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Metabolic N-Hydroxylation of Pentamidine In Vitro

BRADLEY J. BERGER,[1] RICHARD J. LOMBARDY,[2] G. DEAN MARBURY,[3] CONSTANCE A. BELL,[1] CHRISTINE C. DYKSTRA,[4] JAMES EDWIN HALL,[1] AND RICHARD R. TIDWELL[4]*

Departments of Parasitology and Laboratory Practice[1] and Environmental Science and Engineering,[3] School of Public Health, Department of Medicinal Chemistry, School of Pharmacy,[2] and Department of Pathology, School of Medicine,[4] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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By using high-performance liquid chromatography, the in vitro conversion of pentamidine to the corresponding amidoximes (*N*-hydroxypentamidine and *N,N'*-dihydroxypentamidine) was studied in supernatants of rat liver homogenate centrifuged at 9,000 × *g*. The presence of the two amidoxime peaks in chromatograms was confirmed by liquid secondary ion mass spectrometry and by unequivocal synthesis of the suspected metabolites. The metabolic reactions were found to be catalyzed by the cytochrome P-450 system (mixed-function oxidases). The formation of the monohydroxylated product was found to have a K_m of 0.48 mM and a V_{max} of 29.50 pmol/min per mg of protein, while the dihydroxylated metabolite had a K_m of 0.73 mM and a V_{max} of 4.10 pmol/min per mg of protein. *N,N'*-Dihydroxypentamidine was found to have highly reduced antiprotozoal activity in vitro relative to that of pentamidine, and neither of the hydroxylated metabolites nor pentamidine was found to be significantly mutagenic by the Ames test. Contrary to previous reports, pentamidine is readily metabolized to at least two hydroxylated products, and this conversion may be relevant to the clinical use of the compound and to future drug design.

Pentamidine [1,5-di(4-amidinophenoxy)pentane] was synthesized in the late 1930s and has been used in the prophylaxis and treatment of African trypanosomiasis and treatment of antimony-resistant leishmaniasis (24, 32). The compound was found to be effective against *Pneumocystis carinii* pneumonia as early as 1957 (22), but it was not used extensively to treat *P. carinii* pneumonia until the acquired immunodeficiency syndrome epidemic (31). Currently, pentamidine is one of the drugs of choice for the treatment of acquired immunodeficiency syndrome-related *P. carinii* pneumonia (24, 31, 32).

While the pharmacological properties of pentamidine have been studied for decades (23, 24, 26, 36, 37), the recent development of sensitive high-performance liquid chromatographic (HPLC) and biological assays has permitted detailed studies of the distribution and pharmacokinetics of the compound (1–3, 12, 13, 16, 17, 27). However, there have been no published attempts to determine the metabolic fate of pentamidine, largely because of the long-held concept that the drug is metabolically inert (18, 24, 32). This conclusion is primarily based on experiments by Launoy et al. (25, 26), who used pentamidine labeled with ^{14}C in either an amidino or an aliphatic position. Recent work by Clement and Zimmermann (5, 9–11) demonstrating that benzamidine is converted to the corresponding benzamidoxime in supernatants of rat liver homogenate centrifuged at 9,000 × *g* (9,000 × *g* supernatants) strongly suggested that pentamidine could undergo a similar metabolic transformation. In this report we describe the isolation and characterization of two N-hydroxylated metabolites of pentamidine from incubations of the drug in 9,000 × *g* rat liver preparations. Four additional putative metabolites were observed, but they have not yet been characterized. While several investigators have commented on the possibility of pentamidine metabolism (2, 12, 38), this study constitutes the first identification of any metabolites of the drug.

* Corresponding author.

MATERIALS AND METHODS

Compounds. Pentamidine isethionate was obtained from LyphoMed Inc. (Rosemont, Ill.), and the mono- and dioximes of pentamidine and benzamidoxime were synthesized in the laboratories of R. R. Tidwell by the method of Tiemann (35). The structures of the compounds are shown in Fig. 1. Benzamidine was acquired from Eastman Organic (Rochester, N.Y.). NADPH tetrasodium salt and 1-heptanesulfonic acid sodium salt were obtained from Sigma Chemical Co. (St. Louis, Mo.), tetramethylammonium chloride (TMAC) was obtained from Fluka (Zurich, Switzerland), and aniline was obtained from Allied Chemical Co. (Morristown, N.J.). HPLC-grade acetonitrile was acquired from Fisher Scientific Co. (Fairlawn, N.J.), and all water was deionized and filtered through a water purification system (Darco, Durham, N.C.).

Experimental incubations. (i) Preparation of liver homogenates. Barrier-raised male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, Pa.) were euthanized; and the livers were removed immediately, rinsed with phosphate buffer (pH 7.4; 8.7 mM KH_2PO_4, 30.4 mM Na_2HPO_4), weighed, and placed on ice. In a 4°C room, the livers were minced with scissors and homogenized with approximately 2 volumes of 1.15% KCl (0.154 M) by using a motorized Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle glass tube homogenizer (A. H. Thomas Co., Philadelphia, Pa.). The homogenates were transferred to polycarbonate tubes and centrifuged at 9,000 × *g* for 30 min in a centrifuge (RC-2B; Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a GSA rotor. The supernatant was carefully decanted and used as the source of enzyme activity. The protein content in the supernatant was determined by the method of Lowry et al. (28). Microsomes were prepared by centrifuging the 9,000 × *g* supernatant at 105,000 × *g* for 60 min in an ultracentrifuge (L3-50; Beckman Instruments, Inc., Fullerton, Calif.) with a type 40 rotor. The pelleted microsomes were suspended in phosphate buffer by using the tissue homogenizer.

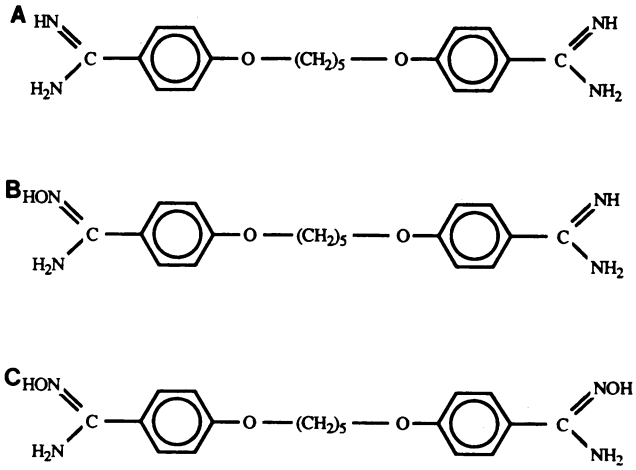

FIG. 1. Structures of the compounds examined in this study. (A) Pentamidine; (B) monooxime of pentamidine (*N*-hydroxypentamidine); (C) dioxime of pentamidine (*N*,*N*′-dihydroxypentamidine).

(ii) Incubation conditions. Two sets of incubations were carried out under similar conditions. For the first experiment, 1.0 ml of liver supernatant and 1.0 ml of cofactor solution (2.0 mg of NADPH and 1.9 mg of $MgCl_2$) were added to 3.0 ml of phosphate buffer (pH 7.4) in duplicate 50-ml Erlenmeyer flasks at 37°C in a shaking water bath. One milliliter of pentamidine isethionate (2.5 mM stock solution in phosphate buffer) was added to each flask to start the incubation. Incubations of individual samples were stopped every 15 min from 0 to 120 min by placing them on ice. The samples were then stored at −70°C until extraction and analysis.

The second experiment was identical to the first one, except that the incubation time of all duplicate samples was 30 min and pentamidine was added to give the following final concentrations: 8.3, 16.7, 83.3, 166.7, 333.3, 500.0, 666.7, and 833.3 μM. Control incubations of 30 min each were performed on samples containing an extra 1.0 ml of phosphate buffer (pH 7.4) in place of 833.3 μM pentamidine, liver supernatant, or cofactor solution.

Microsomal incubations were performed as described above for the 9,000 × *g* supernatant incubations, except that the cofactor mixture contained 2 mg of NADPH per ml, 1.9 mg of $MgCl_2$ per ml, 5 mM glucose-6-phosphate, 0.7 mg of NADP per ml, and 0.1 U of glucose-6-phosphate-dehydrogenase per ml.

(iii) Confirmation of enzyme activity. The presence of mixed-function oxidase enzyme activity was confirmed by measuring aniline hydroxylase activity as described by Imai et al. (21). Briefly, 1.0 ml of 10 mM aniline instead of pentamidine was added to an incubation mixture and was shaken at 37°C for 30 min. The incubation was stopped by adding 1.67 ml of 0.3 M $ZnSO_4$–0.3 N $Ba(OH)_2$ to the incubation mixture and centrifuging the mixture at 1,500 × *g* for 5 min in a clinical centrifuge. One milliliter of supernatant was mixed with 1.5 ml of 5% Na_2CO_3–0.2 N NaOH–4% phenol and incubated at 37°C for 30 min, with the subsequent color being measured at 630 nm in a spectrophotometer.

HPLC assay. Individual incubation samples were extracted over solid-phase columns (Prep-Sep C-18; Fisher Scientific Co.) as described previously (1). Samples were added under gentle vacuum conditions and eluted with 1.0 ml of 95% CH_3CN–10 mM heptanesulfonate–10 mM TMAC–4.2 mM H_3PO_4 in H_2O. The eluates were then brought to dryness under a gentle air stream and suspended in 200 μl of elution buffer for HPLC analysis.

A liquid chromatograph (model 1084B; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a reporting integrator (model 79850B; Hewlett-Packard Co., Boblingen, Federal Republic of Germany) was used for all analyses. We used a reverse-phase diisopropyl C-8 column (25 cm by 4.6 mm; particle size, 5 μm; Zorbax RX; Dupont Co.) that was kept at 40°C. The matrix (RX) was manufactured to be effective for highly basic compounds and was used when the standard octadecylsilane C-18 column (Zorbax ODS; Du Pont) used in previous studies failed to resolve pentamidine and the oxime standards. The mobile phase was CH_3CN–10 mM heptanesulfonate–10 mM TMAC–4.2 mM H_3PO_4 in H_2O with a linear 22.5 to 45% CH_3CN gradient over 25 min. A 25-μl portion of each sample was injected, and detection was performed with a variable-wavelength ultraviolet spectrophotometer (model 79875A; Hewlett-Packard Co.) set at 265 nm with 540 nm as a reference wavelength.

Standard curves for the mono- and dioximes of pentamidine were constructed by adding the standard compound (93.5 ng to 9.35 μg for the monooxime and 49 to 980 ng for the dioxime) to incubation mixtures of liver homogenates, in which 1.0 ml of phosphate buffer was added in place of the cofactor solution. These samples were then immediately extracted and analyzed as described above. The peak areas were plotted against the amounts that were added to give a standard curve. The r^2 values were 0.99 for the monooxime curve and 0.98 for the dioxime curve.

Mass spectrometry. A large-scale incubation was set up by using 500 mg of pentamidine isethionate in 400 ml of phosphate buffer (pH 7.4) containing 50 ml of cofactor solution (50 mg of NADPH, 47.5 mg of $MgCl_2$ in phosphate buffer) and 80 ml of the 9,000 × *g* liver homogenate supernatant. The incubation was carried out for 6 h at 37°C in a shaking water bath. Half of this sample was stored at −70°C, and the other half was extracted over C-18 solid-phase extraction columns as previously described. A total of 32 ml of column eluate was dried to approximately 500 μl under a gentle air stream. Standards were prepared by dissolving several crystals of each standard oxime in 1.5 ml of 95% CH_3CN–10 mM heptanesulfonate–10 mM TMAC–4.2 mM H_3PO_4 in H_2O.

Mass spectra were obtained by using a tandem hybrid mass spectrometry-mass spectrometry system (VG 70-250 SEQ; VG, Manchester, United Kingdom) operated in the liquid secondary ion mass spectrometry (LSIMS) mode. Samples were introduced via direct probe by using glycerol as the matrix, a Cs ion gun set at 35 kV, and an 8-kV source potential. Argon was used as the collision gas in an *Rf*-only collision cell, with the collision energy set at 4 to 7 eV (laboratory reference). Data were acquired by using multichannel acquisition and were not centroided.

Mutagenicity assay. The mutagenic potential of pentamidine, *N*-hydroxypentamidine, *N*,*N*′-dihydroxypentamidine, benzamidine, benzamidoxime, and a 30-min incubation of 1.25 mg of pentamidine per ml in 9,000 × *g* liver homogenate supernatant (with cofactors) was measured by the method of Maron and Ames (29). Various amounts of individual compounds were suspended in 10% dimethyl sulfoxide, and 0.1 ml was added to 0.1 ml of *Salmonella typhimurium* TA100 (*hisG428 rfa ΔuvrB*; grown to 10^8 cells per ml) and 0.5 ml of phosphate buffer (pH 7.4). Two milliliters of top agar at 45°C (0.55% agar, 0.46% NaCl, 45 μM L-histidine, 45 μM biotin) was added, mixed, and poured onto agar plates [30 ml of 1.5% agar, 2% glucose, 0.6% $MgSO_4 \cdot 7H_2O$, 6% citric acid, 30% K_2HPO_4, 10.5% $NaHNH_4(PO_4) \cdot 4H_2O$]. The plates

were incubated for 48 h at 37°C, and the His^+ revertants were enumerated. Control plates that lacked any amidine, amidoxime, or 9,000 × *g* liver homogenate supernatant were also incubated.

Antiprotozoal activity of *N,N'*-dihydroxypentamidine. (i) Antigiardial assay. The assay for the activity of the dioxime in vitro was performed as described by Boreham et al. (4). Serial dilutions of *N,N'*-dihydroxypentamidine were added to a 96-well micro-dilution plate; then, 2.5×10^4 organisms of logarithmically growing *Giardia lamblia* WB (ATCC 30957) trophozoites in a modified Keister medium containing 5% fetal bovine serum were added to each well. The plate was then placed in an anaerobic chamber and incubated at 37°C under nitrogen. After 24 h, 1.5 to 2.0 μCi of [^{3}H] thymidine was added to each well. Eighteen hours later, the cells were harvested onto glass microfiber filters, dried, and analyzed in a scintillation counter to measure [^{3}H]thymidine incorporation. Data on the uptake of radiolabeled thymidine were fitted to a logistic-logarithmic concentration response function by a nonlinear regression method using a non-weighted least-squares criterion (15, 30). The concentration of drug necessary to inhibit 50% incorporation of the labeled substrate was calculated.

(ii) Antileishmanial assay. The activity of *N,N'*-dihydroxypentamidine against *Leishmania mexicana amazonensis* clone WR669C45 was determined as described in Grogl et al. (19). Serial dilutions of the drug were added to a 96-well microdilution plate; then, 1.0×10^6 promastigotes in 200 μl of Schneider drosophila medium containing 10% heat-inactivated fetal bovine serum were added to each well. The plate was incubated in air at 25°C for 24 h before the addition of 1.0 to 2.0 μCi of [^{3}H]thymidine per well. After an additional 18 h of incubation, the cells were harvested, incorporation was determined, and data were analyzed as described above.

(iii) Antiplasmodial assay. The activity of *N,N'*-dihydroxypentamidine against *Plasmodium falciparum* clones W2 (chloroquine resistant, mefloquine susceptible; Indochina III/CDC) and D6 (chloroquine susceptible, mefloquine resistant; Sierra Leone I/CDC) was determined by the method of Oduola et al. (30). Serial dilutions of the drug were added to a 96-well micro-dilution plate; then, 200 μl of RPMI 1640 medium containing human type A-positive erythrocytes at a parasitemia level of 0.2% and a hematocrit level of 1.5% was added. The plate was incubated at 37°C for 24 h in 5% O_2–5% CO_2–90% N_2, before the addition of 1.0 to 2.0 μCi of [^{3}H]hypoxanthine per well. After an additional 18 h of incubation, the cells were harvested, incorporation was measured, and data were analyzed as described above.

(iv) DNA-binding activity. The DNA binding of *N,N'*-dihydroxypentamidine and pentamidine was measured by the method of Cory et al. (14). Sonicated calf thymus DNA was added to either compound at a 1:10 drug to base ratio, and the change in the midpoint of the thermal denaturation curve (ΔT_m) was measured under low ionic conditions.

RESULTS

Pentamidine incubations. Standard samples of pentamidine, monooxime, and dioxime were analyzed by HPLC to determine the relative retention times of the compounds. Pentamidine was found to have a retention time of approximately 12.5 min, while those of the mono- and dioximes of pentamidine were found to be approximately 11.9 and 11.6 min, respectively (Fig. 2). The aniline hydroxylase activity of the liver preparation was 0.54 ± 0.09 nmol converted per min per mg of protein, which demonstrates that microsomal enzymes were both present and active.

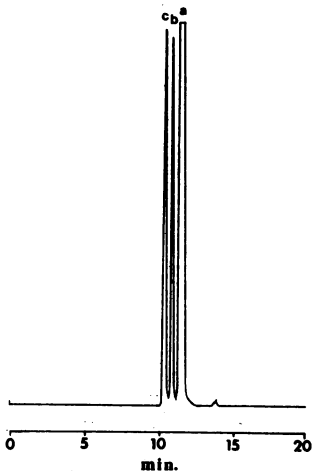

FIG. 2. Chromatogram of standard pentamidine and the corresponding amidoximes. Separation was performed with a diisopropyl C-8 column (Zorbax RX) with 22.5 to 45% CH_3CN–10 mM heptanesulfonate–10 mM TMAC–4.2 mM H_3PO_4 in H_2O graded over 25 min (as described in the text). Pentamidine had a retention time of 12.5 min (peak a), the monooxime had a retention time of 11.9 min (peak b), and the dioxime had a retention time of 11.6 min (peak c).

Control incubations were set up in which pentamidine, cofactor solution, or the 9,000 × *g* liver homogenate supernatant was excluded from the normal 30-min incubation mixture. When the 9,000 × *g* liver homogenate supernatant or cofactor was excluded, only very small peaks could be detected in the region of interest, and no peaks were detected when pentamidine was excluded (Fig. 3). In the experimental incubations, when 41.7 μM pentamidine was present in the 9,000 × *g* liver homogenate supernatant preparation with cofactor solution, peaks which coeluted with the mono- and dioximes could be detected by HPLC in the sample at 15 min and increased in concentration with each subsequent sample obtained every 15 min (data not shown). Chromatograms of the samples obtained at 15 and 105 min are shown in Fig. 4. It is of interest to note that while the two oximes were present in the incubations, there were four other peaks that were not present in the control incubations (Fig. 4, peaks c, d, e, and f).

To determine the enzyme(s) in the 9,000 × *g* supernatant that was responsible for the metabolism of pentamidine, a series of incubations was performed with both 9,000 × *g* rat liver homogenate supernatants and microsomes isolated from these supernatants (Table 1). All putative metabolites were detected in both 9,000 × *g* supernatants with cofactor and microsomes with cofactor, but not in the 105,000 × *g* supernatant (supplied with the microsomal cofactor). Additionally, the addition of carbon monoxide or the cytochrome P-450-inactivating agent SKF-525A to microsomal incubations prevented the metabolism of pentamidine. These results indicate that the cytochromes P-450 (mixed-function oxidases) are responsible for the metabolism of pentamidine.

Kinetics of oxime production. To determine the enzymatic kinetics of production of the oximes of pentamidine, 30-min incubations were performed by using concentrations of pentamidine that ranged from 8.3 to 833.3 μM. The samples were extracted and analyzed by HPLC, and the peak areas were converted to nanomoles by using standard curves.

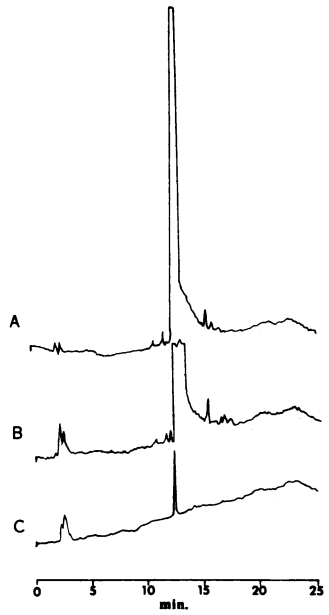

FIG. 3. Chromatograms of control liver homogenate incubations. Thirty-minute incubations were performed at 37°C by using 3.0 ml of phosphate buffer, 1.0 ml of the 9,000 × *g* liver homogenate supernatant, 1.0 ml of cofactor solution (2.0 mg of NADPH and 1.9 mg of $MgCl_2$), and 833.3 μM pentamidine. (A) Liver supernatant was not included; (B) cofactor solution was not included; (C) pentamidine was not included. Samples were extracted and chromatographed as described in the text.

Lineweaver-Burk plots were then constructed for both the mono- and dioximes (Fig. 5). The monooxime of pentamidine was found to have a K_m of 0.48 mM and a V_{max} of 29.50 pmol/min per mg of protein, while the dioxime was found to have a K_m of 0.73 mM and a V_{max} of 4.10 pmol/min per mg of protein. Incubation with 833.3 μM pentamidine was not included in the kinetic plots because of problems with drug solubility at that concentration.

TABLE 1. Incubation of pentamidine with fractions of rat liver homogenates[a]

Sample	Detection of the following metabolite peaks by HPLC[b]:					
	a	b	c	d	e	f
9,000 × *g* supernatant with cofactor	+	+	+	+	+	+
9,000 × *g* supernatant without cofactor	−	−	−	−	−	−
105,000 × *g* supernatant with cofactor	−	−	−	−	−	−
Microsomes with cofactor	+	+	+	+	+	+
Microsomes without cofactor	−	−	−	−	−	−
Microsomes with cofactor and 100% carbon monoxide	−	−	−	−	−	−
Microsomes with cofactor and 0.83 mM SKF-525A	−	−	−	−	−	−

[a] Pentamidine (final concentration, 0.167 mM) was incubated with 1.0 ml of the indicated fractions of rat liver homogenates. All incubations were performed at 37°C for 30 min. The samples were then extracted and analyzed as outlined in the text. Peaks a through f indicate putative metabolite peaks as labeled in Fig. 4.

[b] +, A particular peak was detected by HPLC analysis of a particular sample; −, the peak was not detected.

Mass spectrometry. To confirm the results obtained by HPLC, it was necessary to detect the presence of the two oximes of pentamidine by mass spectrometry. Standard mass spectra were obtained by performing LSIMS on the synthesized oxime standards diluted in the same matrix as the experimental sample (C-18 elution buffer). Since the background level from the ion pair agents in the buffer was high, it was necessary to use mass spectrometry-mass spectrometry. The ion corresponding to the known molecular weight of the standard compound was selected for secondary fragmentation (the monooxime had an M+1 ion of 357, and the dioxime had an M+1 ion of 373). The spectra of the standard compounds are shown in Fig. 6. The experimental sample was a larger-scale liver incubation that was extracted over C-18 columns and concentrated 64-fold. LSIMS mass spectrometry-mass spectrometry was performed on this sample by again focusing on the monooxime M+1 ion of 357 and the dioxime M+1 ion of 373. The secondary mass spectra of the peaks at 357 and 373 from the experimental

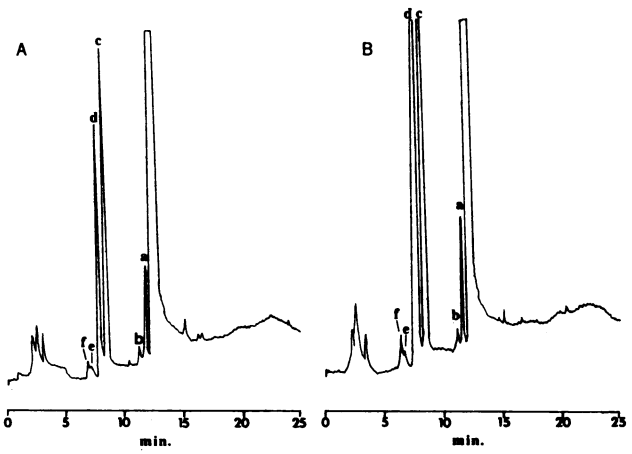

FIG. 4. Chromatograms of experimental liver homogenate incubations. The incubations were performed at 37°C by using 3.0 ml of phosphate buffer, 1.0 ml of the 9,000 × *g* liver homogenate supernatant, 1.0 ml of cofactor solution (2.0 mg of NADPH and 1.9 mg of $MgCl_2$), and 41.7 μM pentamidine. (A) Incubation was terminated after 15 min; (B) incubation was terminated after 105 min. Peak a, monooxime; peak b, dioxime; peaks c through f, unknown putative metabolites. Extraction and chromatography of samples were described in the text.

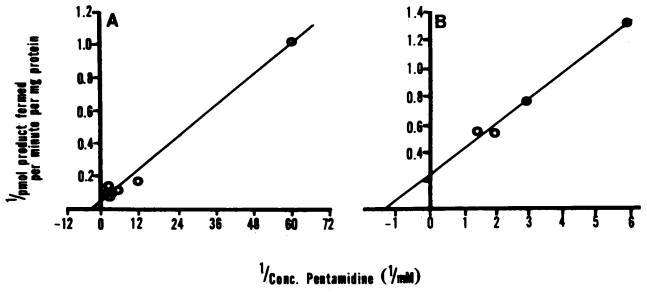

FIG. 5. Lineweaver-Burk plots of the formation of pentamidoxime from pentamidine during a rat liver homogenate incubation. (A) Production of *N*-hydroxypentamidine over the course of a 30-min rat liver homogenate incubation. (B) Production of *N*,*N*′-dihydroxypentamidine over the course of a 30-min incubation. The final concentration of pentamidine was 8.3 to 666.7 μM; however, no *N*,*N*′-dihydroxypentamidoxime was detected in incubations with less than 166.7 μM pentamidine. See the text for more details and for K_m and V_{max} values.

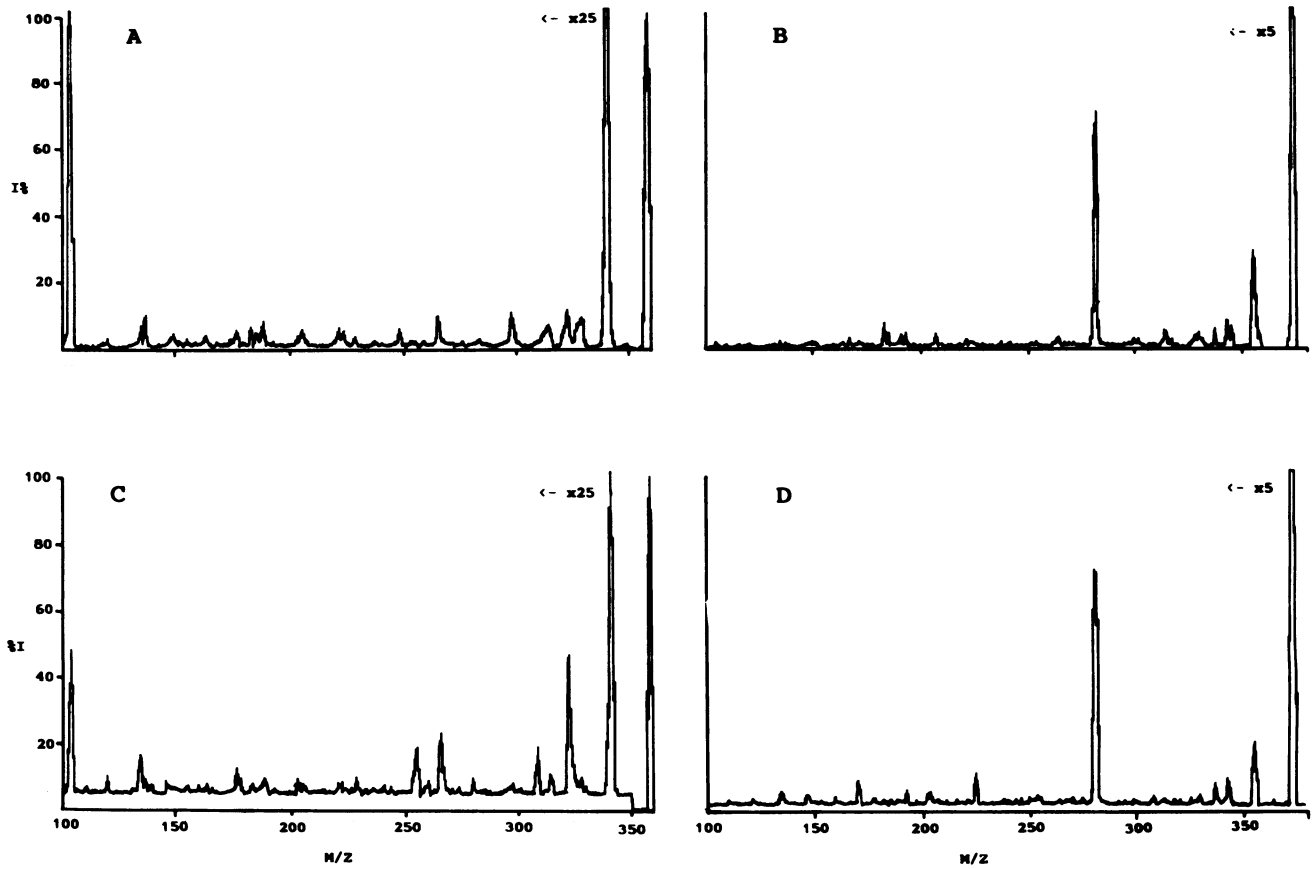

FIG. 6. Secondary mass spectra of the mono- and diamidoximes of pentamidine from standards and experimental incubations. LSIMS mass spectrometry-mass spectrometry was performed by direct-probe bombardment of the sample with cesium ions to produce molecular ions. The molecular ion corresponding to the compound of interest was then selected for secondary fragmentation by collision with argon gas. These secondary fragments are shown for the ion at 357 from a liver homogenate incubation of pentamidine (A), the ion at 373 from the same liver homogenate incubation (B), standard monooxime (M+1 = 357) (C), and standard dioxime (M+1 = 373) (D). Incubation and extraction conditions are outlined in the text. Each spectrum is displayed as percent intensity (I) against mass/charge ratio.

sample are shown in Fig. 6 with the standard spectra. The number and intensity of fragment peaks matched well, thus confirming the presence of the mono- and dioximes of pentamidine in the 9,000 × *g* liver homogenate preparation incubations.

Mutagenicity. Several concentrations of pentamidine, *N*-hydroxypentamidine, *N*,*N*′-dihydroxypentamidine, benzamidine, benzamidoxime, or 9,000 × *g* liver homogenate supernatant incubation of 1.25 mg of pentamidine per ml were analyzed for mutagenicity by the test of Maron and Ames (29). Induction of His^+ revertants of *S. typhimurium* TA100 measured the relative mutagenicity of each of the compounds (Table 2). None of the compounds tested appeared to be strongly mutagenic, but the 30-min liver supernatant incubation of pentamidine was more mutagenic than any of the five drugs at all doses higher than 0.1 μg per plate. Pentamidine appeared to be toxic to the cells at doses higher than 2.5 μg per plate, and *N*-hydroxypentamidine appeared to be toxic at doses higher than 0.1 μg per plate. While neither benzamidine nor benzamidoxime was highly mutagenic, benzamidoxime appeared to be more genotoxic than benzamidine, confirming the results of Clement et al. (7). It is clear from the data in Table 2 that neither *N*-hydroxypentamidine nor *N*,*N*′-dihydroxypentamidine is more mutagenic than pentamidine.

Antiprotozoal activity. *N*,*N*′-Dihydroxypentamidine was tested in vitro for activity against *G. lamblia*, *L. mexicana amazonensis*, and two strains of *P. falciparum* (W2, chloroquine resistant, mefloquine susceptible; D6, chloroquine susceptible, mefloquine resistant). The drug was tested from 1.372 to 1,000 μM against *G. lamblia*, from 0.137 to 100 μM

TABLE 2. Mutagenic activity of amidines and amidoximes in *S. typhimurium*[a]

Treatment	No. of His^+ revertants of *S. typhimurium*/plate (minus control value) with the following concn (μm) of drug/plate[b]:					
	100	10	5	2.5	1	0.1
Pentamidine	0	0	0	45	43.5	37.5
Pentamidine in 6-h liver incubation	46	74	74	65.5	69.5	13.5
N-Hydroxypentamidine	3.5	0	0	0	0	14.5
N-*N*′-Dihydroxypentamidine	19	13.5	25	7	24.5	0
Benzamidine	15	24	11	1.5	26	13.5
N-Hydroxybenzamidine	27.5	41	33.5	15.5	20	65

[a] Various amounts of the indicated compounds or a 6-h incubation of pentamidine in 9,000 × *g* rat liver homogenate supernatant were added to 10^6 *S. typhimurium* TA100 (His^-) cells and plated as described in the text. After incubation for 48 h at 37°C, the resulting colonies (His^+ revertants) were enumerated (29).

[b] All drug concentrations were tested on duplicate plates. The control His^+ revertant value was 367 ± 18.7.

against *L. mexicana amazonensis*, and from 0.001 to 0.1 μM against both strains of *P. falciparum*. Under these conditions, *N,N'*-dihydroxypentamidine had no activity. By contrast, the concentrations of pentamidine necessary to inhibit 50% incorporation of the labeled substrate were 21.24 μM against *G. lamblia*, 0.79 μM against *L. mexicana amazonensis*, 0.12 μM against *P. falciparum* W2, and 0.06 μM against *P. falciparum* D6 (R. R. Tidwell, S. K. Jones, J. D. Geratz, K. A. Ohemeng, C. A. Bell, B. J. Berger, and J. E. Hall, Ann. N.Y. Acad. Sci., in press). The lack of activity displayed by the dioxime indicates that the N-hydroxylation of pentamidine results in a loss of in vitro antiprotozoal activity.

A proposed mode of action for the antiprotozoal activity of pentamidine is by its strong DNA-binding property (14). The ability of the *N,N'*-dihydroxy metabolite to bind DNA similarly was investigated by determining the effect of the drug on the thermal denaturation curve of sonicated calf thymus DNA. *N,N'*-dihydroxypentamidine was found to have a ΔT_m of 0.2°C. In comparison, pentamidine was found to have a ΔT_m of 10.7°C. Therefore, the N-hydroxylation of pentamidine results in a corresponding loss of almost all the DNA-binding ability of the parent compound.

DISCUSSION

The long-held conception that pentamidine is not metabolized (18, 24, 32) appears to have originated from work reported in 1960 by Launoy et al. (25, 26). Approximately 80% of the radiolabel was recovered from the urine of rats and mice injected with [^{14}C]pentamidine (labeled in either the amidino moiety or the terminal carbons of the aliphatic chain). While the results demonstrate that pentamidine is not metabolically degraded with the subsequent loss of carbon atoms, they do not address the possibility of nondegradative metabolism. The results of our study show that pentamidine can be readily metabolized in vitro by rat liver preparations and that two of the six putative metabolites detected by HPLC were found to be *N*-hydroxypentamidine and *N,N'*-dihydroxypentamidine.

Further studies are being performed to determine the identities of all the remaining pentamidine metabolites and to investigate metabolism in vivo. Several possibilities for the structures of the remaining pentamidine metabolites exist (such as chain or ring hydroxylations), and these compounds are being synthesized. HPLC analysis of urine from a rat given a daily dose of 10 mg of pentamidine per kg per day for 14 days showed that several of the putative metabolites were detected in urine on day 14, but neither of the *N*-hydroxy metabolites was detected (data not shown). Similar analyses of urine from patients with acquired immunodeficiency syndrome being treated with pentamidine have been inconclusive because of a lack of proper control samples. Complete elucidation of the in vitro and in vivo metabolism is under way with [^{14}C]pentamidine.

Of obvious medical importance are the efficacy and toxicity profiles of any pentamidine metabolites relative to those of the parent compound. The relative mutagenicities of *N*-hydroxypentamidine, *N,N'*-dihydroxypentamidine, and pentamidine were determined by using the test of Maron and Ames (29). Neither pentamidine nor the two pentamidoximes were found to be highly mutagenic. The 9,000 × *g* liver homogenate supernatant incubation of 1.25 mg of pentamidine per ml was found to be more mutagenic than pentamidine alone was, suggesting that one or more of the unidentified putative metabolites may be more mutagenic than pentamidine.

The two pentamidoximes have been synthesized previously and tested in vivo against African trypanosomal and leishmanial infections (6). In all cases, it was found that the amidoximes were active, but they were less active then pentamidine. However, we determined that *N,N'*-dihydroxypentamidine displays highly reduced antiprotozoal activity in vitro when compared with that of pentamidine. In fact, the activity of the diamidoxime in vitro against all the organisms tested was so low that the concentration necessary to inhibit 50% incorporation of the labeled substrate could not be calculated for the compound. It is likely that the difference seen between in vivo and in vitro activity is due to the occurrence of the reverse reaction (amidoxime to amidine) in vivo. This reaction has been demonstrated previously (8, 20) and could convert enough of the pentamidoxime to pentamidine to produce the low levels of activity seen in vivo. Additionally, *N,N'*-dihydroxypentamidine was found to have no significant DNA-binding ability (ΔT_m, 0.2°C). Since the strong DNA binding of pentamidine (ΔT_m, 10.7°C) is a postulated mode of action for the drug, this result may explain the relative inactivity of the amidoxime. The N-hydroxylation pathway of pentamidine appears to cause a significant loss of antiprotozoal activity and, therefore, may be of consequence in the treatment of *P. carinii* pneumonia. The full impact of metabolism on the biological activity of pentamidine will not be known until all metabolites have been identified, characterized, and tested for antiprotozoal activity.

The observation that pentamidine is readily metabolized requires reexamination of current pentamidine detection methods. Our previous HPLC method for detecting pentamidine is inadequate for detecting any of the putative metabolites. A comparatively less acidic reversed-phase column is effective in separating both the parent diamidine and its corresponding amidoximes (33). HPLC methods for the detection of pentamidine used by other investigators operate on principles similar to those of our original procedure (16, 27) and should be subject to the same problems. The chemical detection method used by several investigators (24, 36, 37) is based on the conversion of pentamidine to a fluorogenic compound with glyoxal and benzaldehyde, and the bioassay used by Bernard et al. (3) is based on the ability of pentamidine to inhibit *Candida tropicalis* ATCC 28707 growth. While it is likely that the pentamidoximes would not react in either of these assays, it is not possible to predict the reactivities of the putative metabolites. Therefore, it is difficult to assess the effectiveness of these two assays in the pharmacokinetic study of pentamidine.

With respect to future antimicrobial drug development, it will be necessary to design analogs of pentamidine with consideration of the metabolic fate of the drug. Several novel pentamidine analogs with increased anti-*P. carinii* activity and lowered toxicity have been synthesized in our laboratories (34), and the relationship between these properties and metabolism must be thoroughly investigated. Only when the metabolic fate of pentamidine is fully determined will it be possible to fully characterize the pharmacology of the compound and begin a logical search for safer, more effective anti-*P. carinii* agents.

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