ISOLATION AND IDENTIFICATION OF URINARY METABOLITES OF PORFIROMYCIN IN DOGS AND HUMANS

WENSHENG LANG, JOHN MAO, TERRENCE W. DOYLE, AND BIJAN ALMASSIAN

Department of Development, Vion Pharmaceuticals, Inc., New Haven, Connecticut

(Received December 7, 1999; accepted April 19, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

Porfiromycin (PM), a bioreductive alkylating agent, is currently under development for the treatment of head and neck cancers as an adjunct to radiation therapy in phase III clinical trials. After i.v. administration of a single dose of PM to patients at 40 mg/m², urinary metabolites were isolated by HPLC and identified by atmospheric pressure chemical ionization mass spectrometry. In dogs, [methyl-³H]PM was administered i.v. to three Beagle dogs at a single dose of 2 mg/kg. Urinary excretion of radioactivity and PM at different times was determined by liquid scintillation counting and by HPLC, respectively. An average of 48.0% of total radioactivity given to the dogs was cumulatively excreted in urine over a period of 7 days. Unchanged parent drug excreted in urine accounted for 10.8% of the administered dose over the same period of time. The results indicated that the majority of excreted dose in dog urine was in the form of metabolites. Three phase I and four phase II metabolites of PM were identified in human and dog urine. The phase I metabolites are 2-methylamino-7-aminomitosene, 1,2-cis and 1,2-trans-1-hydroxy-2-methylamino-7-aminomitosenes. The phase II metabolites are a pair of isomeric N-acetylcysteine-S-conjugates and a pair of isomeric cysteine S-conjugates of mitosenes at the C-1 and C-10 positions. Most of the identified metabolites were confirmed by comparison with synthetic reference standards using HPLC and liquid chromatography/mass spectrometry (LC/MS). The identification of mercapturic acids and cysteine S-conjugates in urine indicates that the metabolism of PM may be through GSH conjugation.

Porfiromycin (PM, Fig. 1), the aziridine N-methyl analog of mitomycin C (MC), is a bioreductive alkylating agent that has been shown to be preferentially cytotoxic to hypoxic tumor cells in vitro and in vivo (Iyer and Szybalski, 1964; Keyes et al., 1985; Fracasso and Sartorelli, 1986; Marshall and Rauth, 1988; Rockwell et al., 1993). Interestingly, PM generates a greater differential toxicity to hypoxic EMT6 and Chinese hamster ovary tumor cells versus their aerobic counterparts than that of MC (Rockwell et al., 1988; Belcourt et al., 1996). Furthermore, human phase I and phase II clinical trials have demonstrated that PM is tolerated at a dose 3-fold higher than that of MC (Foley et al., 1967; Loo et al., 1967; Izicki et al., 1972; Grage et al., 1975; Baker et al., 1976). The findings indicate that PM is potentially superior to MC in clinical applications for the treatment of solid tumors. Currently, PM is under development for the treatment of head and neck cancers as an adjunct to radiation therapy in phase III clinical trials.

In vitro metabolism and bioreductive activation of MC under hypoxic conditions have been extensively studied (Schwartz, 1962; Tomasz and Lipman, 1981; Kennedy et al., 1982; Pan et al., 1984). The enzymes responsible for the reductive metabolism of MC are NADPH:cytochrome c (P-450) reductase (Keyes et al., 1984; Pan et al., 1986), xanthine oxidase (Pan et al., 1984, 1986), xanthine dehydrogenase (Gustafson and Pritsos, 1992), DT-diaphorase (Siegal et al., 1990; Pan et al., 1992, 1995; Robertson et al., 1992, 1995; Beall et al., 1994), and NADH:cytochrome b₅ reductase (Hodnick and Sartorelli, 1993). In contrast, the information on the metabolism and disposition of PM is limited. To date, three metabolites of PM resulting from biotransformation at the C-1 position in the presence of purified NADPH:cytochrome c reductase and xanthine oxidase have been identified (Pan and Iracki, 1988; Pan, 1990). Recently, we reported the identification of several C-1 and C-10 metabolites of PM arising from a rat liver preparation under aerobic conditions (Lang et al., 2000). However, the in vivo metabolism and disposition of PM under physiological conditions are still unclear.

To understand the metabolic fate of PM, we conducted the studies on the metabolism and disposition of PM in dogs and humans. In this article, we present: 1) urinary and fecal excretion of radioactivity derived from [methyl-³H]PM in dogs; 2) isolation and identification of urinary metabolites of PM in dogs and humans; 3) comparison of urinary metabolite profiles between the two species; 4) chemical syntheses of the major urinary metabolites; and 5) postulation of metabolic pathways of PM on the basis of the metabolite profiles.

Experimental Procedures

Materials. Porfiromycin injection, 15 mg, (Promycin) was manufactured at Chesapeake Biological Lab., Inc. (Owings Mills, MD). [Methyl-³H]PM (1.4 mg/ml of ethanol), specific activity 235 mCi/mmol, was purchased from Moravek Biochemicals, Inc. (Brea, CA). Methanol and potassium phosphate monobasic were of HPLC grade, and were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific Co. (Pittsburgh, PA), respectively.

Send reprint requests to: Bijan Almassian, Ph.D., Department of Development, Vion Pharmaceuticals, Inc., Four Science Park, New Haven, CT 06511.

E-mail: balmassi@vionpharm.com
Ammonium acetate, potassium hydroxide, and 37% hydrochloric acid were of American Chemical Society reagents and were purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Tris(hydroxymethyl)-aminomethane, 1-cysteine, and N-acetyl-l-cysteine were purchased from Aldrich Chemical Co. Milli-Q Plus ultra pure water was used throughout the study.

**Animals, Dosing, and Sample Collection.** Three adult male Beagle dogs (Marshall Laboratories of North Rose, NY), weighing from 8.4 to 9.2 kg, were used in this study. A dosing solution was prepared by dissolving six vials of porfirocinoinjection (15 mg) and [methyl-1H]PM (0.7 mg) into a final volume of 45 ml of water to produce a 2 mg/ml [methyl-1H]PM solution (specific activity 5.46 μCi/ml). The dogs were given a single i.v. dose of PM dosing solution at 2 mg/kg (~40 mg/ml) and housed individually in metabolism cages with access to water and food. Urine and feces of the test animals were collected at 0 to 4, 4 to 8, and 8 to 24 h, and at 24-h intervals thereafter for 7 days. The samples were stored immediately at −20°C.

**Human Urine Sample.** A human urine sample obtained from a patient who received a PM radiation therapy trial at Yale New Haven Hospital is presented in this article. The patient was given a single i.v. dose of PM at 40 mg²/m. The urine sample was collected at 0 to 2.5 h after the dose and stored at −80°C until used.

**Urine Sample Preparation.** After thawing, each 10-ml urine sample was filtered through a 0.45-μm nylon filter. The filtrate was loaded onto two Waters Sep-Pak Plus C18 cartridges connected in tandem (sorbent weight of 360 mg each). The cartridge set, preconditioned with 5 ml of methanol and 10 ml of water sequentially, was washed with 10 ml of water, and then eluted with 5 ml of methanol/water (2:1, v/v). The methanolic effluent was collected and concentrated under a stream of nitrogen. Aliquots of 100 μl of the resulting solution were repeatedly injected onto HPLC for isolation.

**HPLC.** A Hewlett-Packard 1100 liquid chromatography system (Wilming-
ton, DE) was used for isolation of urinary metabolites and the determination of PM in dog urine. The LC system consisted of a quaternary pump G1311A, an autosampler G1313A, a diode array detector G1315A, and a column thermo-
stat G1316A. Hewlett-Packard LC3D ChemStation software was used for system control and data processing. HPLC isolation and analysis were carried out on a Supelcosil LC-18 column, 5 μm, 150 × 4.6 mm (Supelco, Bellefonte, PA) at 40°C. Detection wavelength was set at 550 nm for the isolation of metabolites and at 360 nm for the quantitation of PM. The mobile phases were methanol (A) and 50 mM potassium phosphate buffer, pH 7.0. The solution was deaerated by bubbling He gas for 5 min, then 2.1 mg of platinum oxide was added. The reaction was carried out by bubbling H2 gas at 60–65°C for 2 h. The reaction mixture was then exposed to air, filtered, and concentrated in vacuo. The product was purified by reverse-phase high performance liquid chromatography (RP-HPLC). Electrospray ionization-mass spectrometry (ESI-MS) was used to detect and identify the compounds. The HPLC system was equipped with a diode array detector (model FC204; Gilson, Middleton, WI) at 0.25 ml/vial intervals. Fractions containing the product were purified by C18 cartridges to remove salt.

**LC/MS Analysis.** Mass spectra of metabolites were obtained on a Naviga-
tron single quadrupole mass spectrometer equipped with an atmospheric press-
ure chemical ionization (APCI) probe (Finnigan, San Jose, CA). An HP 1100 LC system was interfaced to the MS detector via a heated nebulizer. Before analysis of metabolites, instrumental parameters were optimized for detection and fragmentation via infusion of 2 μg/ml PM in methanol/water (1:1, v/v) at a speed of 10 μl/min. The APCI source was operated at 150°C with the corona pin voltage of 2.6 kV and cone voltage of 10 to 30 V. The flow rate of drying gas (N2) was 5 liter/min. The nebulizer coil temperature was set at 550°C. MS spectra were acquired over m/z 100 to 800 at 2.5 s/scan in the positive ion mode. HPLC was performed on a BDS Hypersil C8 column, 3 μm, 100 × 2 mm, (Keystone Scientific, Bellefonte, PA) at 40°C. An isotropic elution was carried out with 15% (v/v) methanol in an aqueous 20 mM ammonium acetate solution (pH 6.1) at a flow rate of 0.3 ml/min.

**Liquid Scintillation Counting.** Total radioactivity in dog urine was as-
sayed directly on a LC 6000TA or a LS 6000IC Liquid Scintillation Counter (Beckman, Fullerton, CA) in 5 ml of Ready Value cocktail (Beckman, Palo Alto, CA). All urine samples were counted in duplicate to a statistical accuracy of 2% or for a maximum of 10 min. For comparison of the radioactivity profiles of HPLC with those by UV detection for dog urine, the effluent collected from HPLC at 0.25 ml/vial was counted after mixing with 3 ml of ScintiSafe Gel cocktail (Fisher Scientific, Pittsburgh, PA).

**1H NMR Analysis.** 1H NMR spectra were recorded in deuterated methanol (CD3OD) were recorded with a Bruker AC300 NMR spectrometer at 300 MHz. Either internal tetramethylsilane or residual methanol signal was used as a chemical shift reference. The following terminology is used: δ, chemical shift in parts per million; s, singlet; d, doublet; dd, doublet of doubles; q, quartet; m, multiplet; and J, coupling constant in hertz.

**Chemical Syntheses of Metabolites.** 10-Decarbomethyl-2-methylamino-10-
(t-cysteine-S-yl)-7-aminitomosene (M1). L-Cysteine (17.8 mg) and PM (5.1 mg) were dissolved in 2.0 ml of an aqueous 0.1 M Tris/160 HCl buffer, pH 7.0. The solution was deaerated by bubbling He gas for 5 min, then 2.1 mg of platinum oxide was added. Hydrogenation proceeded by bubbling H2 gas at 60–65°C for 2 h. The reaction mixture was then exposed to air, filtered, and concentrated in vacuo. The product was purified by reversed-phase high performance liquid chromatography (RP-HPLC). The effluent was collected with a Gilson fraction collector. Fractions containing the same product were pooled and concentrated in vacuo. The final solution of each product was purified using Sep-Pak Plus C18 cartridges to remove salt. The yield was 18% for M2 and 50% for M4. M2—ESI-MS: m/z (intensity %) 379 (MH, 100), 258 (56); 1H NMR (methanol-d4): δ 1.78 (s, C(6)CH3), 2.42 (s, NCH3), 2.72 (dd, J = 16.4, 4.8 Hz, C(1)H2), 2.77 (dd, J = 14.4, 9.9 Hz, C(1)H3), 3.14 (dd, J = 14.5, 3.7 Hz, C(1)H1), 3.18 (dd, J = 16.5, 7.3 Hz, C(1)H2), 3.75 (dd, J = 9.7, 3.7 Hz, C(2)H), 3.83 (1/2ABq, J = 13.3, C(10)H), 3.94 (m, C(2)H2), 3.96 (1/2ABq, J = 13.4, C(10)H), 4.02 (dd, J = 13.1, 4.6 Hz, C(3)H3), and 4.37 (dd, J = 12.1, 6.7 Hz, C(3)H2).

1.2-trans and 1.2-cis-1-Hydroxy-2-methylamino-7-aminitomosene (M2 and M4). PM (14.0 mg) was dissolved into 10 ml of an aqueous 0.05 N HCl solution and stirred at room temperature for 3 h. The reaction solution was neutralized with a saturated aqueous NaHCO3 solution and concentrated in vacuo. The crude diastereomers (M2 and M4) were separated and purified by semi-preparative HPLC. The purification was performed on a Ultragard ODS (30) column, 5 μm, 250 × 10 mm, at 30°C. The column was eluted with methanol/50 mM KH2PO4, pH 6.0, (3:7, v/v) at a flow rate of 2.0 ml/min. The effluent was collected with a Gilson fraction collector. Fractions containing the same product were pooled and concentrated in vacuo. The final solution of each product was purified using Sep-Pak Plus C18 cartridges to remove salt.

**Chemical Structure of Metabolites.** The structure of the metabolites was determined by 1H NMR analysis. The N-acyl-l-cysteine-derived metabolites were assigned by comparing their NMR spectra with those of the precursor. The chemical shifts and coupling constants of the metabolites are given in Table 1. The structure of the metabolite was confirmed by 2D NMR spectroscopy, specifically 1H-1H COSY and HSQC experiments. The assignments were based on the comparison of the NMR spectra of the metabolites with those of the precursor. The assignments were confirmed by 2D NMR spectroscopy, specifically 1H-1H COSY and HSQC experiments. The assignments were based on the comparison of the NMR spectra of the metabolites with those of the precursor.
and H NMR. The synthetic compounds were used for structural solution as described by Hong and Kohn (1991). The attempt to a single step by acidic activation of PM in an aqueous 0.05 N HCl diastereomeric 1-hydroxymitosenes (M2 and M4) were synthesized in catalytic hydrogenation under a mildly acidic condition (pH 4.55). functionality of PM due to its thermal lability. M6 was prepared by platinum oxide. The procedures for synthesis of M1 and M5, in which prepared under a reductive condition using catalytic hydrogenation by M6 were synthesized in analytical or semipreparative scales using PM eluted with MeOH/50 mM KH2PO4, pH 7.30 (45:55, v/v) at a flow rate of 2.0 ml/min. ESI-MS: 

\[ m/z \] 1.78 (s, C(6)CH3), 2.40 (s, NCH3), 2.71 (dd, 16.5, 7.3 Hz, C(1)H2), 3.17 (dd, J = 16.5, 4.8 Hz, C(1)H2), 3.90 (m, C(2)H2), 3.98 (dd, J = 12.9, 4.7 Hz, C(3)H2), 4.37 (dd, J = 12.8, 6.7 Hz, C(5)H2), 5.14 (s, C(10)H2).

Results

Chemical Syntheses. Urinary metabolites M1, M2, M4, M5, and M6 were synthesized in analytical or semipreparative scales using PM as a starting material. Of these compounds, M1, M5, and M6 were prepared under a reductive condition using catalytic hydrogenation by platinum oxide. The procedures for synthesis of M1 and M5, in which an S-nucleophile displaced the C-10 carbamate moiety of PM at 50–65°C, were novel and efficient. The higher temperatures (50–65°C) were applied to facilitate the removal and activation of the C-10 functionality of PM due to its thermal lability. M6 was prepared by catalytic hydrogenation under a mildly acidic condition (pH 4.55). Tomasz and Lipman (1981) have previously described the mechanism of the formation of M6 via an internal redox reaction. A pair of diastereomeric 1-hydroxymitosenes (M2 and M4) were synthesized in a single step by acidic activation of PM in an aqueous 0.05 N HCl solution as described by Hong and Kohn (1991). The attempt to synthesize metabolites M3 and M7 was unsuccessful. All final products were purified by RP-HPLC and characterized by UV-Vis, MS, and 1H NMR. The synthetic compounds were used for structural confirmation of the metabolites identified in urine by comparison of their HPLC retention times (Rds) and MS fragmentation patterns.

Urinary Excretion in Dogs. Cumulative urinary excretions of radioactivity derived from [methyl-3H]PM and the recoveries of PM in three Beagle dogs after a single i.v. dose over 7 days are given in Table 1. An average of 48.0% of total radioactivity given to the dogs accounted for 10.8% of the administered dose over 7 days. The findings indicated that the majority of excreted dose in dog urine was in the form of metabolites, and urinary excretion of the metabolites was rapid and intensive in dogs.

Identification of Urinary Metabolites. Representative HPLC profiles showing human and dog urinary metabolites are given in Fig. 2. PM and its metabolites were selectively detected at 550 nm due to their characteristic indoloquinone chromophores. Therefore, PM metabolites could be readily differentiated from other complex components present in urine matrices. A total of seven major metabolites M1 to M7 were observed in dog urine (Fig. 2A), and a similar HPLC pattern was obtained for dog urine (Fig. 2B). A major peak M1 at Rf 12.70 min observed in both human and dog urine had a typical mitosene UV absorption pattern (\( \lambda_{max} \) at 250, 310, and 550 nm). The APC1 mass spectrum of M1 showed a cluster of molecular ions at m/z 379 (M+H)+, 401 (M+Na)+, and 417 (M+K)+ in Fig. 3A. The observation of two fragment ions at m/z 336 (M–CO2)+ and 317 (MH–COOH–NH2)+ suggested the existence of carboxylic acid and amino groups in the molecule. A base peak at m/z 292 (MH–C(NH2)COOH)+ and two related fragment ions at m/z 260 (MH–HSCH=C(NH2)COOH)+ and 258 (MH–cysteine)+ indicated that M1 was a cysteine conjugate. Further confirmation of the structure of M1 was obtained by comparison with our synthetic standards. It was found that the HPLC Rf and MS fragmentation pattern of M1 matched those of 10-decarbamoyl-2-methylamino-10-(l-cysteine-S-yl)-7-aminomitosene. The findings supported our structural assignment of M1.

Metabolites M2 and M4 appeared at \( R_f \) 13.28 and 14.65 min, respectively, by HPLC analysis. Identical UV spectra and similar mass spectra were obtained for the two compounds, indicating that the two metabolites are isomers. The APC1-mass spectra showed a cluster of molecular ions at m/z 335 (M+H)+, 357 (M+Na)+, and 373 (M+K)+ in Fig. 4. A series of fragment ions at m/z 288 (MHOC(O)NH2–H2O+MeOH)+, 274 (MHOC(O)O)NH2)+, 258 (MHOC(O)NH2–NH2), and 256 (MHOC(O)O)NH2–H2O) were also observed. The MS fragmentation pattern indicated that the two metabolites contained the C-10 carbamate group and a hydroxyl group. HPLC Rf and MS fragmentation patterns of M2 and M4 matched those of the reference standards of 1,2-trans and 1,2-cis-1-hydroxy-2-methylamino-7-aminomitosene, respectively. Thus, M2 was assigned as 1,2-trans-hydroxy-2-methylamino-7-aminomitosene and M4 as 1,2-cis-hydroxy-2-methylamino-7-aminomitosene.

### Table 1

Recoveries of radioactivity and PM in urine and feces after i.v. administration of a single dose of [3H]PM to three Beagle dogs at 2 mg/kg

<table>
<thead>
<tr>
<th>Time</th>
<th>Percentage of Total Radioactivity in Urine</th>
<th>Percentage of Total Radioactivity in Feces</th>
<th>Percentage of PM Recovered in Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–4</td>
<td>10.7 ± 0.4%</td>
<td>12.9 ± 1.2%</td>
<td>6.2 ± 0.4%</td>
</tr>
<tr>
<td>4–8</td>
<td>21.7 ± 18.0%</td>
<td>NA</td>
<td>3.4 ± 1.8</td>
</tr>
<tr>
<td>8–24</td>
<td>11.2 ± 15.4%</td>
<td>NA</td>
<td>1.2 ± 1.9</td>
</tr>
<tr>
<td>24–48</td>
<td>2.5 ± 0.3%</td>
<td>16.1 ± 13.2%</td>
<td>ND</td>
</tr>
<tr>
<td>48–72</td>
<td>0.7 ± 0.1%</td>
<td>1.2 ± 0.2%</td>
<td>ND</td>
</tr>
<tr>
<td>72–96</td>
<td>0.6 ± 0.1%</td>
<td>0.5 ± 0.3%</td>
<td>ND</td>
</tr>
<tr>
<td>96–120</td>
<td>0.5 ± 0.1%</td>
<td>0.6 ± 0.4%</td>
<td>ND</td>
</tr>
<tr>
<td>120–144</td>
<td>0.5 ± 0.2%</td>
<td>0.3 ± 0.1%</td>
<td>ND</td>
</tr>
<tr>
<td>144–168</td>
<td>0.5 ± 0.2%</td>
<td>0.2 ± 0.2%</td>
<td>ND</td>
</tr>
<tr>
<td>Total</td>
<td>48.0 ± 2.2%</td>
<td>31.8 ± 2.0%</td>
<td>10.8 ± 8.6</td>
</tr>
</tbody>
</table>

* NA, not applicable because no sample was collected for one dog during the period (n = 2).
  * ND, not detectable.

HPLC conditions are described in Experimental Procedures.
Metabolite M5 appeared at R_T 14.85 min in human urine (Fig. 2A) and gave a typical mitosene UV spectrum (λ_{max} at 250, 310, and 550 nm). The mass spectrum of M5 showed a cluster of molecular ions at m/z 421 (M+H)^{+}, 443 (M+Na)^{+}, and 459 (M+K)^{+} (data not shown). The major fragment ions of M5 at m/z 292 (MH-CH_2=C(NHAc)COOH)^{+}, 258 (MH-CH_2=C(NHAc)COOH-H_2S)^{+} were similar to those of M1 (Fig. 3A). On the basis of the MS data, we assigned M5 as a mercaptoacid derivative, in which an N-acetyl-cysteine residue conjugated at the C-10 position. The HPLC R_T and MS fragmentation pattern of M5 matched those of the synthetic compound of 10-decarbamoyl-2-methylamino-10-(N-acetyl-L-cysteine-S-yl)-7-aminomitosene (see structure in Fig. 5).

Metabolite M6 was observed at R_T 15.84 min in both human and dog urine. The mass spectrum of M6 showed a strong potassium adduct ion at m/z 357 and a sodium adduct peak at m/z 341 although the protonated molecular ion at m/z 319 was not observed (data not shown). Two fragment ions at m/z 275 (MH-CO_2H)^{+} and 258 (MH-HOCH(O)NH_3)^{+} corresponded to the losses of carbon dioxide and carboxylic acid, respectively. The MS fragmentation pattern, HPLC R_T, and UV spectrum of M6 were consistent with those of the reference standard of 2-methylamino-7-aminomitosene.

The mass spectrum of M7 showed a cluster of molecular ions at m/z 421 (M+H)^{+}, 443 (M+Na)^{+}, and 459 (M+K)^{+}. The major fragment ions appeared at m/z 292, 258, and 229 (Fig. 6), which were comparable with those of M5. Thus, M7 is believed to be an isomer of M5. The absence of the thermolabile fragment ion at m/z 260 for M7 indicated that the N-acetylcysteine moiety could be attached to the C-1 position. According to the mechanism of the formation of M1 and M3, M7 could be assigned as 10-decarbamoyl-2-methylamino-1-(N-acetyl-L-cysteine-S-yl)-7-aminomitosene (Fig. 5).

**Discussion**

Recently, we reported the identification of metabolites of PM formed in the presence of a rat liver preparation (Lang et al., 2000). Several C-1 and C-10 metabolites of PM were characterized as mitosene analogs in the in vitro system. The findings suggest that the formation of the metabolites is initiated by enzymatic activation of PM to a reactive intermediate with two reactive centers, which subsequently link with environmental nucleophiles in the aqueous medium. The current in vivo studies were our continuing effort to explore the metabolic fate of PM.

Tritium labeled to the N-methyl group of PM was used in the dog study. The isotope is not exchangeable with mobile protons in an aqueous medium. The potential loss of the label by demethylation catalyzed by demethylases has been previously investigated in P388 cell homogenates and liver microsomes (Pan, 1990). The results have demonstrated that PM is a poor substrate for the enzymes. So far, no demethyl metabolites of PM have been found. During the dog study, the effluents from HPLC column and Sep-Pak Plus C18 cartridges were collected, and were subjected to liquid scintillation counting for evaluation of the recoveries of radioactivity in each step. The radiochromatograms of the dog urine samples (data not shown) matched the metabolite profiles of HPLC monitored at 550 nm.

Three phase I metabolites (M2, M4, and M6) were identified in both human and dog urine. The findings are consistent with those previously obtained in vitro (Pan and Iracki, 1988; Lang et al., 2000). Most importantly, we found that M6 is a reactive metabolite that can be directly converted to cysteiny1 S-conjugates (M1 and M5) via
further biotransformation at the C-10 position (Fig. 5). The further metabolism of M6 is believed to be through the loss of the C-10 carbamate group to generate a positive charge, which is stabilized by the indole nitrogen (Iyer and Szybalski, 1964). The confirmation of the existence of such a metabolic pathway of M6 was conducted in vitro by direct incubation of M6 with L-cysteine to produce M1 under a reductive condition via catalytic hydrogenation (data not shown). No corresponding C-10 cysteine S-conjugates for 1-hydroxy-mitosenes (M2 and M4) were identified under the same conditions.

A pair of isomeric phase II metabolites (M1 and M3) as cysteine S-conjugates of mitosene analogs at C-1 and C-10 were identified in both human and dog urine, whereas the pair of metabolites (M5 and M7) as N-acetylcysteine S-conjugates (mercapturic acids) were only observed in human urine (Fig. 2). The formation of mercapturic acids in general is believed to be through N-acetylation of cysteine conjugates in the kidney. The reaction may be catalyzed by the cysteine conjugate N-acetyl-S-transferase (Jakoby, 1978; Elfarra and Anders, 1984). The observation of the differences in the formation of mercapturic acids between humans and dogs indicates that the enzymatic activity present in humans may be much higher than that in dogs. In addition, the identification of the phase II metabolites in urine suggests that GSH conjugation may be involved in the biotransformation of PM. As expected, the significant amount of total radioactivity (31.8%) was found in feces after i.v. administration of [methyl-H3]PM to three dogs (Table 1) because GSH conjugates are commonly excreted bile by a carrier-mediated transport system (Ketterer et al., 1983). Two cysteine S-conjugates of PM identified in both dog and human urine may be the products of subsequent metabolism of the GSH conjugates (Igwe, 1986). In summary, the identification of the urinary metabolites of PM linked to the thiol species in dog and human urine at the C-1 and C-10 positions is a novel finding. On the basis of the metabolite profiles, we propose the in vivo metabolic pathways of PM in Fig. 5. The identification of the urinary metabolites of PM is important to understand its in vivo metabolic fate and disposition of PM in dogs and humans.

Acknowledgements. We thank Dr. Roger Dinallo and Dr. James J.
Keirns of Boehringer Ingelheim Pharmaceuticals, Inc. for their efforts in LC/MS/MS analysis of dog urinary metabolites. We thank Dr. Bruce G. Haffty of Yale New Haven Hospital for providing human samples, and Xenobiotic Laboratories, Inc. for providing the dog urine samples. We also thank Scott Barrows for the HPLC quantitation of PM in dog urine samples.

References


