METABOLISM AND DISPOSITION OF [14C]1-NITRONAPHTHALENE IN MALE SPRAGUE-DAWLEY RATS

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ABSTRACT:

In rats and mice, 1-nitronaphthalene (1-NN) produces both lung and liver toxicity. Even though these toxicities have been reported, the metabolism and disposition of 1-NN have not been elucidated. Therefore, studies were performed to characterize its fate after i.p. and i.v. administration to male Sprague-Dawley rats. After i.p. administration of [14C]1-NN (100 mg/kg; 60 μCi/kg), 84% of the dose was eliminated in the urine and feces by 48 h. At 96 h, 60% of the dose was recovered in the urine, 32% in the feces, and 1% collectively in the tissues, blood, and gastrointestinal contents. Interestingly, 88% of the dose was secreted into bile by 8 h. The \( k_{\text{term}} \) was 0.94 h\(^{-1} \) and the terminal phase half-life (\( T_{1/2,\text{term}} \)) was 3.40 h, and the systemic bioavailability was 0.67. When administered i.v. (10 mg/kg; 120 μCi/kg), 85% of the dose was eliminated in the urine and feces by 24 h. At the end of the study (96 h), 56% of the dose was recovered in the urine, 36% in the feces, and 1% collectively in the tissues, blood, and gastrointestinal contents. Interestingly, 88% of the dose was secreted into bile by 8 h. The \( k_{\text{term}} \) was 0.94 h\(^{-1} \) and the \( T_{1/2,\text{term}} \) was 0.77 h. The major urinary metabolite after both routes of administration was \( N \)-acetyl-(hydroxy-1-nitro-dihydronaphthalene)-L-cysteine. Other urinary metabolites identified include hydroxylated, dihydroxylated, glucuronidated, sulfated, and reduced metabolites, as well as dihydrodiol. The major biliary metabolite was hydroxylglutathionyl-1-nitro-dihydronaphthalene. These data show that 1-NN undergoes extensive metabolism and enterohepatic recirculation, and the majority of the dose is eliminated in the urine.

1-Nitronaphthalene (1-NN)\(^1 \) has various commercial and industrial uses. It is used as a chemical intermediate in the production of dyes, a wood preservative, a fungicide, and a component in firework powder (Kasperczak and Lutomski, 1973; Dominik, 1978; Ying et al., 1986). 1-NN is an environmental contaminant in urban areas of the United States and Europe because of its presence in diesel engine exhaust in ambient airborne particulates (Nishioka et al., 1982; Rasmussen, 1986). Rodent toxicity studies have shown that the major target organ for 1-NN-induced toxicity is the lungs. After a single i.p. injection, 1-NN causes a severe respiratory distress syndrome, acute loss of both ciliated and nonciliated cells, necrosis of the bronchiolar epithelium, and cytotoxicity to tracheal epithelium (Johnson et al., 1984; Paige et al., 1997). In vitro studies have shown that 1-NN is metabolized by cytochrome P-450 to a reactive metabolite(s) that binds to lung microsomes, lung slices, and isolated lung cells (Rasmussen, 1986; Price et al., 1995). With specific inhibitors of pulmonary cytochrome P-450 isoforms, Verschoyle and Dinsdale (1990) reported that cytochrome P-450 2B1 activity is correlated with 1-NN toxicity. Sauer et al. (1995) have shown that pulmonary toxicity is marked by infiltration of inflammatory cells into the interstitial areas around damaged bronchioles. Thus, the pulmonary toxicity caused by 1-NN administration to rats appears to involve both P-450-mediated bioactivation and a distinct inflammatory response.

In addition to lung injury, hepatotoxicity also develops after 1-NN administration. Sauer et al. (1997) reported hepatocellular necrosis consisting of damage to the centrilobular and perportal hepatocytes, as well as bile duct epithelial cells. The hepatic toxicity induced by 1-NN is enhanced with phenobarbital pretreatment and diminished with SKF 525-A pretreatment, suggesting that cytochrome P-450 2B1 bioactivates 1-NN to reactive metabolites in the induced liver (Johnston et al., 1984).

Although biotransformation of 1-NN has been implicated in its toxicity, little effort has been directed to elucidating the metabolism and disposition of 1-NN. Therefore, studies were performed to characterize the fate of 1-NN after a single i.p. or i.v. bolus dose to male Sprague-Dawley rats. Excretion of 1-NN and metabolites via urine, feces, and bile were monitored with particular emphasis on the identification of the major urinary and biliary metabolites. Because previous studies have characterized the lung and liver toxicity after i.p. administration in the rat, this route of administration was used so that the metabolism and disposition data could ultimately be related to the

\(^1\) Abbreviations used are: 1-NN, 1-nitronaphthalene; JVC, jugular vein cannula; AUC, area under the blood concentration-time curve from zero to time infinity; CL, apparent clearance; F, bioavailability; CID, collision-induced dissociation; GC, gas chromatography; MS, mass spectrometry; MS-MS, tandem mass spectrometry; \( R_t \), retention time; LC, liquid chromatography.
toxicity studies. Administration of 1-NN by a single i.v. injection was used for toxicokinetic analysis.

**Experimental Procedures**

**Chemicals.** The following chemicals were purchased from the vendors indicated: 1-NN, 99% pure (Aldrich Chemical Company, Inc., Milwaukee, WI); universally ring-labeled [14C]-naphthalene, specific activity 18.6 mCi/mmol (California Bionuclear Corp., Sun Valley, CA); 1-naphthylamine chemical standard (98% pure), bovine liver β-glucuronidase type B-10, Helix pomatia sulfatase type H-1, and n-saccharic acid 1,4-lactone (Sigma Chemical Co., St. Louis, MO); ethylene glycol and 2-methoxyethanol ether (Mallinckrodt Chemical, Paris, KY); CarboSorb E and Flo-Scint III (Packard, Meriden, CT); Universal cocktail (ICN Radiochemicals, Irvine, CA); and HPLC grade acetone and ethyl acetate (Burdick & Jackson Laboratories Inc., Muskegon, MI).

**Synthesis of [14C]-1-NN.** [14C]-1-NN was prepared by the Synthetic Chemistry Core of the Southwest Environmental Health Sciences Center from [14C]-naphthalene. [14C]-1-NN was synthesized by nitrating [14C]-naphthalene as described in detail by Kameo and Hirashima (1986). The radiochemical purity of [14C]-1-NN was determined by HPLC to be 99%.

**Animal Studies. Animals.** Male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Male Sprague-Dawley rats fitted with an indwelling jugular vein cannula (JVC) were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA). Rats were purchased with a weight range of 175 to 199 g and naturally increased to an average of 216 g by the end of the studies. On arrival to the animal facility, the animals were allowed to acclimate for 5 to 7 days in a temperature-controlled room (20–22°C) with a 12-h light/dark cycle before any treatment. Food (Teklad 4% mouse-rat diet; Harlan Teklad, Madison, WI) and water were provided ad libitum.

**Routes of elimination study.** [14C]-1-NN (100 mg/kg; 60 μCi/kg) was administered i.p. in a peanut oil vehicle (2 ml/kg). Rats were immediately placed individually into sealed glass metabolism cages maintained with a constant inflow of ambient air. Urine (6, 12, 24, and 48 h), feces (24, 48, and 48 h), and exhaled radioactivity (0.5, 1, 2, 4, 6, 8, 10, 12, 24, and 48 h) were collected at the times indicated over a 48-h period. Total airflow through each cage was passed through a series of three traps. The first trap contained 2-methoxyethanol ether for collection of expired [14C]organic, and the remaining two traps contained a mixture (2:1 v/v) of CarboSorb E and ethylene glycol for collection of expired [14C]CO2. All trapping solvents were collected and changed at selected times and measured for total radioactivity by liquid scintillation counting (Beckman LS5000TD Liquid Scintillation Counter; Beckman Instruments, Fullerton, CA).

**Interperitoneal toxicokinetic study.** [14C]-1-NN (100 mg/kg; 60 μCi/kg), in peanut oil, was administered i.p. as described above to jugular vein-cannulated Sprague-Dawley rats. Rats were immediately placed individually into Nalgene metabolism cages to allow collection of urine (6, 12, 24, 48, 72, and 96 h) and feces (24, 48, 72, and 96 h) throughout the 96-h study. Serial trapping solvents were collected and changed at selected times and measured for total radioactivity by liquid scintillation counting (Beckman LS5000TD Liquid Scintillation Counter; Beckman Instruments, Fullerton, CA).

**Metabolism and Disposition of 1-NN**

**Bile study.** The jugular vein and common bile duct were cannulated in rats anesthetized with urethane/water (1:1 v/v; 2 ml/kg i.p.) as described by Jaeschke (1992). [14C]-1-NN (10 mg/kg, 120 μCi/kg), in emulphor/ethanol/saline (3:4:12 v/v/v), was administered as a single i.v. injection into the JVC at 2 ml/kg. Serial bile samples were collected from the bile duct cannula at 0, 0.5, 1, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 8.5 h. These samples were measured for total radioactivity by direct liquid scintillation counting of bile in Universol cocktail.

**Data analysis.** The blood concentration-time data after i.p. and i.v. bolus dosing were analyzed by compartmental methods. Several models were analyzed statistically to determine the appropriate model to fit the data. Toxicokinetic parameter values best describing a linear one-compartmental model for the i.p. study and a two-compartmental model after i.v. administration, and assuming first-order kinetics for all processes, were determined by nonlinear regression analysis (WinNonlin; Pharsight Corporation, Mountain View, CA). The parameters of the model were used to calculate values for the first-order rising phase rate constant (k_rise) after i.p. administration, rising phase half-life (T_1/2,rise) after i.p. administration, terminal phase rate constant (k_term), terminal phase half-life (T_1/2,term) under the blood concentration-time curve from zero to time infinity (AUC), apparent clearance (CL), apparent volume of distribution (V_d), apparent volume of distribution under steady-state conditions (V_SS), time to maximum blood concentration of 1-NN (T_max), and maximum blood concentration of 1-NN (C_max). Bioavailability (F) was determined by dividing the total AUC after i.v. administration by the area after i.v. administration, appropriately correcting for dose, and assuming that clearance remained constant (Rowland and Tozer, 1995). The average blood concentration-time data also were fit to the appropriate model to provide a graphical display of the data.

**Analytical Methods.** HPLC analysis of 1-NN and its metabolites. 1-NN was extracted from 150 μl of blood with 300 μl of ethyl acetate and 150 μl of 0.1 N HCl. The samples were thoroughly mixed, centrifuged, and the organic extract removed. The organic portion of the extraction procedure was repeated and the extracts pooled. Samples were dried by vacuum centrifugation and reconstituted with acetonitrile. After the extraction of 1-NN with this method, all samples were corrected to account for the extraction efficiency of 83%. The reconstituted samples (100 μl) were injected onto a 4.6 mm × 250 mm Partisil 10 ODS-2 WCS analytical column (Whatman Inc., Clifton, NJ), eluted with a mobile phase of water/acetonitrile both containing 0.1% acetic acid, a flow rate of 1 ml/min, and a total run time of 80 min. The mobile phase gradient was run from 95% water and 5% acetonitrile for 5 min, then to 30% acetonitrile over 30 min, and to 100% acetonitrile over 15 min with these final conditions held for 5 min. The column was then brought back to initial conditions over 25 min. The HPLC system used throughout this work was composed of a SP8800 ternary HPLC pump and SP8775 autosampler (PharSyx Analytical, San Jose, CA). The column effluent was monitored in tandem with a UV-visible detector (PharSyx SF-450) at a wavelength of 340 nm and radiochemically with a β-Ram Flow-Through Monitor System (IN/US Systems, Inc., Tampa, FL). 1-NN eluted at 51 min under these conditions.

For analysis of urinary and biliary metabolites, samples were pooled, diluted (1:1 v/v) with distilled water, acidified, thoroughly mixed, and centrifuged at low speed to remove any precipitate. Prepared urine and bile samples (100 μl) were injected onto a 4.6 mm × 250 mm Partisil 10 ODS-2 WCS Analytical Column (Whatman Inc.), and metabolites were eluted under the same conditions and equipment as described previously. Samples were subjected to enzymatic hydrolysis to identify possible glucuronide or sulfate conjugates of 1-NN metabolites. Samples were incubated for at least 24 h with β-glucuronidase (2000 U/ml) or sulfatase (100 U/ml) according to the method of Peters and Caldwell (1994), and analyzed by HPLC with the same method and equipment as described previously.

**Metabolite identification with mass spectrometry (MS) and tandem mass spectrometry (MS-MS) analysis.** Urinary metabolites were isolated by semi-preparatory HPLC and concentrated by lyophilization (VirTis, Gardiner, NY). The purity of the concentrated samples was determined by HPLC before identification on a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an atmospheric pressure source. Samples were introduced into the mass spectrometer ion source by direct flow
injection at 0.3 ml/min. Ions with m/z values corresponding to putative metabolites were subjected to collision-induced dissociation (CID) with Ar gas within a tandem mass spectrometer and the subsequent product ion signal masses analyzed to produce a product ion mass spectrum. Logical fragmentation patterns observed in the resulting MS-MS spectrum provided further evidence as to metabolite identity.

Biliary metabolites were separated via HPLC with the above-mentioned conditions on the Finnigan TSQ 7000 triple quadrupole mass spectrometer. After on-column separation, samples were introduced into the mass spectrometer ion source. The ions with m/z values corresponding to putative metabolites were subjected to CID as described above. The fragmentation patterns observed in the resulting MS-MS spectrum provided further evidence as to metabolite identity.

Extracted urinary metabolites were separated with a Fisons GC-8000 gas chromatograph (GC) coupled to a Fisons MD8000 quadrupole mass spectrometer (Fisons Instruments, Beverly, MA) to produce a distinct electron ionization mass spectrum. Samples (1 μl) were injected onto a DB5-MS capillary column (0.25-μm film thickness, 0.25-mm diameter, 30 m; J & W Scientific, Folsom, CA). The oven temperature was initially maintained at 50°C for 5 min, then increased at 10°C/min for the next 25 min to a final temperature of 300°C, and maintained at 300°C for 5 min. The injector, source, and interface temperatures were 250, 250, and 275°C, respectively.

Results

Intraperitoneal Administration. A one-compartmental model with apparent first-order input adequately described the i.p. blood concentration-time profile. The maximum blood concentration (C\(_{\text{max}}\)) of 1-NN (3.94 μg/ml) was obtained at 91 min (T\(_{\text{max}}\)) after i.p. administration, and declined slowly until the values were below the limit of detection by 24 h (Fig. 1). Interestingly, the blood concentration-time profile showed a paradoxical reversal in which the terminal phase was determined by the absorption rate rather than by the elimination rate. The absorption of 1-NN from the peritoneal cavity into the systemic circulation was the rate-limiting step. The mean ± S.D. k\(_{\text{term}}\) and T\(_{1/2,\text{term}}\) values after i.p. administration were 0.21 ± 0.04 h\(^{-1}\) and 3.40 h ± 0.63 h, respectively. The F was 0.67 ± 0.21. These toxicokinetic parameters are listed in Table 1.

By 96 h, >90% of the administered dose was recovered in the urine and feces (Fig. 2A). The radioactivity associated with exhaled carbon dioxide and organic vapors was at or below background levels (0%). Oxidation of selected tissues, blood, and gastrointestinal contents indicated that little radioactivity was retained in the rat 96 h after treatment (0.68%). At 96 h, only 0.10 and 0.21% of the administered dose remained in the liver and blood, respectively (data not shown). The total recovery of administered radioactivity (at 96 h) in the urine, feces, selected tissues, blood, and gastrointestinal contents was 93%.

Intravenous Administration. The blood concentration-time profile was best described by a biexponential equation, consistent with a linear two-compartmental model. After a single i.v. bolus dose, the concentration of 1-NN in blood declined rapidly within 15 min, and was below the limit of detection by 12 h (Fig. 1). The mean ± S.D. k\(_{\text{term}}\) and T\(_{1/2,\text{term}}\) values were 0.94 ± 0.22 h\(^{-1}\) and 0.77 ± 0.21 h, respectively. These toxicokinetic parameters are listed in Table 1.

After i.v. administration of [\(^{14}\)C]1-NN, the majority of the radiolabel was eliminated in urine; 52% of the dose within 24 h. Cumulative excretion of total radioactivity in the urine and feces is shown in Fig. 2B. As with i.p. administration, nearly all of the i.v. administered dose was recovered in urine and feces at 96 h. Selected tissues, blood, and gastrointestinal contents retained little radioactivity 96 h after treatment (0.96%). At 96 h, only 0.53 and 0.14% of the administered dose remained in the liver and blood, respectively (data not shown).

Essentially, the entire dose was recovered in urine and feces, with the total recovery of the administered radioactivity (at 96 h) in the urine, feces, selected tissues, blood, and gastrointestinal contents being 94%.

Bile Study. The significant amount of radioactivity recovered in the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Kinetic Parameter</th>
<th>i.p.</th>
<th>i.v.</th>
</tr>
</thead>
<tbody>
<tr>
<td>k(_{\text{term}}) (h(^{-1}))</td>
<td>1.55 ± 0.30</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>T(_{1/2,\text{term}}) (h)</td>
<td>0.46 ± 0.08</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>k(_{\text{lim}}) (h(^{-1}))</td>
<td>0.21 ± 0.04</td>
<td>0.94 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>T(_{1/2,\text{lim}}) (h)</td>
<td>3.40 ± 0.63</td>
<td>0.77 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>AUC (mg h/l)</td>
<td>25.93 ± 8.10</td>
<td>3.85 ± 2.21</td>
<td></td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>2.59 ± 0.00</td>
<td>3.11 ± 1.36</td>
<td></td>
</tr>
<tr>
<td>V(_{\text{dis}}) (l/kg)</td>
<td>n/a</td>
<td>3.17 ± 0.82</td>
<td></td>
</tr>
<tr>
<td>V(_{\text{ss}}) (l/kg)</td>
<td>n/a</td>
<td>2.79 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.67 ± 0.21</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>T(_{\text{max}}) (h)</td>
<td>1.52 ± 0.19</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>C(_{\text{max}}) (μg/ml)</td>
<td>3.94 ± 1.34</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

* n/a, not applicable.
Fig. 2. Cumulative recovery of total radioactivity in urine (■), feces (●), and expired [14C]organics and [14C]CO₂ (●) after i.p. (100 mg/kg; 60 µCi/kg) (A) and i.v. (10 mg/kg; 120 µCi/kg) (B) administration of [14C]1-NN to male Sprague-Dawley rats.

C, cumulative recovery of total radioactivity in bile of two individual rats [rat 1 (■) and rat 2 (●)] after i.v. (10 mg/kg; 120 µCi/kg) administration of [14C]1-NN to male Sprague-Dawley rats. A and B, data expressed as mean percentage of dose ± S.D. (N = 3 per time point for both the i.p. and i.v. studies). C, data expressed as percentage of dose for two individual rats.
feces following both routes of administration indicated that biliary secretion would be an important route of elimination of 1-NN metabolites. This was confirmed when 88% (N = 2) of the administered i.v. bolus dose was secreted in bile over an 8-h period (Fig. 2C). This amount exceeded the percentage of dose recovered in feces (33% of the dose between 0 and 24 h).

Determination of Urinary Metabolites. HPLC analysis of pooled urine from rats treated either i.p. or i.v. with [14C]1-NN revealed major radioactive peaks and similar metabolite profiles (Fig. 3, A and B, respectively). A quantitative summary of each urinary metabolite at 96 h after each route of administration is shown in Table 2.

Peak I (retention time (R_T): 51 min) was determined to be 1-NN. This peak had the identical HPLC and GC R_T and electron ionization mass spectrum with a molecular ion at m/z 173 as the 1-NN standard. The signal at m/z 127 resulted from the loss of NO_2, and the signal at m/z 115 was assigned to a structure resulting from the loss of NO_2 and one carbon (Table 3).

The major urinary metabolite (D, R_T: 29 min), regardless of route of administration, was identified as N-acetyl-S-(hydroxy-1-nitrodihydronaphthalene)-L-cysteine (Fig. 4 and Table 4). The deprotonated molecule (MH^-, m/z 351) of this metabolite was subjected to CID within a tandem mass spectrometer. The resulting product ion spectrum contained signals at m/z 333, 204, and 162. In this spectrum, a signal was observed at m/z 162, which is characteristic of an
N-acetyl-L-cysteine fragment. Another product ion signal at \( m/z \) 333 resulted from the loss of water. The major product ion signal at \( m/z \) 204 most likely resulted from the loss of water and a portion of the N-acetyl-L-cysteine moiety fragmented at the methylene-sulfur bond.

Metabolites A (R_T: 19 min) and B (R_T: 21 min) were determined to be dihydroxy-1-nitro-dihyronaphthalene-O-glucuronide and hydroxy-1-nitronaphthalene-O-glucuronide (Table 4). The CID-MS-MS spectra of these metabolites contained product ion signals at \( m/z \) 174 and 176, which represent glucuronide fragments. Their presence was confirmed by enzymatic hydrolysis of the conjugates with \( \beta \)-glucuronidase (data not shown). In this assay, peaks A and B disappeared in the urine sample treated with \( \beta \)-glucuronidase and peaks H and E increased by the same amount, respectively.

Metabolite C (R_T: 25 min) was identified as dihydroxy-1-nitro-dihyronaphthalene-O-sulfate by liquid chromatography (LC)-MS-MS (Table 4). The resulting CID production ion spectrum of the \( MH^+ \) ion at \( m/z \) 286 contained signals at \( m/z \) 97 and 188. The major product ion signal observed at \( m/z \) 97 resulted from the O-sulfate, which is a diagnostic signal for sulfates. The signal observed at \( m/z \) 188 resulted from the loss of O-sulfate. The presence of this sulfate metabolite was confirmed by enzymatic hydrolysis of the sulfate conjugate with sulfatase (data not shown). Peak C in the sulfatase-treated urine sample disappeared and peak H increased by the same amount.

Metabolite H (R_T: 48 min) was determined to be dihydroxy-1-nitro-dihyronaphthalene (Table 4). The resulting CID-MS-MS spectrum of the \( MH^+ \) ion at \( m/z \) 206 contained signals observed at \( m/z \) 166 and 59. The major production was at \( m/z \) 166 was assigned to a structure resulting from the loss of a portion of the naphthalene ring system. Another signal was observed at \( m/z \) 59, which represented the fragment of the two hydroxyl moieties and a portion of the naphthalene ring system.

Urinary metabolite E (R_T: 31 min) was identified as hydroxy-1-nitronaphthalene by LC-MS-MS and GC-MS. Important evidence for this assignment is found in the electrospray ionization (ESI)-LC-CID production ion spectrum of the \( MH^+ \) ion at \( m/z \) 188 (Table 4). The product ion spectrum contained signals at \( m/z \) 158 and 142, which resulted from the loss of NO and NO_2, respectively. Another signal was observed at \( m/z \) 46, which represented the NO_2 fragment.

The electron ionization mass spectrum resulting from urinary metabolite E contained a signal at \( m/z \) 189 (Table 3). The signal in the same spectrum at \( m/z \) 131 was assigned to a structure resulting from the loss of NO_2 and one carbon. The signal at \( m/z \) 115 was due to the loss of NO_2 and ring collapse releasing neutral CO. The product ion spectrum of \( MH^+ \) ion at \( m/z \) 188 and the electron ionization spectrum at \( m/z \) 189 provide evidence for this urinary metabolite.

Metabolite F (R_T: 37 min) was determined to be dihydroxy-1-nitronaphthalene based on the CID-MS-MS spectrum (Table 4). The protonated molecule (\( MH^+ ; m/z \) 206) produced, on collision, a product ion spectrum containing a major signal at \( m/z \) 106. This signal represented the NO_2, a portion of the rings, and hydroxyl moiety fragment.

Metabolite G (R_T: 44 min) was determined to be 1-naphthylamine. This peak had the same HPLC and GC R_T and electron ionization spectrum with a major signal at \( m/z \) 143 as the 1-naphthylamine standard. The signal at \( m/z \) 115 was assigned to a structure resulting from the loss of NH_3 and one carbon (Table 3).

Three minor metabolites were not identified by either LC-MS-MS

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**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>HPLC R_T</th>
<th>Peak Identification</th>
<th>% of Total Dose in Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>m/z</td>
<td></td>
</tr>
<tr>
<td>Dihydroxy-1-nitro-dihyronaphthalene-O-glucuronide</td>
<td>19</td>
<td>A</td>
<td>2.4</td>
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<tr>
<td>Hydroxy-1-nitronaphthalene-O-glucuronide</td>
<td>21</td>
<td>B</td>
<td>3.0</td>
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<tr>
<td>Dihydroxy-1-nitro-dihyronaphthalene-O-sulfate</td>
<td>25</td>
<td>C</td>
<td>5.3</td>
</tr>
<tr>
<td>N-acetyl-S-(hydroxy-1-nitro-dihyronaphthalene)-L-cysteine</td>
<td>29</td>
<td>D</td>
<td>29.6</td>
</tr>
<tr>
<td>Hydroxy-1-nitronaphthalene</td>
<td>31</td>
<td>E</td>
<td>0.1</td>
</tr>
<tr>
<td>Dihydroxy-1-nitronaphthalene</td>
<td>37</td>
<td>F</td>
<td>4.1</td>
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<tr>
<td>1-Naphthylamine</td>
<td>44</td>
<td>G</td>
<td>0.4</td>
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<tr>
<td>Dihydroxy-1-nitro-dihyronaphthalene</td>
<td>48</td>
<td>H</td>
<td>2.2</td>
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<tr>
<td>1-Nitronaphthalene</td>
<td>51</td>
<td>I</td>
<td>6.9</td>
</tr>
<tr>
<td>Other</td>
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<td></td>
<td>7.4</td>
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<tr>
<td>Total Dose in Urine (0–96 h)</td>
<td>60.5%</td>
<td>56.4%</td>
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* Average reversed-phase HPLC retention time.
* Numbers in parentheses, relative abundance (percentage of highest peak).

---

**TABLE 3**

<table>
<thead>
<tr>
<th>Peak Identification</th>
<th>HPLC R_T</th>
<th>Molecular Ion</th>
<th>Fragmentation Ions</th>
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<tbody>
<tr>
<td></td>
<td>min</td>
<td>m/z</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>31</td>
<td>189 (52)*</td>
<td>159 (15), 131 (50), 115 (100), 89 (12), 77 (8), 63 (8)</td>
</tr>
<tr>
<td>G</td>
<td>44</td>
<td>143 (100)</td>
<td>115 (49), 89 (8), 63 (6)</td>
</tr>
<tr>
<td>I</td>
<td>51</td>
<td>173 (50)</td>
<td>143 (28), 127 (90), 115 (100), 101 (17), 89 (9), 77 (22), 63 (12)</td>
</tr>
</tbody>
</table>

* See Fig. 3.
* Average reversed-phase HPLC retention time.
* Numbers in parentheses, relative abundance (percentage of highest peak).
These represented between 7 and 12% of the i.p. or i.v. dose, respectively.

**Determination of Biliary Metabolites.** HPLC analysis of bile from rats treated i.v. with [14C]1-NN revealed major radioactive peaks (Fig. 3C). A representative radiochromatogram of pooled bile samples compared with urine samples showed similar but not identical metabolite profiles.

The identity of the major biliary metabolite (X, R_T: 25 min) was hydroxy-glutathionyl-1-nitro-dihydronaphthalene (Fig. 5 and Table 4). This is based on the mass of the MH⁻ ion and the product ion spectrum generated by CID. In this spectrum, a signal was observed at m/z 477 resulting from the loss of water. Another product ion signal was observed at m/z 272, which resulted from a portion of the glutathione conjugate fragmented at the methylene-sulfur bond. Other biliary metabolites were putatively identified by R_T coelution with the identified urinary metabolites (labeled A–I).

**Discussion**

The blood concentration of 1-NN could be fitted to a two-compartment model after i.v. administration, with the initial distribution phase essentially completed by 15 min. The blood concentration of 1-NN at the end of the first sampling point (1 min) was only 0.12 mg, or 5% of the 10 mg/kg administered dose. The relatively large apparent volume of distribution under steady-state conditions (2.79 l/kg) indicated the extensive movement of 1-NN out of the blood. After the distribution of 1-NN into the tissues, the subsequent decline in blood concentration was due to elimination of 1-NN by metabolic and excretory processes. The CL of 11 ml/min for 1-NN correlated with
the reported hepatic blood flow of 13 to 20 ml/min in the rat, suggesting efficient hepatic clearance of 1-NN (Derelanko and Hollinger, 1995).

After i.p. administration, the blood concentration–time curves of 1-NN were substantially different from those obtained after i.v. administration. A substantially longer terminal phase half-life with an extended systemic exposure indicated a slow rate of absorption of 1-NN from the peritoneum. Furthermore, the blood concentration of 1-NN could be fitted to a one-compartment toxicokinetic model. The prolonged absorption of 1-NN is probably attributed to the lipophilic nature of the peanut oil vehicle, which appears to act as a depot for 1-NN, and substantially slows its absorption. The absolute F of the i.p. dose was 0.67. Several factors probably influence the systemic exposure of 1-NN, including its hepatic metabolism during first pass, presystemic biliary elimination, the completeness of absorption from the peritoneal cavity, as well as the enterohepatic recirculation of parent 1-NN.

After both i.p. and i.v. administration of [14C]1-NN, the majority of the radiolabel was excreted into the urine. Although the bulk of the radiolabeled was associated with metabolites of 1-NN, a measurable amount of parent 1-NN (2–7% of the total dose) was cleared from the systemic circulation by renal elimination. The feces represented a secondary route of excretion, resulting from biliary elimination of metabolites, as well as intact 1-NN. Thus, regardless of the route of administration, 1-NN is primarily cleared from the systemic circulation by metabolism and the majority of its metabolites are ultimately excreted into the urine. However, a substantial portion of the radiolabeled dose undergoes enterohepatic recirculation before excretion in the urine. From the bile cannulation experiments, it appears that the majority of the conjugated metabolites eliminated into the bile undergo further hydrolysis. These products are subsequently reabsorbed, further processed, and excreted in the urine. The bulk export of 1-NN metabolites in the bile may represent a mechanism by which 1-NN causes bile ductule cell injury.

When administered systemically to rats, 1-NN causes acute bronchiolar epithelial injury in the lung, as well as parenchymal and bile duct epithelial cell injury in the liver (Johnson et al., 1984; Sauer et al., 1995). However, the Clara cells in the lung appear to be the primary target cell for 1-NN in the rat, and this cell-specific toxicity appears to be associated with the localization of cytochrome P-450 isoforms. Previous studies have indicated that oxidative metabolism of 1-NN is essential in the hepatic and pulmonary toxicity of 1-NN. In vitro studies with lung microsomes, lung slices, and isolated lung cells have shown that 1-NN is metabolized by cytochrome P-450 isoforms via an oxidative pathway that results in macromolecular binding (Rasmussen, 1986; Price et al., 1995). In addition, Verschoyle and Dinsdale (1990) have shown 1-NN toxicity to be correlated with cytochrome P-450 2B1 activity in the lung with inhibitors of specific cytochrome P-450 isoforms. In the noninduced rat liver, it appears that 1-NN is bioactivated by cytochrome P-450 1A1 and 1A2 (Verschoyle et al., 1993). However, in rats pretreated with phenobarbital, bioactivation by the liver is associated with cytochrome P-450 2B1. Induction of this isoform in the liver most likely explains the shift in target organ toxicity because the liver becomes the target tissue (Johnson et al., 1984).

From the metabolic data presented herein, it appears that the 1-NN-induced toxicity is probably mediated by the formation of a reactive epoxide intermediate (Fig. 6). The formation of such a reactive intermediate can result in covalent binding to tissue macromolecules and tissue injury. Although not directly identified, the formation of 1-nitronaphthalene-oxide has been ascertained from its detoxification products. The primary biliary metabolite after 1-NN administration, hydroxy-glutathionyl-1-nitro-dihydronaphthalene (metabolite X), probably results from the nucleophilic attack of glutathione on 1-ni-
This metabolite is further modified and ultimately appears in the urine as N-acetyl-S-(hydroxy-1-nitro-dihydronaphthalene)-L-cysteine (metabolite D). Spontaneous or enzymatic hydrolysis of 1-nitronaphthalene-oxide would result in dihydroxy-1-nitro-dihydronaphthalene, a metabolite found in the urine (metabolite H). Watt et al. (1998, 1999) incubated liver and lung microsomes with 1-NN and identified the major metabolites as 1-nitro-5,6-dihydroxy-dihydronaphthalene, 1-nitro-5-glutathionyl-6-hydroxy-5,6-dihydronaphthalene, and 1-nitro-5-hydroxy-6-glutathionyl-5,6-dihydronaphthalene by NMR and MS. Thus, it appears that 1-NN is bioactivated by cytochrome P-450, resulting in the formation of an epoxide intermediate that is either detoxified by glutathione conjugation or epoxide hydrolase.

After both i.v. and i.p. administration, 1-naphthylamine was detected in the urine. Because a small amount of 1-NN was excreted into the bile, 1-naphthylamine most likely was formed as a result of nitro-reduction in the gut (Poirier and Weisburger, 1974; Johnson and Cornish, 1978). This metabolite also may contribute to 1-NN-induced hepatotoxicity. Amine-containing compounds formed as a result of reduction by intestinal microflora can covalently bind to hepatic macromolecules after reabsorption (El-Bayoumy and Hecht, 1982). Bioactivation of 1-naphthylamine via oxidation of the aromatic rings or the amino group could result in a reactive intermediate capable of binding to macromolecules. Thus, the covalent binding of resorbed amine-containing metabolites, as well as reactive intermediates formed by bioactivation of 1-NN and 1-naphthylamine, may all play a role in the pulmonary and hepatic toxicity observed after 1-NN administration in the rat.

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References


References


