INTRODUCTION

PEN (3,5-dichloro-2,4-dimethoxy-6-(trichloromethyl)pyridine) is a highly substituted pyridine derivative synthesized by Dow Chemical Company and presently under evaluation by the National Cancer Institute as a potential antitumor agent. The mechanism of action of PEN is not known. PEN was selected for study because antitumor activity was observed against s.c. implanted mouse CD8F, mammary adenocarcinoma and human MX-1 mammary carcinoma implanted under the renal capsule following i.p. administration of drug (1). PEN was also active against i.p. P388 leukemia, but not against i.p. B16 melanoma, i.v. Lewis lung carcinoma, or s.c. colon adenocarcinoma 38 following i.p. administration (1); p.o. activity of PEN was observed in several s.c. implanted murine tumor models including advanced stage mouse CD8F; mammary adenocarcinoma, human MX-1 mammary carcinoma, advanced stage human MCF-7 and early stage mouse 16/C breast adenocarcinomas, and advanced stage human H-82 small cell lung carcinoma (1, 2). Therapeutic activity of PEN following p.o. administration was also assessed against several drug-resistant leukemia sublines. Cross-resistance was observed with sublines resistant to alkylating agents melphanal, cyclophosphamide, and Carmustine. Due to limited water solubility of PEN, a parenteral product consisting of an oil/lecithin/water emulsion was developed for i.v. administration of drug. Therapeutic activity of PEN against advanced s.c. or intrathecal MX-1 mammary carcinoma following i.v. administration of the parenteral product was similar to that observed following p.o. administration of the same parenteral product (2). Antitumor activity was not dependent upon schedule of administration (1, 2). While central nervous system side effects have been observed in mice (2), myelosuppression was the major dosing limiting toxicity of PEN in rats and dogs (3).

The goal of our work was to develop a HPLC assay for determination of PEN in biological fluids and to apply the assay to studies on the stability of PEN in biological fluids, to characterize murine pharmacokinetics, and to begin characterizing the in vitro and in vivo metabolism of PEN.

MATERIALS AND METHODS

PEN, [trichloromethyl-14C]PEN (24.5 mCi/mmol), and formulated PEN (7.5 mg/ml in a 10% oil/lecithin/water emulsion) were provided by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Pentachloropyridine was obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents were HPLC grade. Human tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD) and maintained as monolayer cultures in DMEM containing 10% fetal calf serum in an incubator at 37°C under 5% CO2 and 100% relative humidity.

HPLC Analysis of PEN. Reverse phase HPLC analyses were performed on a Hewlett-Packard 1090M Liquid Chromatograph equipped with a diode array detector. Separations were achieved on an IBM octadecyl column (250 x 4.5 mm inside diameter, 5 µm) and a Brownlee RP18 guard column (15 x 3.2 mm inside diameter, 7 µm). The mobile phase, consisting of acetonitrile/water (85/15), was delivered at a flow rate of 1.0 ml/min. PEN and the internal standard pentachloropyridine were detected by UV at A214 and A345, respectively.

For isolation of PEN, diethyl ether (4.0 ml) was added to buffer, plasma, or urine samples (0.5 ml) containing drug and internal standard, and shaken on a mechanical shaker (15 min). Following centrifugation (15 min x 1500 rpm), 2.5 ml of the organic layer were transferred to a conical centrifuge tube containing n-butanol (100 µl). The organic mixture was concentrated under a gentle stream of nitrogen until only 20 µl of this mixture were injected for HPLC analysis.

Standard curves were prepared by adding known amounts of PEN (3-300 µM) and internal standard (40 µM) to buffer, plasma, or urine. After analysis, drug/internal standard peak area ratios versus drug concentration data were fitted by linear least squares regression. Concentrations of PEN in unknown samples were calculated from the unknown/internal standard peak area ratios using the appropriate standard curve.

Growth Inhibition Assay. Growth-inhibitory activity of PEN was assessed by a colony formation inhibition assay. Log-phase tumor cells (500 cells/dish) were added to plastic cell culture dishes (Falcon 3002, 60 mm) containing DMEM with 10% fetal calf serum, and placed in an incubator (37°C, 5% CO2/95% air, 100% relative humidity) for 48 h. Medium was then removed from culture dishes by aspiration and replaced with fresh medium containing PEN. Following 1- or 48-h
incubation (37°C, 5% CO2/95% air, 100% relative humidity), the medium containing drug was removed and the dishes were washed twice with prewarmed DMEM. Fresh medium (3 ml) was added to each dish and the cells were incubated (37°C, 5% CO2/95% air, 100% relative humidity) for 7 days. Medium was removed by aspiration and the dishes washed twice with 1–2 ml prewarmed saline. Colonies were fixed and stained with 3 ml Coomassie blue reagent for 15 min. The dishes were rinsed with tap water and the colonies counted with the aid of a colony counter.

Each experiment included 3 dishes per concentration of drug and 6 control dishes, and experiments were repeated 2 to 3 times. Fifty % inhibitory concentration values were calculated from the experimental data as the concentration of drug required to cause 50% inhibition of colony formation by drug-treated cells compared to colony formation by untreated cells following 1- or 48-h exposure.

In Vitro Metabolism. Microsomes were obtained by differential centrifugation of freshly prepared liver homogenates from nontumored male CD2F1 mice (4). For some experiments, cytochrome P-450 enzymes were induced by pretreatment with phenobarbital (80 mg/kg/day i.p. x 3 days). Mice were sacrificed on the fourth day. PEN (0.5 mM) was incubated with microsomes (3 mg protein/ml) in the presence of an NADP-generating system containing NADP+ (0.4 mM), glucose-6-phosphate (23.6 mM), glucose-6-phosphate dehydrogenase (0.47 units/ml), magnesium chloride (5 mM), and pH 7.4 Tris buffer (50 mM). Protein content was determined by the Lowry (5) method. To detect the presence of formaldehyde in incubation mixtures, 0.5-ml aliquots were placed in 15-ml centrifuge tubes containing 0.17 ml of 15% ice cold trichloroacetic acid. Following centrifugation (10 min, 10,000 rpm), 0.5 ml of the supernatant was added to screw-capped tubes containing 0.5 ml of the Nash reagent (30% w/v ammonium acetate, 0.4% v/v acetylace tone in distilled water). The tubes were shaken, capped, and heated at 55°C for 10 min. After the tubes cooled to room temperature, the A415 was recorded (6).

Metabolite Isolation and Identification. Direct probe electron impact mass spectra were obtained on a VG30-250 quadrupole mass spectrometer (probe temperature ambient, electron energy 25 eV). Metabolites from reductive incubation mixtures were isolated by collecting HPLC effluents from 4–6, 10–12, and 15–17 min following injection of ether extracts. The HPLC mobile phase was removed by lyophilization and the residues analyzed by electron impact mass spectrometry.

Pharmacokinetics. Nontumored male CD2F1 mice (>20 g), supplied by National Cancer Institute, were housed 5 per cage on commercially obtained pure wood shaving bedding in an on-site facility with light provided from 6:00 am to 8:00 p.m. Food (Purina Mouse Chow) and tap water were provided ad libitum.

Formulated PEN (7.5 mg/ml) was administered to mice without dilution that a 25-g mouse received a 330-μl volume for a dose of 300 mg/m2. For i.v. drug administration, mice were placed in standard Broome restrainers and PEN was injected over 30 s into the lateral tail vein using tuberculin syringes fitted with 27-gauge 0.5-in. sterile disposable needles. PEN was administered p.o. to mice using a tuberculin syringe fitted with a 20-gauge straight 1.5-in. Harvard ball-tipped needle. Whole blood was obtained by cardiac puncture from mice anesthetized under ether vapors according to the following schedule: p.o. for 10, 20, 40, 60, 80, and 100 min; 2, 4, 6, 16, 24, and 48 h; and i.v. for 5, 15, 30, and 60 min; 2, 4, 6, 16, 24, and 48 h. Abbott midline incision was made to expose the peritoneal cavity, and blood was removed from the heart with citrated syringes containing 150 μl citrate, phosphate, dextrose, adenine anticoagulant per ml whole blood. Plasma was isolated following centrifugation (10,000 rpm x 3 min) and stored frozen (–20°C) until analysis.

Urine and feces were collected from mice (5 mice/cage) in glass metabolism cages during a 48-h period after i.v. or p.o. drug administration. Urine and feces were collected in Erlenmeyer flasks kept on dry ice. At the end of each collection period, the volume and quantity of urine and feces, respectively, were recorded and the samples were stored frozen (–20°C) until analysis.

Analysis of Pharmacokinetic Data. Plasma elimination data were fit by nonlinear regression using the program PCNONLIN (7). AUC values were calculated by trapezoidal approximation (8), and bioavailability was determined by comparison of AUC values after p.o. and i.v. administration.

RESULTS

HPLC Assay, PEN Stablility. Successful chromatography of PEN and internal standard were achieved on an octadecyl column eluted with a high concentration of acetonitrile (85%) as illustrated for plasma extracts (Fig. 2). Due to the volatility of PEN, efficient extraction (90%) from diethyl ether required concentration in the presence of n-butanol rather than concentration to dryness. Plasma standard curves were linear (r² > 0.99) over the concentration range 0–300 μM. The detection limit of PEN in plasma was 0.3 μM (10 pmol on-column). The within-day reproducibilities of the assay, based on the coefficient of variation values of peak area ratios at 300, 30, and 3 μM, were 6.0% (n = 4), 2.4% (n = 5), and 15% (n = 5). The day-to-day reproducibility of the assay, as determined from the coefficient of variation of the slope, was 3.1% (n = 5).

PEN was stable for at least 48 h at 37°C in human and mouse whole blood, plasma, and microsomal incubation solutions. Approximately 90–95% of the PEN in whole blood was sequestered by RBCs.

Growth Inhibition. We assessed growth-inhibitory activity of PEN against A204 (rhabdomyosarcoma), A375 (melanoma), A549 (alveolar cell carcinoma), and MCF-7 (breast carcinoma) human tumor cell lines. Following 1- and 48-h exposure of cells to PEN, 50% growth inhibition values were 163–249 and 107–147 μM, respectively. Thus, only a modest increase (~2-fold) in growth inhibition was observed when the same concentrations of drug were incubated with cells for 48 h rather than 1 h.

Fig. 2. Chromatograms of plasma extracts of blank mouse plasma (A) and mouse plasma containing PEN (154 μM) and internal standard (40 μM) (B). Elution of PEN was monitored at 214 nm (top panels). Elution of the internal standard was monitored at 243 nm (bottom panels). * peak that coelutes with the internal standard when monitored at 214 nm, but not when monitored at 243 nm.
Murine Pharmacokinetics. Plasma elimination of PEN was characterized following i.v. and p.o. administration to mice because the drug was active in vivo following administration by both routes to tumored animals. Following i.v. administration of the parenteral formulation of PEN (300 mg/m²), plasma disappearance of PEN was biphasic (Fig. 3A). Values of the distribution and elimination phase half-lives, total body clearance, and steady state volume of distribution were 12 min, 69 min, 114 ml/min/m², and 4800 ml/m², respectively. Analysis of PEN in the RBC fraction of whole blood obtained from mice following administration of PEN showed that concentrations in RBCs were 60–70-fold greater than those in plasma (data not shown). Following p.o. administration of 300 mg/m², PEN plasma concentrations (Fig. 3B) were substantially lower than those observed in plasma following i.v. administration. Peak plasma concentrations (1.8 μM) were observed 20–60 min following administration of PEN. The p.o. bioavailability of PEN, based on AUC values obtained following p.o. (121 μM · min) and i.v. (8502 μM · min) drug administration was <2%.

To further study PEN disposition, radiolabeled PEN (300 mg/m², 0.16 μCi/mg) was administered to mice. Plasma profiles of parent drug and total radioactivity after p.o. and i.v. doses of PEN are illustrated in Fig. 3. Concentrations of radioactivity in plasma, expressed as drug equivalents, were equal to or greater than parent drug concentrations in plasma following i.v. and p.o. drug administration. In contrast to parent drug, total plasma radioactivity was slowly eliminated and levels remained elevated 48 h after drug administration. The AUC values of plasma radioactivity calculated for the 48-h period following i.v. and p.o. drug administration were 856,000 and 541,000 μM · min, respectively. While urinary recovery of parent drug was negligible (<0.2%), urinary recoveries of total radioactivity following i.v. and p.o. administration were 30 and 24%, respectively. A portion of the urinary radioactivity was associated with metabolites that eluted early in HPLC radiochromatograms. PEN was not detected in feces and <14% of the total radioactivity was excreted in the feces following administration by both routes.

Metabolism. The substantial in vivo metabolism of PEN prompted in vitro metabolism studies with murine hepatic microsomes. Metabolism was not observed during oxidative incubation of PEN with murine microsomes in the presence or absence of NADPH. However, approximately 50% of PEN added to incubations was consumed in incubation mixtures containing NADPH-fortified microsomes from phenobarbital-pretreated mice (Fig. 4). No metabolites were detected by UV absorption in HPLC chromatograms of ether extracts or of aqueous residues from incubation aliquots. Positive results were observed when NADPH-fortified microsomal aliquots were incubated with the Nash reagent, consistent with oxidative O-demethylation (data not shown). When radiolabeled drug was incubated with microsomes, oxidative metabolism afforded several early eluting substances observed with the radiochemical detector (data not shown).

The trichloromethyl group of PEN is reminiscent of haloalkanes such as carbon tetrachloride, which are reductively metabolized in the liver (9). Biotransformation of carbon tetrachloride includes one-electron reductive dehalogenation to yield radical species that subsequently dimerize to form hexachloroethane (10, 11). PEN was extensively metabolized (>70%) by NADPH-fortified microsomes from untreated and phenobarbital-pretreated mice when incubations were carried out under a nitrogen atmosphere (Fig. 4). HPLC analysis of ether extracts
revealed 4 metabolite peaks (Fig. 5), each with a UV spectrum similar to that of parent drug (data not shown). A striking feature of preliminary electron impact mass spectra of the 2 metabolites eluted following PEN during HPLC analysis was the presence of ions with masses greater than that of the parent drug (M, 323) and consistent with dimer-like metabolites of PEN. The electron impact mass spectrum for the metabolite eluting at 15 min is shown in Fig. 6. We believe the metabolite to be a molecule (Fig. 7) with M, 576 formed by initial reductive cleavage of chlorine at the trichloromethyl carbon followed by dimerization of the radical intermediates. As is frequently the case for polychlorinated molecules, the parent molecular ion was not observed in the electron impact spectrum (12, 13).

However, as expected for a polychlorinated molecule of M, 576 under conditions of electron impact mass spectrometry, a pseudomolecular ion was observed at m/z 543, representing the M+2 ion in a chlorine cluster following loss of chlorine from the parent molecular ion. The ratios of relative abundance, at m/z 541, 543, 545, 547, 549, 551, 553, and 555, are those expected from a molecule containing 7 chlorine atoms due to the relative abundance (3/1) of 35Cl to 37Cl. Due to low relative abundance, the m/z peaks 553 and 555 are not observed in the mass spectrum (Fig. 7). An analogous loss of chlorine was observed in the electron impact mass spectrum of PEN, which contained a small molecular ion peak (m/z 323) and a major peak at m/z 288 (data not shown). The second major ion cluster observed in the metabolite mass spectrum (Fig. 7), centered around m/z 473, represents loss of 3 chlorine atoms. This is most likely due to loss of Cl2 across the adjacent dichloromethyl moieties and cleavage of ring chlorine. As expected, this pattern of fragmentation was not observed with PEN, which lacks the adjacent CC12 moieties. The third ion cluster centered around m/z 290 is consistent with homolytic cleavage of the carbon-carbon bond that links the monomers through the dichloromethyl moieties.

DISCUSSION

A satisfactory HPLC assay was developed for the hydrophobic PEN utilizing extraction with diethyl ether, and reverse phase HPLC with a mobile phase containing a high concentration of acetonitrile (85%) and water. Due to the volatility of PEN, addition of n-butanol to organic extracts was essential for reproducible recovery of parent drug. PEN was stable in aqueous solutions, plasma, and whole blood.

The present pharmacokinetic studies indicate that PEN undergoes rapid distribution and clearance following i.v. administration to mice. Metabolism was a substantial component of clearance since only a minute fraction of the administered dose was recovered as parent drug in the urine, and when radiolabeled drug was administered, parent drug accounted for a small percentage (<1%) of excreted radioactivity.

Based on several observations, we believe that the low bioavailability (<5%) of PEN observed following p.o. administration was due to extensive presystemic metabolism rather than poor absorption through the gastrointestinal mucosa. Following p.o. administration of radiolabeled PEN, most of the dose was absorbed since only a small portion of total radioactivity (10%) was recovered in the feces, and urinary recoveries of total radioactivity were similar following p.o. and i.v. doses. In addition, systemic exposure to total administered dose represented by the AUC of plasma radioactivity was similar following both p.o. and i.v. administration of radiolabeled PEN. Finally, the urinary excretion data also indicate that extensive metabolism occurred following p.o. administration, since only a small fraction of total plasma radioactivity was due to parent drug.

Harrison et al. (2) recently reported that i.v. and p.o. doses of 60-90 mg/kg (180-270 mg/m2) and 90-135 mg/kg (270-405 mg/m2) PEN, respectively, were effective against advanced s.c. or intrathecally implanted MX-1 carcinoma xenografts in athymic mice when administered daily during a 5-day period. The activity observed following p.o. administration is interesting in view of the poor p.o. bioavailability (<5%) and substantial metabolism of parent drug observed in our studies. It was also noteworthy that PEN displayed relatively poor growth-inhibitory activity against human tumor cells in culture. These data are all consistent with the possibility that metabolism is required for in vivo PEN antitumor activity.

The highly substituted structure of PEN provides several potential routes of metabolism including oxidative O-demethylation of methoxy substituents, N-oxidation of the pyridine nitrogen, and reductive dehalogenation at the trichloromethyl group. The presence of the radioactive label on the trichloromethyl carbon raised the possibility of C-C bond cleavage at the trichloromethyl group and the elimination of the radiolabel. While we recognized this possibility, we are unaware of instances in which a halogenated methyl moiety is cleaved metabolically from an aromatic ring. Oxidative metabolism was observed only in microsomes from mice pretreated with phenobarbital. NADPH-dependent formation of formaldehyde was detected in the incubation mixtures consistent with O-demethylation. Investigations with radiolabeled PEN indicated that polar metabolites were formed that remained in the aqueous phase during extraction procedures. These materials have not yet been identified, but are consistent with O-demethylation and N-oxidation.

The trichloromethyl moiety of PEN provided routes for reductive metabolism as observed for other agents containing

Fig. 6. Electron impact mass spectrum of the 15-min metabolite (see Fig. 7).
haloalkyl moieties. Since there are no carbon-hydrogen bonds in the halogen-substituted α-methyl group of PEN, a mechanism of reductive metabolism similar to that reported for carbon tetrachloride (9, 10) (which also lacks C-H bonds on the carbon bearing the halogen) may be proposed for PEN. Under anaerobic conditions, liver microsomes catalyze an NADPH-dependent one-electron reduction of CCl₄ to form the trichloromethyl radical CCl₃⁻ (10, 11). This radical can undergo a second one-electron reduction of CCl₄ to form the trichloromethyl radical CCl₃⁻. (10, 11). This radical can undergo a second one-electron reduction to form a dichlorocarbene species that, via hydrogen abstraction or dimerization of the dichloroalkyl chloroform (9, 10) or dimerize to form hexachloroethane (10, 11). In vitro metabolism of carbon tetrachloride is inhibited by the presence of oxygen (10).

By analogy, the serial one-electron reduction of PEN may produce a dichloroalkyl radical and a carbonyl chloride species, while hydrogen abstraction or dimerization of the dichloroalkyl radical may yield other products. We clearly observed reductive metabolism based on disappearance of parent drug in the presence of NADPH and appearance of 4 metabolites in incubation extract HPLC chromatograms. Two metabolites were less polar than parent drug and all had UV absorbance spectra similar to parent drug. In addition, mass spectral analysis of the metabolite which eluted at 15 min during HPLC analysis is consistent with formation of the dimer shown in Fig. 7, formed following radical cleavage of chlorine from the trichloromethyl moiety of PEN. Major ions in the mass spectrum of the metabolite are m/z 543 (loss of chlorine from the PEN dimer), m/z 473 (loss of Cl⁻ and further loss of Cl⁻), and m/z 290 (cleavage of the dimer across the carbon-carbon bond).

These studies, as well as murine antitumor activity data reported by Harrison et al. (2), are consistent with a role for metabolism in the antitumor activity of PEN. Preliminary data obtained in our laboratory (14) suggest microsomal conversion of PEN to reactive species that interact with macromolecules.

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REFERENCES

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