

URINARY NAPHTHALENE MERCAPTURATES AS BIOMARKERS OF EXPOSURE AND STEREOSELECTIVITY OF NAPHTHALENE EPOXIDATION

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ABSTRACT:

Previous work has shown that the rate and stereochemistry of naphthalene epoxidation correlates with differences in susceptibility to cytotoxicity. The development of methods for measuring epoxide formation *in vivo* could provide a marker for assessing events critical to naphthalene cytotoxicity that are applicable to humans. Here, urinary diastereomeric mercapturates have been measured in mice (susceptible) and rats (nonsusceptible) after intraperitoneal administration (1.56–200 mg/kg) or inhalation exposures (0.8–110 ppm, 4 h) to naphthalene. No significant differences were observed in the percentage of the dose eliminated as mercapturate in urine between mice (25–34%) and rats (24–35%) or at varying doses after *i.p.* administration. The amounts of urinary mercapturate after 4-h exposures were considerably greater in mice than rats. In mice, the ratio of diastereomeric mercapturates

derived from the 1*R*,2*S*- to 1*S*,2*R*-epoxide was 1:1 at low doses (1–3 mg/kg), increased to 3:1 at intermediate doses (50 mg/kg), and decreased to 2:1 at high doses (100 and 200 mg/kg). In rats, these ratios remained less than 1:1 at all doses. After inhalation, ratios were 5 to 6:1 at low concentrations (less than 15 ppm) and decreased to 3:1 at higher concentrations (15–100 ppm) in mice, whereas in rats, the ratios were 1:1 or less for all concentrations. These studies show that mercapturates provide good assessments of internal dose, that there are not significant differences between mice and rats in the percentage eliminated as mercapturate but that the ratios of mercapturates derived from the 1*R*,2*S*- versus 1*S*,2*R*-epoxide differ markedly and are consistent with previous *in vitro* metabolism studies.

The volatile aromatic hydrocarbon naphthalene is found in dyes, leather tanning agents, moth repellents, mainstream and sidestream cigarette smoke, and is a product of incomplete combustion of both gasoline and diesel fuel (Schmeltz et al., 1976; Agency for Toxic Substances and Disease Registry, 1995). Human populations are exposed to naphthalene as evidenced by the finding of detectable naphthalene concentrations in 75 and 40%, respectively, of human breast milk (Pellizzari et al., 1982) and body fat samples (Stanley, 1986).

Administration of naphthalene by intraperitoneal or inhalation routes results in highly species-, tissue-, and cell-selective cytotoxicity (Mahvi et al., 1977; Plopper et al., 1992; West et al., 2001). Injury occurs primarily to Clara cells in mice and to olfactory epithelial cells of the rat and mouse nose (Plopper et al., 1992). Doses as low as 50 mg/kg *i.p.* in mice produce swelling of Clara cells and focal vacuo-

lation; 200-mg/kg doses result in extensive necrosis and exfoliation of Clara cells. Clara cell toxicity was not observed in rats at any dose of naphthalene tested [up to the LD₅₀ (1600 mg/kg)], whereas nasal epithelium was reasonably susceptible to the compound. In contrast to the high parenteral doses required to produce cytotoxicity in the mouse, inhalation exposures at concentrations well below the current occupational standard (2–5 ppm) resulted in substantial Clara cell injury in mice but not rats (West et al., 2001). Long-term studies in both mice and rats have indicated some oncogenic potential for naphthalene (Abdo et al., 1992; NTP TR 500). Nasal epithelium was a target in both species and, at the high concentration, pulmonary adenomas were observed in female mice.

Murine Clara cells have high concentrations of cytochrome P450 2F2, a protein that metabolizes naphthalene to reactive intermediates, and this likely plays an important role in the susceptibility of this cell type to injury (Ritter et al., 1991; Buckpitt et al., 1995; Shultz et al., 1999). CYP2F2 metabolizes naphthalene rapidly, with a low K_m (~4 μ M) and high degree of stereoselectivity (66:1 ratio of 1*R*,2*S*- to 1*S*,2*R*-naphthalene oxide) (Shultz et al., 1999). The stereoselectivity of epoxidation, relatively low K_m for naphthalene metabolism, and high turnover numbers observed with recombinant CYP2F2 are reflected in murine pulmonary microsomal metabolism studies where the apparent K_m and V_{max} values are 40 μ M and 14 nmol/mg of microsomal protein/min, respectively (Buckpitt et al., 1992). Although the apparent K_m values for microsomal metabolism in rat and hamster lung microsomes were similar to those observed in the mouse, striking differences were observed in the apparent V_{max} and

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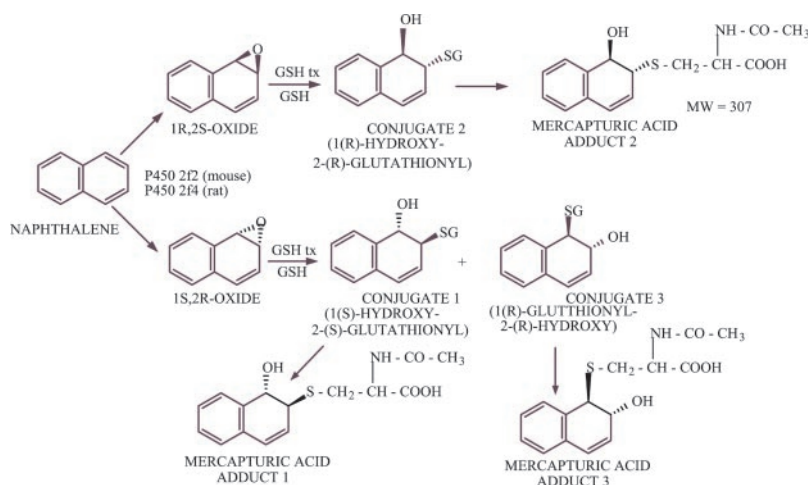


FIG. 1. Pathways of naphthalene metabolism through epoxides to thioether derivatives eliminated in the urine.

stereoselectivity of metabolism (Buckpitt et al., 1992), which correlates with the species differences in susceptibility to naphthalene-induced lung injury. Orthologs of CYP2F2 have been reported in the rat (CYP2F4; Baldwin et al., 1999), goat (CYP2F3; Wang et al., 1998), and human (CYP2F1; Nhamburo et al., 1990). Whether these proteins are catalytically similar to CYP2F2 in metabolizing naphthalene and whether they are present in amounts necessary to generate sufficient quantities of electrophilic naphthalene metabolites to overwhelm the detoxication systems is unresolved. The striking species differences in susceptibility to acute injury resulting from naphthalene exposure raises the question of possible human susceptibility. The high background of lung diseases associated with cigarette smoking combined with the fact that human exposures to naphthalene often occur as multicomponent mixtures account for the fact that epidemiologic studies have provided no clear-cut evidence for or against an association between naphthalene exposure and lung diseases.

One approach, which may provide information regarding the relevance of the animal models for examining pulmonary toxicants such as naphthalene, is based on the development of biomarkers that are tied to critical steps in the mechanism of toxicity of the agent. The correlative association between rates and stereochemistry of naphthalene epoxide formation with tissue susceptibility to cytotoxicity suggests that measurements capable of probing these processes in vivo hold promise as a means of assessing steps critical to toxicity in a number of species, including humans. Accordingly, the work presented here was done to explore the elimination of diastereomeric mercapturic acids in the urine of both susceptible (mice) and nonsusceptible (rats) species by parenteral and inhalation routes of exposure (Fig. 1, metabolic scheme). These studies show: 1) elimination of mercapturates is dose related; 2) there are not substantial species differences in the percentage of dose eliminated as mercapturate; 3) diastereomer ratios differ by species, dose, and route of administration; and 4) diastereomer ratios after inhalation exposures correlate well with rates and stereoselectivity of metabolism in the lung.

Materials and Methods

Animals and Treatment. Male Sprague-Dawley rats (150–200 g) and Swiss-Webster mice (25–30 g) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All animals were housed in HEPA-filtered racks in American Association for the Accreditation of Laboratory Animal Care-accredited animal facilities at the University of California. Food (Purina Rodent Chow; Purina, St. Louis, MO) and water were provided ad libitum. Animals were allowed to acclimate for at least 5 days after receipt from the supplier.

Chemicals. High-pressure liquid chromatography solvents were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade.

Radiochemical. [^{14}C]Naphthalene (52 Ci/mol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The radiochemical purity was shown to be >99.5% by high-pressure liquid chromatography on a C_{18} column by using a mobile phase of 60:40 methanol/ H_2O (v/v).

Chemical Synthesis. Briefly, (\pm)-naphthalene oxide in 0.5% triethylamine/99.5% ethanol was added dropwise with stirring to a 2 M ratio of *N*-acetyl-

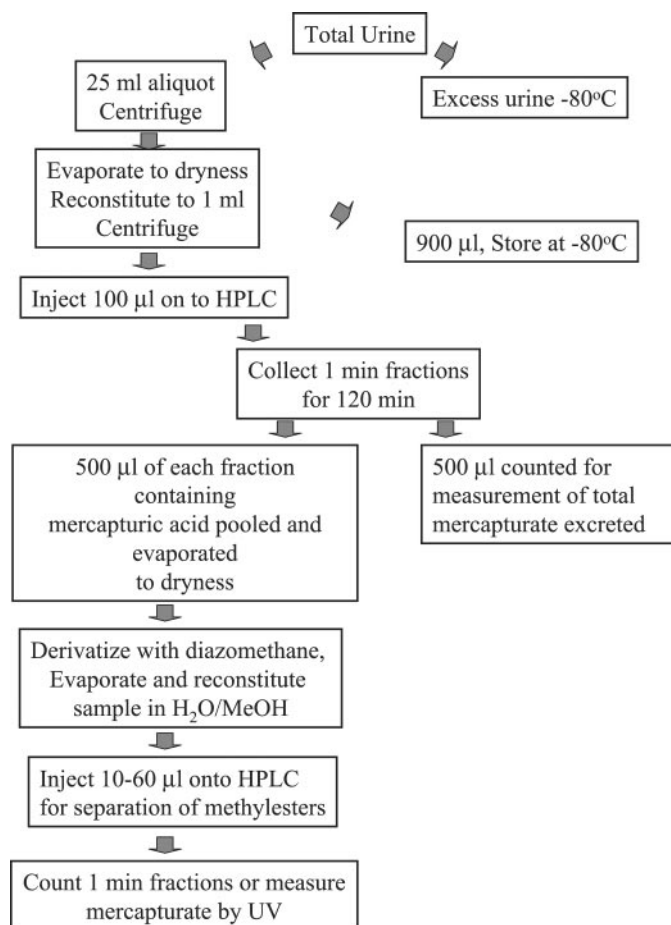


FIG. 2. Schematic diagram of methods used for the isolation and quantitative measurement of naphthalene mercapturates.

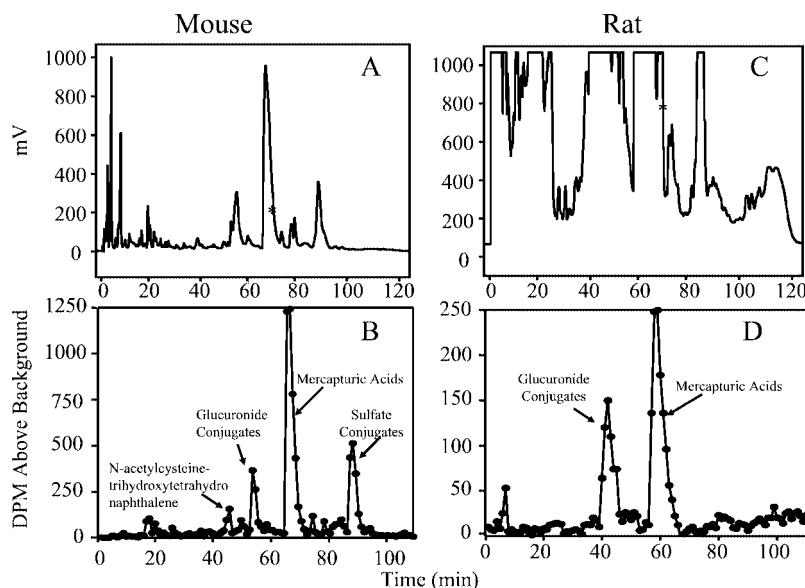


Fig. 3. UV (A and C) and radioactive (B and D) HPLC profiles of mouse (A and B) and rat (C and D) urine extracts obtained from animals treated 24 h earlier with 200 mg/kg [^{14}C]naphthalene.

Twenty-five milliliters of urine was lyophilized and reconstituted in 1 ml of H_2O , and 100 μl was injected onto the HPLC column. Eluant was monitored at 260 nm, and 1-min fractions were collected for radioactive counting.

cysteine dissolved in phosphate buffer at pH 8.5. The mixture was stirred under N_2 for 4 h. The pH was adjusted to 6.0 by the dropwise addition of dilute phosphoric acid and unreacted naphthalene oxide/1-naphthol was removed by extraction with diethyl ether. The remaining aqueous sample was partially evaporated under reduced pressure and the pH was adjusted with acetic acid to 3.0. The mixture was placed on a styrene-divinyl benzene solid phase extraction column (SS-20; Dionex, Sunnyvale, CA) that previously had been washed with acetonitrile and equilibrated with 20 column volumes of 1% acetic acid. The column was washed with 20 bed volumes of 1% acetic acid and the *N*-acetylcysteine conjugates of naphthalene were eluted with 50:50 acetonitrile/water. The pH of the eluant was adjusted to 7.0 with pyridine and solvent was removed by lyophilization. The purity of the final product was confirmed by HPLC³ and the identity by mass spectrometry.

Ethereal diazomethane was generated from diazald (Aldrich Chemical, Milwaukee, WI) and was used to form methyl ester mercapturic acid derivatives.

Naphthalene Administration and Collection of Urine. [^{14}C]Naphthalene was dissolved in corn oil such that 0.1 ml was administered i.p. per 10 g of body weight. Doses ranged from 0 to 200 mg/kg (4 animals/group, specific activity = 5.2–770 dpm/nmol for mice and 0.082–4200 dpm/nmol for rats). Animals were placed in metabolism cages (mice, Teflon cages; rats, silanized glass cages) (Bioserve Biotechnologies, Laurel, MD) immediately after naphthalene administration for separate collection of urine and feces. Air was drawn through the cages at a rate of 1 l/min for mice and 1.5 l/min for rats. Expired radiolabel was trapped in methanol. Urine was collected over dry ice for 24 h.

In the inhalation studies, naphthalene vapor was generated by passing air through crystalline naphthalene packed in a 2.5×70 -cm glass column (same rate as mentioned above). Effluent from the glass column was mixed with fresh air to achieve average air concentrations of 0.8 to 110 ppm for 4 h. Total airflows were as described above. Naphthalene concentrations were measured before and hourly during exposure by withdrawing 10 ml of air from the cage with a gastight syringe. Three milliliters of methanol was drawn into the syringe, the sample was mixed, and concentrations of naphthalene were measured spectrophotometrically ($\lambda = 210$ nm). Naphthalene concentrations also were measured during exposure by online recording with a spectrophotometer equipped with a flow cell. Animals were then transferred to clean cages, and urine was collected for the next 20 h over dry ice. This was combined with the 0- to 4-h urine.

³ Abbreviations used are: HPLC, high-performance liquid chromatography; ESI, electrospray ionization.

Sample Preparation. The overall procedure for separation and measurement of mercapturates is presented in Fig. 2. Collected urine was combined with cage washings (50:50 distilled water/methanol, v/v), transferred to Teflon tubes, and centrifuged for 20 min at 10,000g to remove particulate matter. Twenty-five ml of urine was aliquoted into a centrifuge tube and evaporated to dryness by using a centrifugal evaporator. The residue was reconstituted with 1 ml of water, and particulates were removed by centrifugation. Samples and remaining urine were kept at -80°C until analysis.

Measurement of Total Mercapturic Acid Conjugates. One hundred microliters of reconstituted urine was analyzed with a Phase Sep C_{18} ODS2 column (5 μm , 0.46×22 cm), $\lambda = 260$ nm. A linear 100-min gradient of 100% mobile phase A (0.06% triethylamine in water, v/v, pH 3.1 with phosphoric acid) to 100% mobile phase B (25:75 acetonitrile/mobile phase A, v/v) was started at the injection. The mobile phase flow rate was 1 ml/min. The solvent composition was held at 100% B for 5 min before returning to initial conditions. The mercapturic acid conjugates eluted between 55 and 65 min; conjugate diastereomers could not be separated without derivative formation.

In the urine samples collected from animals treated with [^{14}C]naphthalene, fractions of the column eluate were collected at 1-min intervals and half of each of the fractions was counted for determination of total mercapturate excreted. Those fractions containing mercapturates were pooled for derivatization and further HPLC separation to determine diastereomer ratios (Buonarati et al., 1990). Briefly, pooled mercapturic acid fractions were evaporated to dryness and converted to methyl esters by addition of 5 ml of saturated ethereal diazomethane in silanized glass centrifuge tubes with Teflon-lined caps. After 1 h, the reaction mixture was evaporated under nitrogen, the centrifuge tubes were rinsed with 50:50 methanol/ H_2O (v/v), vortexed, and evaporated to dryness by using a centrifugal evaporator. The residue was reconstituted in 50:50 methanol/ H_2O (v/v). Methyl esters were separated isocratically using a 28:72 methanol/ H_2O (v/v) mobile phase at 1 ml/min. After elution of the methyl esters, a 5-min gradient to 100% methanol followed by 10 min of 100% methanol was programmed to elute nonpolar components. A wavelength of 218 nm was used to detect the esterified adducts.

In animals exposed to naphthalene by inhalation, total mercapturate could not be determined reliably by UV absorbance of the peaks eluting at 55 to 65 min from the initial HPLC step due to interfering peaks in the sample. Accordingly, calculations of the total amounts of mercapturate excreted were derived from samples that were run initially under the gradient conditions described above. Mercapturates were collected, solvent was removed by evaporation, and samples were derivatized with diazomethane. The derivatized

mercapturates were separated by HPLC, and peaks were measured at 218 nm (as described above). The total amount of mercapturate eliminated in the urine was calculated by adding all three mercapturate diastereomers. Synthesized standards were prepared and run with each batch of samples. Signals were linear over the range of sample values (0.008–32 nmol injected).

Mass Spectra. Electrospray (ESI) negative and positive ion spectra were acquired with a VG Quattro BQ (Fisons Instrument, Atrincham, England) tandem mass spectrometer with configuration of QhQ (Q, quadrupole; h, r.f. hexapole) by using a standard ESI source. Some samples were run on a Finnigan LCQ MSⁿ ion-trap mass spectrometer (Thermoquest, San Jose, CA) with a configuration of ooT (o, r.f. only octapole; T, iontrap) by using a standard heated capillary ESI source. The tandem mass spectrometry experiments were all performed on the LCQ ion trap mass spectrometer. The spectra were the result of 5 to 10 consecutive scans.

Statistical Analysis. Statistical differences were determined by one-way analysis of variance and the Bonferroni *t* test with *p* < 0.05 as the level of significance.

Results

HPLC Separation and Identification of Metabolites in Urine of Rats and Mice Treated with Naphthalene. UV and radioactive HPLC profiles of urine from mice (Fig. 3, A and B) and rats (Fig. 3, C and D) treated intraperitoneally with 200 mg/kg [¹⁴C]naphthalene indicated the presence of several UV-absorbing, radioactive metabolites. Major metabolite(s) eluting between 55 and 65 min in both mouse and rat urine was identified by both cochromatography with a synthetic mercapturic acid standard and by mass spectrometry as

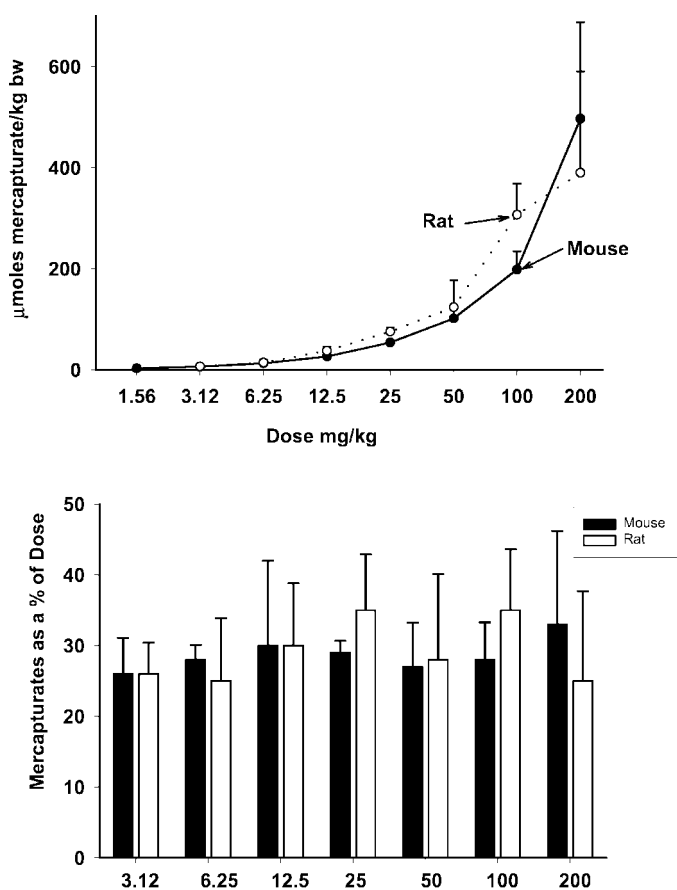


FIG. 4. Elimination of mercapturates in mice and rats treated intraperitoneally with varying doses of naphthalene.

Top, data are presented as total micromoles of mercapturate eliminated per kilogram of body weight at each dose (mean \pm S.D.; *n* = 4) in mice (body weights from 28–34 g; ●) and rats (body weights 229–314 g; ○). Bottom, data are presented as the percentage of the dose eliminated as mercapturate.

hydroxydihydro-mercapturic acids of naphthalene. The molecular ion (M-H)⁻ appeared at *m/z* 306 Th; fragments at *m/z* 288 Th (dehydration product), *m/z* 177 Th (thiolate ion), and *m/z* 159 Th (dehydration product of the thiolate ion) have all been reported previously (Buonarati et al., 1990). In addition, trace quantities of an *m/z* 492 Th signal were consistent with the presence of an *N*-acetylglutathione conjugate (Tsuruda et al., 1995). A product spectrum of the *m/z* 492 Th ion yielded fragments at *m/z* 474 Th (-H₂O) and *m/z* Th 456 (-2 \times H₂O), which were identical to the synthetic standard. The *N*-acetylglutathione conjugate was detected as a contaminating peak in the mercapturic acid fraction only at the 200-mg/kg dose. The peak eluting at 45 min was identified as an *N*-acetylcysteine derivative of trihydroxytetrahydronaphthalene (Tsuruda et al., 1995), whereas the peak eluting at 54 min was a glucuronide of the dihydrodiol. The peak eluting at 85 to 90 min was tentatively identified as a sulfate conjugate, also derived from the dihydrodiol. The profile of metabolites eliminated in rat urine was dominated by mercapturic acids and contained significant quantities of glucuronide.

Urinary Mercapturic Acid Excretion: Dose Dependence after Parenteral Administration and Inhalation Exposures. Mercapturic acids accounted for 24 to 33% (mice) and 25 to 34% (rats) of the administered dose of radioactive metabolites eliminated in the 24 h urine of animals treated with [¹⁴C]naphthalene i.p. (Fig. 4). Statistically significant differences in the percentage dose eliminated as mercapturic acid between doses or species were not observed. As expected, total mercapturic acids (μ mol/kg) eliminated, in both mice and rats, was dose-dependent, and differences between doses were

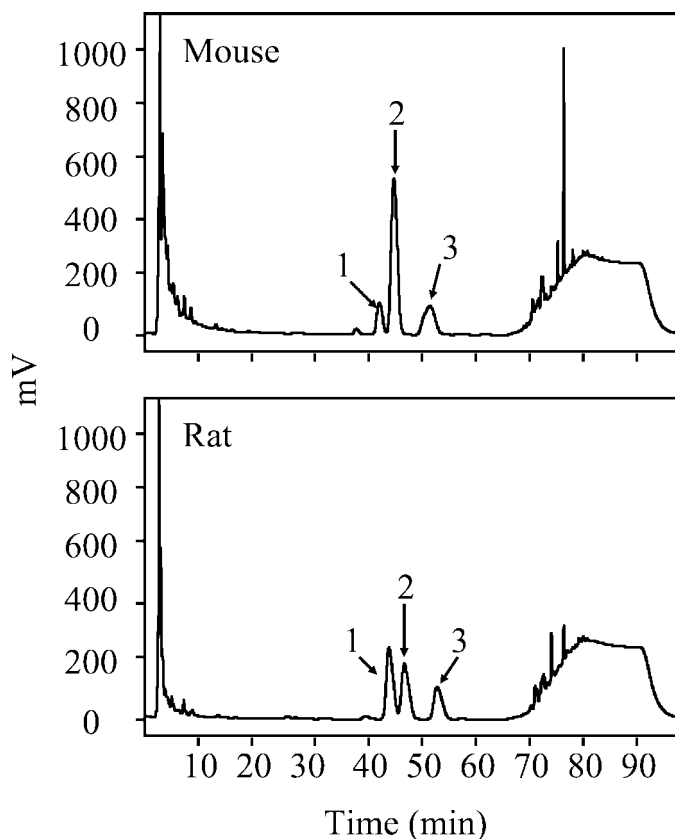


FIG. 5. Typical HPLC-UV profile (218 nm) of mercapturic acid diastereomers derived from urine of a mouse or rat exposed to 107 ppm naphthalene for 4 h.

Urine was lyophilized, reconstituted, and run on the HPLC. Unseparated diastereomers were collected, derivatized with diazomethane, and run on the HPLC, as described under *Materials and Methods*. Peaks 1 and 3 are derived from the 1*S*,2*R*-epoxide; peak 2 is derived from the 1*S*,2*R*-epoxide.

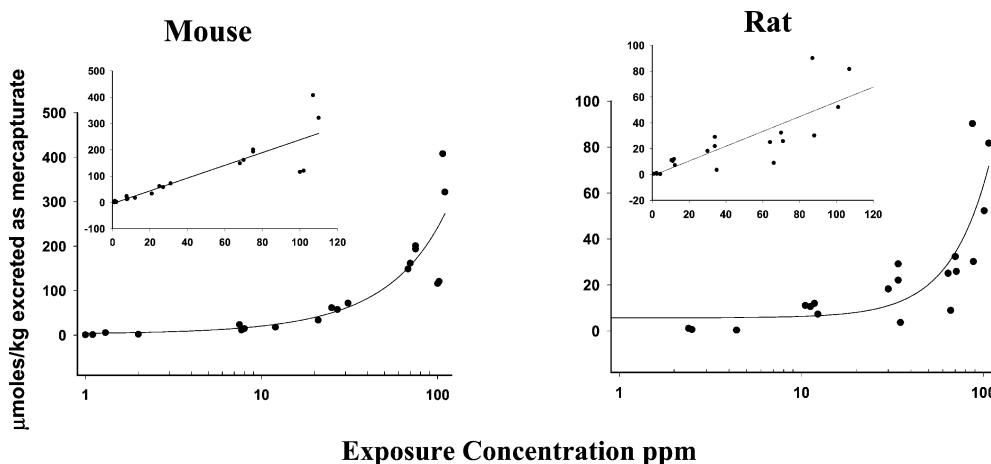


FIG. 6. Amounts of mercapturic acids eliminated in the urine of mice and rats that were exposed to naphthalene for 4 h at varying concentrations.

Each point represents data from a single animal. Inset, data are plotted as a linear relationship.

statistically significant (Fig. 4). In mice, elimination of mercapturic acid varied from 6.5 $\mu\text{mol/kg}$ b.wt. naphthalene mercapturate at 3.12 mg/kg to 497 $\mu\text{mol/kg}$ at 200 mg/kg, whereas rats eliminated from 7.0 $\mu\text{mol/kg}$ to 390 $\mu\text{mol/kg}$ at 3.12 and 200 mg/kg, respectively.

In animals exposed by inhalation, the amounts of naphthalene mercapturic acid eliminated as a percentage of the dose could not be determined because the actual inhaled dose was not determined. Since [^{14}C]naphthalene was not used in these studies and because the mercapturates could not be clearly separated from interfering substances in the urine, a different approach was used for the measurement of total mercapturate eliminated. The amount of urinary mercapturate was measured after collection of the "mercapturate fraction" from the first HPLC step. This fraction was evaporated to dryness under reduced pressure, derivatized with diazomethane, and subjected to further HPLC separation to yield the diastereomers. Figure 5 presents a typical UV chromatogram of derivatized mercapturic acid diastereomers isolated from a mouse or rat exposed to naphthalene by inhalation. In all instances, known standards of naphthalene mercapturic acids were subjected to the same procedures to create standard curves for analysis. In both mice and rats, there was a concentration-dependent increase in the amount of urinary mercapturic acids eliminated (Fig. 6). In mice, total mercapturic acids eliminated ranged from 1 $\mu\text{mol/kg}$ at exposure levels of 1 ppm to an average of 240 $\mu\text{mol/kg}$ at exposure levels of approximately 100 ppm. In comparison, elimination of mercapturate in rats varied from a low of 0.6 $\mu\text{mol/kg}$ at low exposure levels (0.8 ppm) to 67 $\mu\text{mol/kg}$ at high concentrations (\sim 100 ppm). Linear plots of mercapturate versus exposure concentration (Fig. 6, inset) revealed that, although there is considerable animal-to-animal variability in the data, there is a relationship between exposure and amount of mercapturate eliminated. Correlation coefficients were 0.76 in mice and 0.63 in rats.

Ratios of Urinary Mercapturate Diastereomers: Differences of Dose, Route of Administration, and Species. In mice treated intraperitoneally at high doses of naphthalene (100 and 200 mg/kg), the ratio of mercapturates derived from 1*R*,2*S*- to 1*S*,2*R*-naphthalene oxide was 1:1 (Fig. 7, top). As the dose decreased, the ratio was closer to 2 to 3:1 (1.56–12.5 mg/kg), indicating that more mercapturate was derived from 1*R*,2*S*-naphthalene oxide than 1*S*,2*R*-naphthalene oxide. A statistically significant difference between ratios was obtained at 100 and 200 mg/kg in comparison with all doses at or below 50 mg/kg in the mouse. In contrast, the ratios of naphthalene mercapturates in rats treated with naphthalene i.p. remained less than 1:1 at all doses tested. No statistical differences were noted in the ratios between

doses. In mice, after low concentration inhalation exposures (7.5–12 ppm), the ratios of mercapturates derived from the 1*R*,2*S*- to 1*S*,2*R*-epoxide were $>6:1$ (Fig. 7, middle). As the exposure concentrations increased (21–110 ppm), these ratios decreased and averaged 3:1. In comparison, the ratio of mercapturates in rat urine after inhalation exposure varied from approximately 1:1 at low exposure concentrations (0.8–12 ppm) to generally less than 0.5:1 at higher concentrations (>60 ppm) (Fig. 7, bottom).

Discussion

Assessing the potential human health consequences of exposure to a chemical in the absence of solid epidemiologic evidence of adverse effects from that chemical remains a daunting challenge. This is particularly true in those cases where widely divergent responses are observed in rodent species. Accordingly, considerable efforts have been made to develop methods that are capable of assessing internal dose to target tissue of biologically active agents (for review, see Farmer, 1999) in experimental animals for subsequent application in exposed human populations. The studies presented in this article focus on measurements of diastereomeric naphthalene mercapturic acids as a means of monitoring the rates of formation of naphthalene epoxide enantiomers *in vivo*. Substantial evidence suggests that the epoxide plays a major role in naphthalene-induced lung injury (Kaneke et al., 1991; Chichester et al., 1994) and contributes to the overall levels of reactive metabolite bound covalently to proteins (Buonarati et al., 1989).

The ability of measurements of diastereomeric mercapturic acids to provide a reasonable assessment of the formation of naphthalene epoxides is predicated on 1) the demonstration that naphthalene glutathione conjugates, which are directly generated from the epoxide, are eliminated primarily as mercapturic acids in the urine; and 2) the relative amounts of the epoxides that are metabolized to glutathione conjugates versus other metabolites. Previous work (Buonarati et al., 1990) examined the profile of metabolites eliminated in mouse urine after intravenous administration of the diastereomeric glutathione conjugates derived from C1 (benzylic) and C2 (allylic) thioethers of naphthalene. These studies showed that a significant amount of the naphthalene conjugate generated by conjugation of naphthalene with glutathione at C1 (14–25% of the dose, depending on the dose) was eliminated in the urine as a pyruvic acid conjugate after intravenous administration. However, 76 to 84% of both of the conjugates derived from attack of glutathione at C2 of the naphthalene nucleus were eliminated as mercapturates after intravenous administration. Thus,

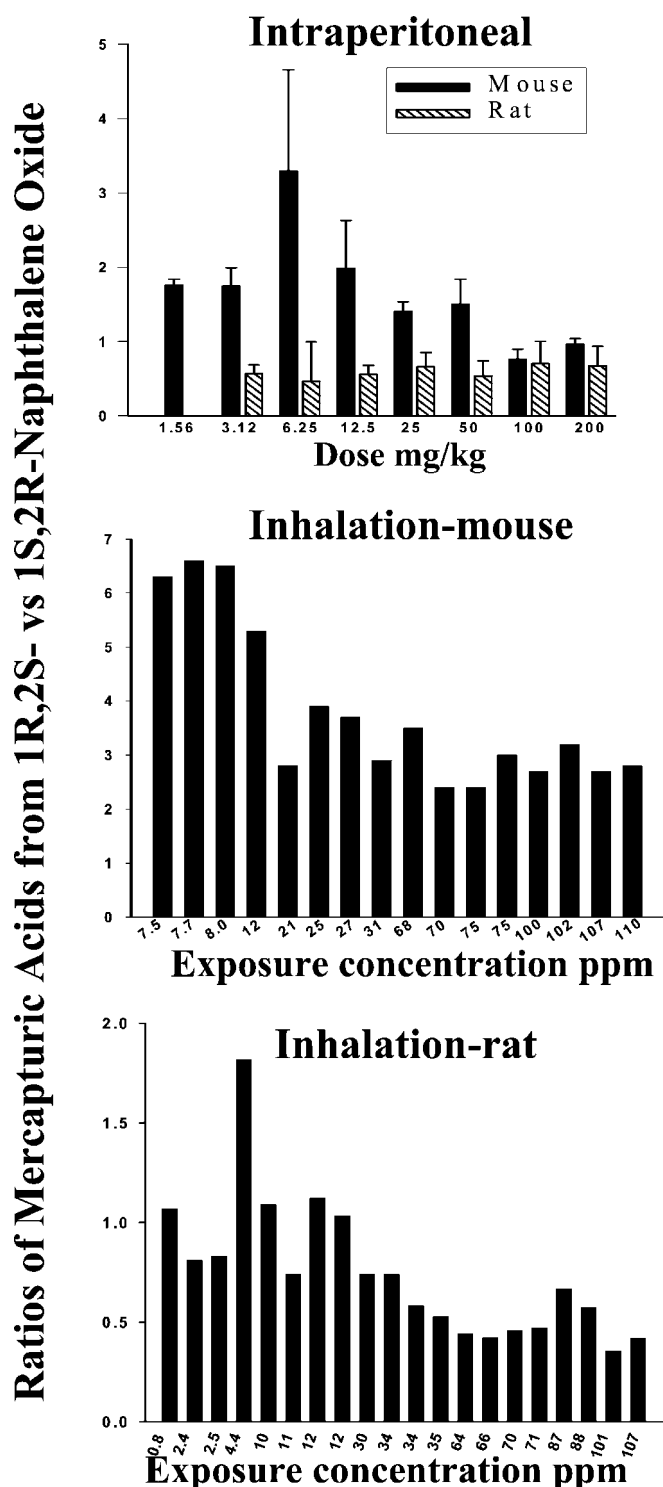


Fig. 7. Ratios of mercapturic acids derived from 1R,2S- to 1S,2R-epoxide in urine of mice and rats after intraperitoneal and inhalation exposures.

Since previous work demonstrated that a significant quantity of the glutathione adduct, which generates peak 3, is metabolized to a thiopyruvic acid adduct (Buonarati et al., 1990), all ratios were calculated with the following formula: $[\text{nmol peak 2}]/[\text{nmol peak 1} \cdot 2]$. Values from animals treated with naphthalene intraperitoneally are the mean \pm S.D. ($n = 4$). Values obtained from both mice and rats exposed by inhalation are from single animals.

measurement of the mercapturates derived from these two metabolites provides a reasonable assessment of the stereoselectivity of epoxide formation.

Dihydrodiols and naphthols are potential products involved in the metabolic disposition of naphthalene epoxide, and as indicated in Fig. 3, glucuronides (mice and rats) and sulfates (mice) are prominent metabolites in our studies. These data confirm previous work in both species (Horning et al., 1980; Stillwell et al., 1982). In addition, naphthols, eliminated in the urine as conjugates, were measured in urine of industrial workers exposed to naphthalene (Bieniek, 1994, 1997) and were found in nearly 80% of urine samples from an adult population (NTP TR 500). Although urinary naphthols are good biomarkers of naphthalene exposure and are capable of providing an indication of the amounts of naphthalene oxide produced, these metabolites are less abundant than the mercapturic acids in rodents (Fig. 3) and provide no information on the stereochemistry of the initial epoxidation step. In addition, urinary naphthols are potentially derived from sources other than naphthalene in exposed human populations. 1-Naphthol is one of the major metabolites of the pesticide, carbaryl, and this metabolite was validated as a biomarker of carbaryl exposure (Shealy et al., 1997).

Another possible confounding issue that must be considered when interpreting the data presented in this article is the possibility that the urinary mercapturic acid ratios could be influenced by the enantioselectivity of epoxide hydrolases or the glutathione transferases. Higher levels of the dihydrodiol and lower levels of the glutathione conjugates were generated in isolated murine hepatocytes from the 1R,2S-compared with the 1S,2R-epoxide (Buonarati et al., 1989). Likewise, glutathione transferase μ , α , and π show substantial enantioselectivity for the two epoxides of naphthalene, but this is considerably more pronounced at high compared with low substrate concentrations (D. Morin, unpublished data).

As indicated in the current studies, the mercapturic acid conjugates are quantitatively important naphthalene metabolites that account for 25 to 35% of the total dose administered. The percentage of the dose eliminated as a mercapturic acid conjugate remained relatively constant over the entire dose range studied in both mice and rats, indicating that it is highly unlikely that the doses used exceeded the glutathione threshold (Fig. 4). The data on the amounts of mercapturate eliminated in the urine of mice and rats after inhalation exposure to the compound indicated a relationship between exposure concentration and amounts of metabolite. As noted in Fig. 6, insets, these relationships are not directly linear. This is consistent with the recent kinetic studies showing that blood levels of naphthalene in mice and rats were not strictly proportional to the exposure concentration (NTP TR 500). The amounts of mercapturate eliminated per kilogram of body weight in the urine of mice at the same exposure concentration were considerably higher than the corresponding quantities of mercapturates in rats. This is consistent with the data showing higher levels of the parent compound in mouse than in rat blood taken immediately after a 6-h exposure to 10 ppm (NTP TR 500). These data also are consistent with higher rates of naphthalene metabolism in mouse compared with rat lung microsomes (Buckpitt et al., 1992). The amounts of mercapturate eliminated in the urine of mice and rats treated i.p. are similar when corrected for body weight. This is probably a reflection of the similarity in the rates of hepatic metabolism in the two species as well as the fact that a major portion of a dose of naphthalene is metabolically cleared by the liver after parenteral administration.

Like the naphthols, the mercapturic acid metabolites of naphthalene are capable of indicating both the extent of exposure to the parent compound and the amounts converted to naphthalene oxide. With the caveats discussed above, the added advantage of measuring these metabolites is that they offer the possibility of examining the initial stereochemistry of epoxidation. This is demonstrated with the data in

Fig. 7. After intraperitoneal administration of naphthalene at most of the dose levels studied, the ratio of mercapturates derived from the 1*R*,2*S*- compared with the 1*S*,2*R*-epoxides is 2 to 1 or less. This is likely a reflection that a large percentage of the dose is being metabolized in the liver where the epoxide ratio is 1:1 (Buckpitt et al., 1992). Similarly, in the rat, the ratio of epoxides derived from the 1*R*,2*S*- versus the 1*S*,2*R*-epoxide remains considerably less than 1 at all of the doses tested, and this reflects hepatic metabolism where epoxide ratios of 0.5:1 have been observed in microsomal metabolism experiments (Buckpitt et al., 1987). In contrast to the findings after intraperitoneal administration, ratios of mercapturates derived from the 1*R*,2*S*- to 1*S*,2*R*-epoxides were 5 to 6:1 at low concentrations of naphthalene vapor. As the inhalation exposure concentration increased, these ratios decreased yet remained greater than 2:1 at all exposure levels. This is consistent with the finding that the 1*R*,2*S*-epoxide is the predominant epoxide enantiomer generated in murine lung and that the ratios of 1*R*,2*S*- to 1*S*,2*R*-epoxide are higher at low rather than high substrate concentrations (Buckpitt et al., 1992). This is also consistent with the possibility that at the higher exposure levels, more parent naphthalene is escaping pulmonary metabolism, entering the circulation, and being metabolized by the liver. Not surprisingly, the ratios of 1*R*,2*S*- to 1*S*,2*R*-naphthalene epoxide-derived mercapturates in the urine of rats exposed by inhalation is generally 1:1 or less. Overall, these data support the contention that the ratios of mercapturic acid diastereomers in the urine can be used to monitor the stereoselectivity of epoxidation reactions occurring in the lung in vivo for a volatile aromatic hydrocarbon like naphthalene.

The long-term goal of these studies is to examine the amounts and ratios of mercapturates in human urine after naphthalene exposure either from cigarette smoke or from industrial sources. Work published more than 40 years ago by Boyland and Sims (1958) provided solid evidence that humans are capable of metabolizing naphthalene to urinary mercapturates. The doses used in these studies (500 mg orally or about 5–10 mg/kg) exceed the total dose likely to be taken up during inhalation exposures in humans. Thus, more sensitive methods will be needed before addressing this issue. It is possible that the enzyme-linked immunosorbent assays developed by Marco et al. (1993) can be adapted for this purpose, but the antibody remains to be tested to determine whether it recognizes the methylated mercapturate.

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