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High-performance liquid chromatographic assay detects pentamidine metabolism by Fisher rat liver microsomes

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Abstract

Fisher rat liver microsomes metabolized the antimicrobial drug pentamidine to four new compounds detected by gradient elution reversed-phase high-performance liquid chromatography with variable wavelength detection. Coelution experiments with pentamidine metabolite standards determined the new peaks to be previously identified hydroxylated metabolites of pentamidine, with 1,5-bis(4′-amidinophenoxy)-3-pentanol and 1,5-di-(4′-amidinophenoxy)-2-pentanol formed in the greatest amount. The data contradict a previous report that Fisher rat liver homogenates do not metabolize pentamidine. Pentamidine and its known primary metabolites have almost identical absorption spectra; thus, pentamidine metabolism must be evaluated using gradient elution HPLC to resolve pentamidine from its metabolites. The current assay has now been used to demonstrate that Fisher and Sprague–Dawley rat, mouse, rabbit and human liver microsomes all metabolize pentamidine in vitro.

Keywords: Pentamidine

1. Introduction

The aromatic diamidine pentamidine has been used to treat the tropical protozoal infections African trypanosomiasis and visceral leishmaniasis for over 50 years [1,2]. The drug has been more frequently used in industrialized countries recently because of its good activity against *Pneumocystis carinii* pneumonia, one of the most detrimental opportunistic infections in the AIDS population [1,2,8]. Several pharmacokinetic studies of pentamidine were performed during the past 30 years [6,7,16–22], although most were performed with the assumption that pentamidine was not metabolized. Only during the past decade have methods been developed that permit separation of pentamidine from potential metabolites [3–5,9,10]. Gradient elution reversed-phase high-performance liquid chromatography (HPLC) using ion modifiers, followed by tandem mass spectrometric analysis, or HPLC directly coupled to mass spectrometry have been used to show that pentamidine is readily metabolized to at least seven primary metabolites by mammalian drug metabolizing enzymes [3–5,9,13,15].

A recently published work, however, reported that no pentamidine metabolites could be found after

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incubating the drug with liver homogenates from Fisher rats [11]. Since microsomes from Sprague–Dawley rats, mice, rabbits and humans have all been shown to actively metabolize pentamidine [3–5,9,10,13,15], we were interested to confirm whether Fisher rats indeed could not metabolize pentamidine. Using gradient elution reversed-phase HPLC methods that resolve pentamidine from unequivocally synthesized pentamidine metabolites, we confirm that pentamidine is readily metabolized by Fisher rat liver microsomes. Isocratic HPLC with diode-array detection is apparently insufficient to distinguish among the chemically, chromatographically, and spectrally similar pentamidine and its chain hydroxylated metabolites.

2. Experimental

2.1. Chemicals

All solvents and reagents were of analytical or of HPLC grade. Triethylamine (TEA) and potassium phosphate dibasic and monobasic were obtained from Fisher Scientific (Pittsburgh, PA, USA). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). Pentamidine diisethionate was provided by Fujisawa USA (Deerfield, IL, USA). Pentamidine purity was greater than 99% by HPLC analysis. Water was deionized and filtered using a Dracor water-purification system (Durham, NC, USA). The pentamidine metabolites used — 1,5-bis(4′-amidinophenoxy)-3-pentanol, 1,5-di-(4′-amidinophenoxy)-2-pentanol, 5-(4′-amidinophenoxy)pentanoic acid, and 5-(4′-amidinophenoxy)-1-pentanol — were synthesized as hydrochloride salts, as previously described [3,5] and are labelled I–IV, respectively (see Table 1). The synthesis of the internal standard: 1,4-bis(4′-amidinophenoxy)butane dihydrochloride, or butamidine, was previously reported [12].

2.2. HPLC apparatus and conditions

The chromatography system used was a Hewlett-Packard (HP) 1090 HPLC system equipped with an autosampler, a Zorbax RX diisopropyl 4.6×250 mm C_8 column (Mac-Mod, Chadd's Ford, PA, USA) maintained at 40°C, and a HP 1050 variable-wavelength detector set at 265 nm. The system was controlled by a Vectra 486/66U computer with HP Chemstation software installed. The mobile phase consisted of two solvents: A=100% water with 15 m*M* TEA and 35 m*M* acetic acid, pH 4.0; B=75% acetonitrile and 25% water with 15 m*M* TEA and 35 m*M* acetic acid. The compounds were resolved using the following gradient: 7.5–18.75% acetonitrile for 22 min, then 30% acetonitrile for 25 min, and, finally, sharply increasing the acetonitrile concentration to 67.5% for 35 min before recycling. The solvent flow-rate was 1.5 ml/min and the injection volume was routinely 20 μl.

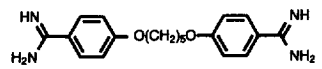
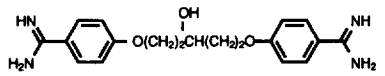
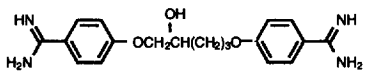
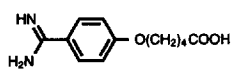
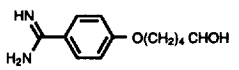
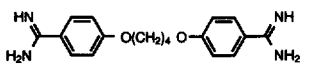
2.3. Rat liver microsomal preparation

Two male and two female Fisher rats (170–180 g) were obtained from Charles River (Wilmington, MA, USA) and housed in quarters in the Department of Laboratory Animal Medicine at the University of North Carolina at Chapel Hill. They were allowed food (Agway, Syracuse, NY, USA) and water ad lib. The rats were killed by decapitation and their livers were removed, washed with ice-cold 50 m*M* potassium phosphate buffer (pH 7.4) and kept ice cold. The livers were suspended in the potassium phosphate (pH 7.4) buffer and were thoroughly homogenized using a Bio-Homogenizer (Bartlettsville, OK, USA). The homogenates were centrifuged in a Sorvall (RC2B; Ivan Sorvall, Norwalk, CT, USA) centrifuge at 9500 *g* for 20 min at 4°C. The supernatants were transferred to thick-walled ultracentrifuge tubes, then centrifuged at 105 000 *g* for 65 min at 4°C in an L3-50 ultracentrifuge (Beckman Instruments, Fullerton, CA, USA). The microsomal pellet was resuspended in a minimal volume of buffer, analyzed for protein content, and used as the enzyme source for in vitro xenobiotic metabolism experiments. The amount of microsomal protein was determined using the standard Bio-Rad (Richmond, CA, USA) Protein Assay following the method of Bradford [14] with bovine γ-globulin as the protein standard.

2.4. Incubation conditions

Stock cofactor solution consisted of 1.6 mg/ml $MgCl_2$, 2 mg/ml NADPH, 1.04 mg/ml glucose-6-

Table 1
Structures of pentamidine and metabolites

Compound label	Structure	Chemical name
P (Pentamidine)		1,5-Bis(4′-amidinophenoxy)pentane
I		1,5-Bis(4′-amidinophenoxy)-3-pentanol
II		1,5-Di-(4′-amidinophenoxy)-2-pentanol
III		5-(4′-Amidinophenoxy)pentanoic acid
IV		5-(4′-Amidinophenoxy)-1-pentanol
B (Butamidine)		1,4-Bis(4′-amidinophenoxy)butane

phosphate and 1.92 U/ml glucose-6-phosphate dehydrogenase in 50 m*M* potassium phosphate buffer. Stock solutions of pentamidine diisethionate (1 m*M*) were dissolved in the above buffer in polypropylene tubes and refrigerated until use. Three ml of 50 m*M* potassium phosphate buffer (pH 7.4) and 1 ml each of cofactor solution, rat liver microsomal preparation and pentamidine as substrate (added last to start reaction) were incubated in a 25 cm^2 open tissue culture flask in a 37°C hot water bath for 30 min. The microsomal and cofactor solutions were maintained at −80°C until used. The final microsomal protein concentration was routinely 0.9 mg/ml. For control incubations, either the substrate or the cofactor was omitted and 1 ml of deionized water was added instead. Samples were extracted immediately over activated solid-phase extraction cartridges.

2.5. Sample extractions

Incubated samples were extracted over activated solid-phase C_{18} Bond Elut (Varian Associates, Sunnydale, CA, USA; 3-ml capacity) extraction cartridges. Samples were extracted slowly under slight vacuum, then washed with 1.5 ml of water and 1.5 ml of 100% acetonitrile, and then eluted with 1.5 ml of HPLC solvent B. The eluate was evaporated to dryness under a gentle stream of nitrogen, resuspended in 200 μl of 10% acetonitrile, then analyzed by HPLC. Quantities of metabolites I and II were determined from standard addition curves using butamidine as the internal standard.

3. Results and discussion

Pentamidine is metabolized in vitro by the liver microsomal fraction of both male and female Fisher rats. Four new peaks (labelled 1–4) were detected, in addition to the substrate pentamidine (P) and the internal standard butamidine (B), upon HPLC analysis of a sample incubate of male rat liver microsomes (Fig. 1A). The new peaks were not formed when either cofactor (Fig. 1B) or pentamidine (Fig. 1C) was removed from the incubation mixture. The four newly formed peaks were not present as impurities

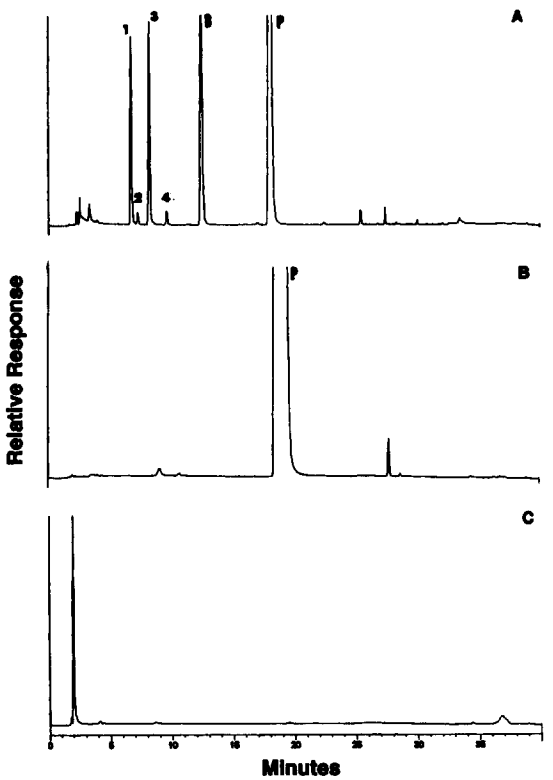


Fig. 1. Metabolism of pentamidine by liver microsomes from male Fisher rats. Incubations were performed at 37°C for 30 min, then extracted and assayed as described in the text. (A) Chromatogram of an experimental incubation containing 167 μ*M* pentamidine, cofactor solution (NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $MgCl_2$) and microsomes in 50 μ*M* potassium phosphate buffer (pH 7.4). Peaks 1–4 are newly formed putative metabolite peaks. (B) Chromatogram of a control incubation lacking cofactor solution. (C) Chromatogram of a control incubation omitting pentamidine. Peaks B and P are butamidine, as internal standard, and pentamidine, respectively.

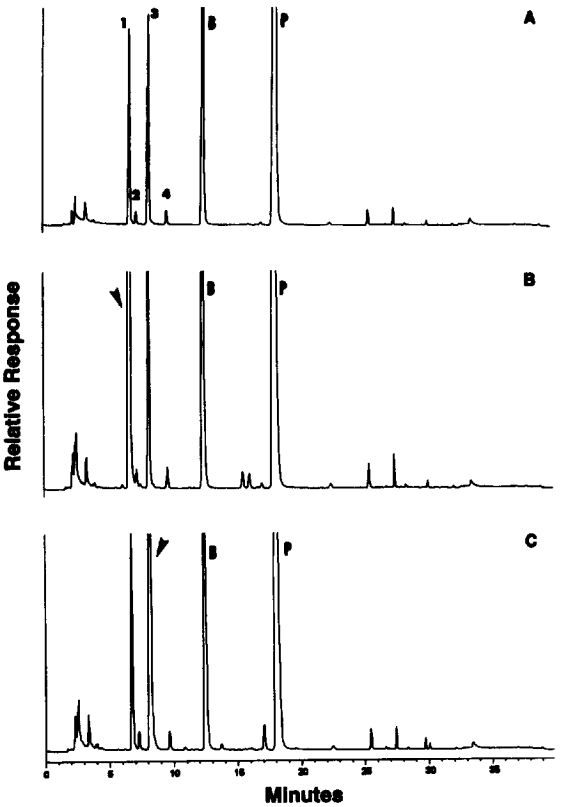


Fig. 2. The coelution of standard 1,5-di(4′-amidinophenoxy)-2-pentanol and 1,5-bis(4′-amidinophenoxy)-3-pentanol with two major metabolite peaks. (A) Chromatogram of an experimental incubation showing four putative metabolite peaks. (B) The same sample after spiking with 1,5-di(4′-amidinophenoxy)-2-pentanol standard, showing coelution with peak 1. (C) The same sample after spiking with 1,5-bis(4′-amidinophenoxy)-3-pentanol standard, showing coelution with peak 3. The arrows indicate those peaks which increased in peak area due to spiking experiments. Peaks B and P are butamidine, as internal standard, and pentamidine, respectively.

of pentamidine, cofactors or the enzyme preparation. Female rat liver microsomal incubations produced the same qualitative results; therefore, all subsequent experiments were done using the liver microsomes from male rats.

Coelution experiments performed by spiking extracts with authentic standards of previously identified pentamidine metabolites [3–5,10,13] suggest the four new peaks were formed by chain hydroxylation of pentamidine. Peak 1 coeluted with compound II (Fig. 2B) and peak 3 coeluted with compound I (Fig. 2C). Additional coelution experiments showed that peaks 2 and 4 are compounds III and IV, respectively (data not shown). The *N*-hydroxylated metabolites found in prior studies of pentamidine metabolism using other mammalian species were not detected. The lack of detection of both mono- and dipentamidoxime in the Fisher rat liver microsomal incubation sample is not surprising given that previous studies of pentamidine hydroxylation by Sprague–Dawley rat liver microsomes reported that very small amounts of the *N*-hydroxylated metabolites were detected [4,5,10,13,15].

Though coelution experiments provide tentative confirmation of a compound's identity, previously reported chromatographic separations of enzyme mixtures and extracts incubated under identical conditions produced chromatographic profiles similar to Fig. 1A [4,5,10,13,15]. The analogous peaks of those chromatograms were analyzed by LC–MS techniques and were positively identified as the four metabolites reported here [13].

In agreement with previous studies with Sprague–Dawley rat liver microsomes [3–5], metabolites I and II were also formed in the greatest amounts by Fisher rat liver microsomes. Quantities of newly formed compounds I and II were calculated from standard addition curves using butamidine as the internal standard. The products of pentamidine C-1 hydroxylation, compounds III and IV, were not quantified. The extraction recoveries for compounds I and II using male liver microsomes were 89% and 83%, respectively. An average of 0.24 nmol/min/mg protein of metabolite I and 0.25 nmol/min/mg protein of metabolite II were produced by male Fisher rat liver microsomes. These two metabolites accounted for the turnover of 9.1% of the available pentamidine under the incubation conditions described. This result compares to 11.3% turnover of pentamidine reported from a mass balance study using radiolabelled pentamidine and male Sprague–Dawley rat liver microsomes [5].

This research thus demonstrates that pentamidine is indeed metabolized to at least four chain-hydroxylated products by Fisher rat liver microsomes. These results are in agreement with previous studies which used microsomes isolated from Sprague–Dawley rats, mice, rabbits and humans [3–5,10,15], and bacterially-expressed human cytochrome P-450 isozymes [15], and showed that those systems all metabolize pentamidine by chain hydroxylation. The current study contradicts a recent report that Fisher rat liver homogenates do not metabolize pentamidine [11]. We do not know why results from the two studies differ. However, we have been able to resolve pentamidine from its primary hydroxylated metabolites in our laboratories only by using gradient elution HPLC methods similar to those reported here or by using HPLC–MS methods [3–5,9,13]. Finally we demonstrate here that pentamidine cannot be distinguished from its primary metabolites by UV

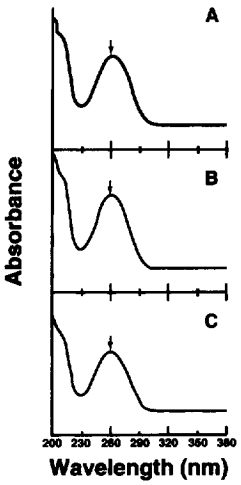


Fig. 3. Ultraviolet spectra of pentamidine and two synthesized metabolite standards. UV spectra of (A) pentamidine, (B) 1,5-di(4′-amidinophenoxy)-2-pentanol, and (C) 1,5-bis(4′-amidinophenoxy)-3-pentanol. The arrows pinpoint the maximum absorbance of each compound. All three compounds were assayed at 10 μ*M* in distilled water using a Shimadzu UV-2101 PC (Columbia, MD, USA) spectrophotometer set to scan from 200–380 nm.

spectral characteristics. As shown in Fig. 3, the UV spectra of pentamidine and metabolites I and II are essentially identical. Very similar UV spectra were also obtained for the two other hydroxylated metabolites, with only very slight differences in the shapes but not the absorption maxima of the curves (data not shown). These results demonstrate that photodiode-array detection techniques cannot discriminate pentamidine from its metabolites in the absence of chromatographic resolution.

Thus, the liver microsomal preparations from Fisher rats, like microsomes from Sprague–Dawley rats, mice, rabbits, and humans, metabolize pentamidine. Although studies of human pentamidine metabolism have thus far been limited to two published reports [10,15], both using human liver microsomes and bacterially-expressed cytochrome P-450 enzymes, pentamidine metabolism appears to be qualitatively similar for the human samples and the animal models examined. The same primary metabolites are formed by microsomes from each species. The rabbit may prove to be a more appropriate animal model of human pentamidine metabolism, however, as the *N*-hydroxylated products are

formed at substantially higher rates by rabbit [15] and human [10,15] liver microsomes. An obvious important question that should now be addressed is whether patients treated with pentamidine metabolize the drug. The methods reported here, particularly when combined with our previously reported mass spectrometric methods [13], can be used to evaluate human pentamidine metabolism in vivo. If human pentamidine metabolism is quantitatively significant, then the pharmacokinetics of pentamidine should be reassessed.

Acknowledgments

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