epoxide hydrase activity in the SWV mouse (20, 21). Characterization of the remaining CBZ urinary metabolites (described in descending order of urinary abundance) was achieved by spectroscopic analyses, because synthetic standards were not available.
characteristic of oxidized metabolites of CBZ. The precursor ion spectrum of \( m/z \) 253 revealed a predominant peak at \( m/z \) 429, consistent with the molecular ion designation of metabolite B (spectrum not shown). LC/MS/MS analysis also yielded a product ion spectrum of \( m/z \) 429 (described in table 3). The spectrum (which was obtained by CID of the \( M^+ \)) is characterized by two fragmentation pathways in which charge retention occurred on either the aglycone or glucuronyl moiety, with respective neutral losses. At low collision energy, facile cleavage of the glucuronyl moiety gave rise to the protonated aglycone at \( m/z \) 253 (base peak). Loss of neutral dehydroglucuronic acid \([M+H-176]^+\) has previously been described as a characteristic ESI fragmentation pathway of glucuronide conjugates (22). Fragmentation of the carbamoyl side chain, characteristic of CBZ and its metabolites, also occurred with ions appearing at \( m/z \) 236 \([M+H-NH_2]^+\) and at \( m/z \) 210 \([M+H-CONH]^+\). Several minor product ions that arose by fragmentation of the conjugated glucuronic acid moiety were also observed, including consecutive losses of the elements of water at \( m/z \) 411 \([M+H-H_2O]^+\) and \( m/z \) 393 \([m/z \ 411-H_2O]^+\), \( m/z \) 319 \([m/z \ 393-C_3H_2O_3]^+\), and \( m/z \) 295 \([M+H-C_4H_6O_5]^+\). In addition, CID fragmentation of \( M^+ \) was associated with loss of the aglycone \([M+H-252]^+\) to afford product ions corresponding to dehydroglucuronic acid at \( m/z \) 177, dehydroxyglucuronic acid substituted with the carbamoyl side chain at \( m/z \) 220, and a related fragment ion at \( m/z \) 159 \([m/z \ 177-H_2O]^+\).

Although the mass spectra were consistent with a glucuronide conjugate, characterization of metabolite B solely by MS was insufficient to discern regiosomeric differences between several possible CBZ phenolic ether glucuronides and a N-linked glucuronide of an oxidized CBZ metabolite. However, assignment of glucuronic acid to the terminal nitrogen atom of the carbamoyl side chain was supported by the resilience of peak B to \( \beta \)-glucuronidase treatment (11). In addition, the absorbance spectrum of peak B, obtained using a diode array detector with chromatographic system 1, displayed a single absorbance maximum near 220 nm, consistent with an aliphatic 10,11-epoxide of CBZ (23, 24). On the basis of these findings, the identity of peak B is most consistent with CBZE-N-glucuronide. This metabolite has also been identified in the urine of rats treated with CBZ (25).

LC/MS analysis of metabolite C resulted in a full mass spectrum (table 2) with protonated and sodium adduct molecular ion species appearing at \( m/z \) 413 and \( m/z \) 435, respectively. An abundant ion fragment was also displayed at \( m/z \) 237, corresponding to protonated CBZ. The structural identity of metabolite C was ascertained by LC/MS/MS under CID conditions identical to those used in the analysis of metabolite B (table 3). As observed for CBZE-N-glucuronide, product ions of \( m/z \) 413 (metabolite C) indicated the existence of two fragmentation pathways that involve charge retention on the aglycone or glucuronyl fragments. Neutral loss of 176 Da (loss of dehydroglucuronic acid) gave rise to the protonated aglycone at \( m/z \) 237. Protonated and deprotonated forms of iminostilbene were also observed in the spectrum at \( m/z \) 194 and \( m/z \) 192, respectively. Product ions of lower abundance that correspond to fragmentation of glucuronic acid, in a pattern identical to that observed for metabolite B, appeared at \( m/z \) 395 \([M+H-H_2O]^+\), \( m/z \) 377 \([m/z \ 395-H_2O]^+\), \( m/z \) 303 \([m/z \ 377-C_3H_2O_3]^+\), and \( m/z \) 279 \([M+H-C_4H_6O_5]^+\). Side chain cleavage also occurred on both sides of the carbonyl functional group to yield two distinct ion fragments appearing at \( m/z \) 220 (base peak). As observed with other metabolites of CBZ, cleavage occurred distal to the carbonyl group to afford a CBZ oxocarbonium ion at \( m/z \) 220. Alternatively, cleavage between the carbonyl carbon and azepine nitrogen occurred with charge retention on the carbamoyl-substituted

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**Fig. 2. Effect of drug pretreatment on CBZ urinary chromatographic profiles.**

HPLC radiometric analyses of urine, hydrolyzed with \( \beta \)-glucuronidase (37°C, pH 4.6, 18–20 hr), collected (0–24 hr) from CBZ 1,000/PB 45 mg/kg/day (a), CBZ 1,500 mg/kg/day (b), and CBZ 1,500/STP 300 mg/kg/day (c)-treated SWV female mice. HPLC separations of CBZ metabolites using chromatographic system 1.

**Peak B and Peak C.** Metabolites B and C (20 min and 25 min, respectively; fig. 2b) collectively accounted for 19–37% of the total urinary radioactivity of each treatment group after \( \beta \)-glucuronidase hydrolysis. Each of these metabolites was successfully isolated from hydrolyzed urine by semipreparative HPLC using chromatographic system 2.

Analysis of metabolite B by full-scan LC/MS using chromatographic system 3 identified a protonated molecular ion at \( m/z \) 429 (table 2). The mass spectrum also displayed a sodium adduct at \( m/z \) 451 and an abundant ion fragment at \( m/z \) 253, an ion fragment
glucuronoyl moiety to yield a second product ion at m/z 220, as observed for metabolite B. In addition to an ESI fragmentation pattern similar to that observed for CBZE-N-glucuronide (metabolite B), other evidence consistent with an N-glucuronide of CBZ included: 1) absorbance maxima at ~220 and 280 nm, 2) resistance to hydrolysis by β-glucuronidase, and 3) HPLC retention time consistent with a less polar species than metabolite B.

Peak A. Isolation of metabolite A (12 min; fig. 2b) from hydrolyzed samples of pooled urine was accomplished by semipreparative HPLC using chromatographic system 2. Full-scan LC/MS of the collected peak A revealed a protonated molecular ion at m/z 416 (as shown in table 2). Abundant ion fragments at m/z 253 (base peak) and m/z 237 were also evident. These ions are commonly observed in the mass spectra of CBZ metabolites and respectively correspond to an oxidized metabolite of CBZ. Additional structural information regarding peak A was obtained using a diode array detector with chromatographic system 1. The spectrum contained absorbance maxima at ~220 and 280 nm. The absorbance maximum near 280 nm is diagnostic of a double bond joining carbons 10 and 11 of the CBZ moiety, and thus suggested that the hydroxyl and NAC substituents are positioned on a nonaromatic 6-membered ring of CBZ.

A product ion spectrum of m/z 416 is shown in fig. 3 with the proposed metabolite structure and origin of product ions resulting from cleavage around the cysteinyl thiol and at the carbamoyl side chain. The low-energy CID spectrum is characterized by two related fragmentation pathways consistent with a CBZ mercapturate conjugate, and together account for a majority of the product ions observed. The first series of product ions arose from loss of the elements of the NAC moiety to afford a product ion at m/z 220 and 280 nm. The absorbance maximum near 280 nm is diagnostic of a double bond joining carbons 10 and 11 of the CBZ moiety, and thus suggested that the hydroxyl and NAC substituents are positioned on a nonaromatic 6-membered ring of CBZ.
protonated hydroxyiminostilbene ion at m/z 210. On the basis of these findings, metabolite A is most consistent with a NAC conjugate of dihydrohydroxy-CBZ.

**Peak D and Peak E.** The urinary excretion of metabolites D and E (34 min and 36 min, respectively; fig. 2b) each accounted for ~1% of the administered CBZ dose (table 1). Relatively low urinary abundance and incomplete chromatographic separation of these metabolites limited the ability to make quantitative comparisons between treatment groups. However, using chromatographic system 3, it was possible to achieve complete resolution of metabolites D and E (8 min and 13 min) under isocratic conditions (35% B), while monitoring at 220 nm (chromatograms not shown). To measure the effects of PB and STP on the excretion of these minor CBZ metabolites using system 3, metabolites D and E were partially purified from hydrolyzed urine samples of each treatment group. Chromatographic system 1 was used to collect a single fraction containing metabolites D and E (33–36 min) from each urine sample. Aliquots collected from two injections per sample (18,000 dpm; 3–25 μl) were lyophilized and resolubilized in ammonium acetate buffer (100 μl). Each reconstituted sample (18 μl) was analyzed using chromatographic system 3 and the peak areas (220 nm) of metabolites D and E were compared. The results of these comparisons indicated that PB coadministration was associated with a 5% greater excretion of metabolite D and a 31% greater excretion of metabolite E, relative to the respective amounts excreted by female mice treated with CBZ alone. Conversely, STP coadministration was associated with the diminished excretion of metabolites D and E (9% and 22%, respectively) relative to the CBZ-only treatment group. These results are consistent with the relative changes in metabolites D and E measured as a function of total urinary radioactivity (table 1).

Analysis of metabolite D by full-scan LC/MS using chromatographic system 3 revealed a protonated molecular ion at m/z 356 accompanied by a sodium adduct at m/z 378 and less abundant ions at m/z 310 and m/z 267 (table 2). The even-mass MH+ of metabolite D (m/z 356) is consistent with a CYS conjugate of CBZ (25). A product ion scan obtained by CID of MH+ at m/z 356 is shown in fig. 4 with a proposed metabolite structure and origins of abundant product ions. Fragmentation occurring at the carbamoyl side chain gave rise to an oxocarbonium ion at m/z 339 [M+H-NH3]+ and protonated cysteiny1-S-iminostilbene ion at m/z 311 [M+H-CONH]+. The spectrum also displayed a series of product ions that corresponded to cleavage of the cysteiny1 moiety, including loss of the terminal carboxylate to give rise to m/z 310 [M+H-COOH]+, cleavage at the cysteiny1 thiol to afford a protonated RS+ at m/z 267 and at m/z 224 (base peak), where R corresponds to CBZ [M+H-C6H5NO2]+ or iminostilbene [m/z 267-CONH]+, respectively. On the basis of these findings, the identity of metabolite D is most consistent with S-cysteiny1-CBZ.

The full mass spectrum of metabolite E, obtained with chromatographic system 3, exhibited a protonated molecular ion at m/z 331 and sodium adduct at m/z 353 (table 2). Also present in the spectrum was an abundant peak at m/z 288, which represents a loss of 43 Da with subsequent protonation and charge retention at the nitrogen of the substituted iminostilbene fragment, [m/z 331-CONH]+. Based on the indicated mass of 330 Da, an elemental composition of C16H14N2O4S was proposed for metabolite E. This lead us to consider that metabolite E was a methylsulfonylhydroxy-CBZ metabolite, previously postulated by Lertratanangkoon and Horning (12). However, the UV absorbance spectrum of metabolite E revealed a single maximum near 220 nm, as determined by diode array detection, indicating the absence of a double bond joining carbons 10 and 11 of the CBZ ring structure. Therefore, a regioisomeric methylsulfonyl-CBZE metabolite is a more likely structural assignment for metabolite E.

A product ion spectrum of metabolite E with the proposed metabolite structure and origin of the base peak ion at m/z 288 are shown in fig. 5. The spectrum [obtained by low-energy CID (20 eV) of m/z 331] exhibited fragment ions characteristic of CBZ metabolites, including an oxocarbonium ion at m/z 314 afforded by loss of the elements of ammonia [M+H-NH3]+ and m/z 288 from elimination of the carbamoyl side chain [M+H-CONH]+. Also present in the ESI spectrum were two groups of related product ions that arose by side chain cleavage and expulsion of CO from the central epoxy-azepine ring of metabolite E. The abundant pair of fragment ions at m/z 180 and m/z 179 correspond to protonated and deprotonated acridine ions ([C13H9N+H]+ and [C14H8N]+), respectively. Acridine ions have also been observed in the product ion spectra of CBZE and the 10-keto-CBZ congener, oxcarbazepine. Moreover, acridine fragments arising from contraction of an azepine ring were also seen with dibenzoazepines by EI fragmentation (27), with LSIMS analysis of CBZ-10-O-glucuronide (28) and EI fragmentation of CBZE (29). Accordingly, the second pair of abundant peaks (fig. 5) correspond to protonated and deprotonated forms of a methylsulfonyl-substituted acridan (9,10-dihydroacridine) ion occurring at m/z 258.
On the basis of the fragmentation pattern observed in the product ion mass spectra and HPLC characteristics, metabolite E is most consistent with a methylsulfonyl-CBZ-10,11-epoxide metabolite.

Peak I. Metabolite I (42 min; fig. 2b) was isolated by HPLC using chromatographic system 1. The presence of 3-hydroxy-CBZ in the fractions was inevitable, because metabolite I was a shoulder peak of the more abundant metabolite H (3-hydroxy-CBZ). Therefore, the full mass spectrum of metabolite I (obtained by LC/MS) was complicated by the presence of predominant ions at m/z 253 (base peak) and m/z 210 that respectively correspond to MH⁺ of 3-hydroxy-CBZ and a protonated 3-hydroxyiminostilbene ion (table 2). Also present in the spectrum were the proposed MH⁺ and sodium adduct of metabolite I appearing at m/z 315 and m/z 337, respectively. An indicated mass of 314 Da was consistent with a postulated elemental composition of C₁₆H₁₄N₂O₃S that suggested metabolite I was a methylsulfonyl ad-
duct of CBZ, a CBZ metabolite previously identified in rat urine by Lertratanangkoon and Horning (12). Furthermore, reanalysis of hydrolyzed urine samples at a higher wavelength (280 nm) indicated the presence of a peak corresponding to metabolite I at 42 min (chromatographic system 1). These data were consistent with absorbance maxima at 220 and 280 nm associated with metabolite I and suggested the methylsulfonyl substituent was attached to an aromatic ring of CBZ. A product ion scan of metabolite I obtained by CID of MH$^+$ \((m/z \ 315)\) is shown in table 3. Class-characteristic cleavage of the carbamoyl side chain afforded an oxocarbonium product ion \((m/z \ 298)\), protonated \((m/z \ 272)\) and deprotonated \((m/z \ 270)\) methylsulfonyl-iminostilbene ions, and a protonated iminostilbene product ion at \(m/z \ 193\). On the basis of these findings, the identity of metabolite I was most consistent with methylsulfonyl-CBZ.

**Discussion**

We have characterized the pathways of CBZ elimination in the SWV mouse model of CBZ teratogenicity and have identified metabolites that are likely to contribute to the teratogenicity observed with the drug. Initial studies demonstrated that the position of the radiolabel used to identify metabolites relevant to teratogenicity was not lost in vivo.

Approximately 75% of the total urinary radioactivity was identified after enzymatic hydrolysis using a combination of spectroscopic techniques. 3-Hydroxy-CBZ and glucuronicides of CBZ and CBZE accounted for the majority of CBZ metabolism in the SWV mouse. Several other previously identified CBZ minor metabolites were also found, including 2-hydroxy-CBZ, CBZE, and methylsulfonyl-CBZ. In addition, two novel CBZ metabolites were tentatively identified, S-cysteinyl-CBZ and dihydrohydroxy-CBZ-NAC, as well as one formed from CBZE, methylsulfonyl-CBZE.

A proposed scheme of CBZ metabolism in the SWV mouse is shown in fig. 6. The primary pathways of CBZ elimination included: (i) N-glucuronidation of CBZ, (ii) epoxidation of CBZ with (iii) subsequent N-glucuronide formation, (iv) ring-oxidations to form CBZ phenols, and (v) phenol conjugation with glucuronic acid. In addition, a series of minor pathways exist (vi) and (vii) that share common intermediate steps in the formation of CBZ thioether metabolites. In addition to results presented herein, the suggestion that phenol formation involves arene oxide intermediates is based on the identification of CBZ phenols, catechols, dihydrodiols and thioether metabolites present in urine, have been postulated to account for the majority of CBZ metabolism in the SWV mouse. The mercapturic acid and CYS-β-lyase pathways of GSH adduct catabolism in the context of CBZ elimination are shown in fig. 7. A general scheme is depicted to illustrate the possible formation of the CBZ thioether metabolites A, E, D, and I by a common mechanism, as well as to acknowledge the possibility of alternate metabolic routes. The nonenzymatic dehydration of dihydrohydroxy-S-glutathionyl adducts of CBZ or CBZE (xi) yields the respective S-glutathionyl conjugates that may undergo further catabolism. Alternatively, the dihydrohydroxy-S-glutathionyl conjugates may be metabolized directly by enzymes of the CYS-β-lyase pathway. Similar biotransformations of the dihydrohydroxy-S-glutathionyl adducts of naphthalene (33) and bromobenzene (34) have been reported. Thus, formation of the respective CYS conjugates may occur by sequential metabolism of the S-glutathionyl or dihydrohydroxy-S-glutathionyl conjugates by γ-glutamyltranspeptidase (xii) and CYS-glycine transpeptidase (xiii). The acetylation of CYS conjugates is catalyzed by N-acetyltransferase (xiv), whereas soluble decactylases located in the gastrointestinal tract may catalyze the reverse reaction (xv) (35). Alternatively, CYS conjugates are also substrates for CYS-β-lyase (xvi) to form a corresponding thiol. Aromatic thiols may be subsequently methylated by thiol-methyltransferases (xvii) and sequentially oxidized to the respective methylsulfinyl (xviii) and methylsulfonyl (xix) conjugates (36–38). In addition, it should be mentioned that nonenzymatic dehydration of the dihydrohydroxy-S-linked adducts could occur at any of the steps (xii–xix); however, for simplicity, only step (xi) is shown.

Aren oxide intermediates, that could lead to the formation of thioether conjugates present in urine, have been postulated to account for CBZ-related hypersensitivity reactions (39) and teratogenicity (3, 6). Hypersensitivity and teratogenic effects associated with CBZ exposure may share a common underlying mechanism related to CBZ metabolism. Hypersensitivity is thought to occur secondary to hapten formation by an electrophilic species formed from CBZ and an endogenous protein. Teratogenicity may occur by the binding of a CBZ electrophile to a critical protein essential for normal embryonic development. The extensive body of evidence suggesting that CBZ is eliminated through an arene oxide has lead to the hypothesis that an inherited defect in epoxide hydrolases may account for an unusual susceptibility for hypersensitivity reactions (39), as well as for congenital malformations (40). However, attempts to correlate epoxide hydrolase activity with the incidence of CBZ hypersensitivity have not been successful (41, 42), leading one group of investigators to conclude that patient variability in epoxide hydrolase activity may not account for the observed adverse reactions (41).
teratogen in this model (17). CBZE seems to be an important first step in forming the ultimate significant, because we recently provided evidence that formation of treatments. The identification of methylsulfonyl-CBZE is particularly involving the formation of electrophiles are modulated by PB and STP metabolism in the SWV mouse revealed that the relative urinary excretion of three CBZ metabolites is consistent with the modulation of teratogenicity by these three pretreatments as reported previously (13). Our microsomal studies suggested that CBZ may also form a quinone intermediate (30). Moreover, the identification of thioether metabolites of CBZ and CBZE is consistent with maternally generated metabolites that may be transported to the conceptus, whereas if an arene oxide accounted for the teratogenicity, it may have to be formed by the fetus. The likelihood of transport and teratogenic potential of CBZ thioether metabolites is consistent with the recently reported transplacental transfer, irreversible binding, and developmental toxicity in mice of 3-methylsulfonyl-2,2-bis(4-chlorophenyl)-1,1-dichloroethene, a metabolite of the chlorinated insecticide DDT (43).

Comparison of the effects of PB and STP cotreatments on CBZ metabolism in the SWV mouse revealed that the relative urinary excretion of three CBZ metabolites is consistent with the modulation of teratogenicity by these pretreatments as reported previously (13). The relative abundance of dihydroxy-CBZ-NAC,S-cysteinyl-CBZ, and methylsulfonyl-CBZ (peaks A, D, and E, respectively) were increased by PB and decreased by STP coadministrations in a manner consistent with a contribution to teratogenicity by these three metabolites, their precursors, or subsequent products. Of particular interest is the finding that metabolic pathways of both CBZ and CBZE involving the formation of electrophiles are modulated by PB and STP treatments. The identification of methylsulfonyl-CBZE is particularly significant, because we recently provided evidence that formation of CBZE seems to be an important first step in forming the ultimate teratogen in this model (17).

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References


