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High-performance liquid chromatographic determination of pentamidine in plasma

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ABSTRACT

This report describes the analysis of pentamidine by isocratic reversed-phase high-performance liquid chromatography (HPLC) using a commercially available compound (melphalan) as the external standard. Previously described assays use ion-pairing HPLC, an internal standard (hexamidine) that is not readily available, and require a relatively large sample size. In the present assay, pentamidine was extracted from plasma using solid-phase extraction and was analyzed using a C_{18} column and a mobile phase containing 18% acetonitrile, 2% methanol, 0.2 *M* ammonium acetate and 0.5% triethylamine. The identity of the eluting peaks was verified using a diode array detector. The extraction yield of pentamidine was 82%. The limit of detection was 8.6 ng/ml with a sample size of 100 μl. The inter-day and intra-day coefficients of variation ranged between 0.3% and 10% with an average of 5%. This method was applied to study the pharmacokinetics of pentamidine in rodents.

INTRODUCTION

Pentamidine, an aromatic diamidine with antiprotozoal activity, has been used in the treatment of trypanosomiasis, leishmaniasis, and *Pneumocystis carinii* pneumonia (PCP) [1–3]. PCP is a major cause of death in patients with acquired immunodeficiency syndrome (AIDS). Thus, the use of pentamidine for prophylaxis and treatment of PCP in these patients has increased dramatically. However, the usefulness of this drug is limited by its side effects including nephrotoxicity, hypotension, abnormal liver function, hypoglycemia and pancreatic toxicity [4–6]. AIDS patients often receive other drugs. Among these, 2′,3′-dideoxyinosine, a reverse transcriptase inhibitor, has been shown to cause acute, life-threatening pancreatitis [7]. Numerous reports have shown that up to 80% of AIDS patients receiving concurrent pentamidine and 2′,3′-dideoxyinosine therapy develop clinical pancreatitis [7–9], suggesting an interaction between these two drugs. To evaluate the potential pharmacokinetic interaction in experimental animals such as rodents, a sensitive assay is needed to analyze pentamidine in a limited blood volume.

A number of analytical methods for pentamidine in plasma, serum and urine have been reported [10–13]. Bernard *et al.* [10] reported a bioassay where pentamidine concentrations were quantitated by growth inhibition of *Candida tropicalis*. The detection limit is *ca.* 80 ng/ml using 100 μl plasma. Several ion-pair high-performance liquid chromatographic (HPLC) assays have been described for pentamidine in human plasma [11–16]. Although these assays have a high sensitivity of *ca.* 1 ng/ml, they require a relatively large sample size, *i.e.* 500 μl, which is

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not readily obtainable in rodents. In most of the reported HPLC methods, the internal standard was hexamidine, which is not commercially available. The present report describes an isocratic reversed-phase HPLC assay with fluorescence detection to analyze pentamidine in small samples (100 μl). A commercially available compound, melphalan, was used as the external standard.

EXPERIMENTAL

Chemicals

All HPLC solvents and reagents were of analytical or HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pentamidine (Lot No. 29F5603), melphalan (Lot No. 79F07001), acetophenetidin (phenacetin) (Lot No. 82H0748) and triethylamine (Lot No. 50H0805) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC analysis showed that pentamidine and melphalan were >95% pure. Both chemicals were used as received. Storage of solutions of pentamidine in water and melphalan in methanol at −70°C for up to five months gave negligible (<5%) degradation.

Apparatus

The HPLC system consisted of a solvent pump (Spectroflow 400, Applied Biosystem, Foster City, CA, USA), an automated injector (WISP 712, Waters Assoc., Milford, MA, USA), a scanning fluorescence detector (Model 470, Waters Assoc.) and an HP 3396A integrator (Hewlett-Packard, Menlo Park, CA, USA). The fluorescence detector was set at an excitation wavelength of 265 nm and emission wavelength of 345 nm. The UV spectra of the eluting peaks were determined using a diode array detector (Model 1040A, Hewlett-Packard), placed in sequence to an UV detector. The stationary phase was a reversed-phase C_{18} column (Resolve, 150 mm × 3.9 mm I.D., 5 μm particle size, Waters Assoc.). The aqueous mobile phase consisted of 18% acetonitrile, 2% methanol, and 0.5% triethylamine in 0.2 *M* ammonium acetate buffer. The pH of the mobile phase was adjusted to 3.8 with acetic acid. The solvent flow-rate was maintained at 1.4 ml/min. All analyses were carried out at ambient temperature.

Animal study

A six-month-old female Fisher rat (Charles River Breeding Lab., Kingston, NJ, USA), weighing 202 g, was used for the pharmacokinetic study. One day prior to the experiment, the rat was anesthetized with ether and a permanent catheter was inserted into the right external jugular vein for blood sampling. A second catheter was inserted into a lateral tail vein for drug administration. The animal was housed in a metabolic cage with access to food and water *ad libitum* overnight. On the day of the experiment, a 7.3 mg/kg dose of pentamidine (0.4 mg/ml dissolved in physiologic saline) was administered by intravenous infusion over 3 h. Constant rate infusion was achieved using a syringe pump (Model 940, Harvard Apparatus, South Natick, MA, USA) calibrated to deliver 0.0206 ml/min. Serial blood samples (200 μl each) were collected over 24 h through the venous catheter. After each sampling, an equal volume of heparinized physiologic saline was infused to replace the lost fluid volume. Blood samples were placed in heparinized tubes and kept on ice to minimize clotting and/or pentamidine decomposition. Blood samples were centrifuged at 13 000 *g* for 1 min at 0°C and the plasma fraction transferred to a separate tube and stored at −70°C until analysis.

Sample extraction

Plasma sample clean-up was performed using solid-phase extraction (SPE-C_8 and SPE-C_{18}) columns (Bond Elut, Jones Chromatography, CO, USA). The SPE columns were preconditioned with two 1-ml aliquots of 100% methanol, two 1-ml aliquots of eluting buffer (acetonitrile–1.0 *M* ammonium acetate, pH 3, 75:25, v/v), and five 1-ml aliquots of distilled water. Each fraction of the conditioning solvents was allowed to equilibrate with the SPE column for 1 min and was then pulled through the column under suction. Care was exercised so that the solid-phase material was immersed in solvent at all times. Plasma (100 μl) was mixed with 100 μl of distilled water and added to the

preconditioned SPE-C_8 column. After equilibration for 1 min, the solution was pulled through under suction. After the column was washed with three 1-ml aliquots of methanol, the external standard melphalan (40 ng in 80 μl of methanol) was added to the SPE-C_8 column. Pentamidine and melphalan were eluted with 1 ml of eluting buffer. The eluent was dried in a SpeedVac (SC 210A, Savant Ins., Farmingdale, NY, USA) and then reconstituted with 200 μl of the mobile phase. Fifty μl was analyzed by HPLC.

Because extraction and washing of the plasma and the liver homogenates using the SPE columns may lead to the loss of the more polar pentamidine metabolites, the liver homogenates and some of the plasma samples were not extracted, and directly analyzed by HPLC. The external standard (0.4 μg/200 μl melphalan) was added to some of the samples.

Detection of pentamidine metabolites

One group of investigators reported the isolation and identification of pentamidine metabolites generated using liver homogenates or isolated perfused livers [17–20]. To evaluate the elution of these metabolites in the HPLC system and whether these metabolites may potentially interfere with the pentamidine elution, we examined the metabolism of pentamidine using liver homogenates.

Five months old female Fisher rats, weighing 220 ± 15 g, were used for the *in vitro* metabolism studies. Animals were fasted for 24 h prior to the studies. Tissue homogenates were prepared by established methods [21]. In brief, the liver was removed from an animal and homogenized in 1.15% KCl in 10 m*M* phosphate buffer, pH 7.4 using a motor-driven pestle tissue homogenizer. The homogenate was centrifuged for 20 min at 9000 *g* in a refrigerated centrifuge. The supernatant fraction was carefully transferred and was used as the enzyme source. The incubate contained 1.0 ml of liver homogenate supernatant, 1.0 ml of cofactor solution (2.0 mg of NADPH and 1.9 mg of $MgCl_2$) and 3.0 ml of 50 m*M* phosphate buffer (pH 7.4) preincubating at 37°C in a shaking water bath for 15 min, as previously described [17–19]. The metabolism experiments were conducted at 37°C in a shaking water bath. One ml of pentamidine stock solution was added to initiate the reaction. Aliquots (200 μl) of the incubation mixture were taken at different time intervals (every 30 min up to 240 min). The samples were then stored at −70°C until analysis. Control experiments to measure the nonenzymatic degradation were done simultaneously using enzyme solution pre-boiled at 100°C for 3 h.

To confirm the enzymatic activity of the liver homogenates, the metabolism of phenacetin to acetaminophen was measured in simultaneous experiments. Briefly, 1 ml of 20 m*M* phenacetin was incubated with the liver homogenates. The final concentration was 3.3 m*M* phenacetin. The analysis of phenacetin and acetaminophen was similar to the previously described methods [30,31] with some modification. After extraction by ethyl acetate, phenacetin and acetaminophen were analyzed by isocratic reversed-phase HPLC and UV detection at 254 nm. The stationary phase was an Ultraphere-I.P. C_{18} column (250 mm × 4.6 mm I.D., 5 μm particle size, Beckman, Fullerton, CA, USA), and the mobile phase was methanol–0.1 *M* KH_2PO_4–0.01% acetic acid (23:74:3, v/v/v).

RESULTS AND DISCUSSION

Sample extraction and recovery

We compared the retention of pentamidine on two different SPE columns, *i.e.* SPE-C_8 and SPE-C_{18}. SPE-C_8 and SPE-C_{18} retained 88% and 78% of pentamidine, respectively, after washing with three 1-ml aliquots of 100% methanol. The SPE-C_8 column showed a higher retention of pentamidine, and was selected for subsequent use. Washing the SPE-C_8 column with 100 m*M* phosphate buffer (pH 6.9) or 50% methanol instead of 100% methanol yielded several interferences in the HPLC chromatographs, indicating insufficient sample clean-up. With the 100% methanol wash there was one interference coeluting with pentamidine. We subsequently discovered that this interference originated from the extraction column. This interference was eliminated by washing the SPE-

C_8 column with eluting buffer and distilled water during the preconditioning step.

The recoveries of pentamidine for SPE-C_8 columns using ammonium acetate buffer at pH 3, 4, and 5 were 90, 88, and 78%, respectively ($n = 3$ at each pH value). At pH 3, the extraction yields at four different concentrations, *i.e.* 29, 57, 144 and 287 ng/ml, were 81.2, 81.9, 82.5 and 83.3%, respectively ($n = 3$ for each concentration), with an overall average of 82%. In the final procedures, 100% methanol was used in the preconditioning washing step and 1 *M* ammonium acetate of pH 3 was used for elution.

Selection of external standards

Several compounds were evaluated as potential internal or external standards. The ideal standard should have similar retention and extraction characteristics as pentamidine, show fluorescence at the same excitation and emission wavelengths and be readily available. Diminazene, a commercially available structural analog of pentamidine, was not adequately retained on the C_{18} analytical column. Disopyrimide, used in a previous study [16], did not show fluorescence. Melphalan was selected due to its fluorescence and retention on reversed-phase columns [22].

However, melphalan was readily eluted from the SPE-C_8 extraction column by the preconditioning methanol washes and could not be used as an internal standard. On the other hand, the high and reproducible extraction yield of pentamidine suggested that an external standard, instead of an internal standard, could be used in the analysis. Melphalan was used as an external standard and was added between the clean-up and the elution steps.

Selection of mobile phase

We examined the use of acetonitrile, methanol, and mixtures of acetonitrile and methanol to separate interfering endogenous plasma components from pentamidine and melphalan and to develop the final assay conditions. Methanol and acetonitrile affected the retention and elution of the interferences, pentamidine and melphalan to different extents. For the interferences, the decreases in retention by increasing methanol concentration were greater than by increasing acetonitrile concentration, while the reverse was true for pentamidine and melphalan. By using a combination of methanol and acetonitrile in the mobile phase, the interfering plasma peaks were separated from pentamidine and melphalan. Several buffers including sodium phosphate, sodium acetate and ammonium acetate were evaluated. Pentamidine was eluted by 0.2 *M* ammonium acetate buffer but was not eluted with sodium phosphate and sodium acetate at the same concentration. The band symmetry of pentamidine was improved by increased ammonium acetate concentration. Amine modifiers such as triethylamine in the mobile phase may prevent the tailing phenomenon of basic compounds [20]. We found that the inclusion of triethylamine in the mobile phase eliminated peak tailing. The selected mobile phase was 18% acetonitrile, 2% methanol, 0.5% triethylamine and 0.2 *M* ammonium acetate (pH adjusted with acetic acid to 3.8). The elution volumes of pentamidine and melphalan were 8.7 and 12.9 ml, respectively. Representative chromatograms of pentamidine in rat plasma samples are shown in Fig. 1.

Standard curve and precision

The lower detection limit of this assay was 8.6 ng/ml, using a 100-μl plasma sample. The standard curve of pentamidine in rat plasma was linear over a concentration range of 8.6 to 574 ng/ml, with a coefficient of determination of 0.9998. Table I shows the inter-day and intra-day precision and coefficients of variation at a concentration range of 17.2 to 287 ng/ml. The average coefficient of variation was *ca.* 5%.

Lack of pentamidine metabolism by rat liver homogenates

Metabolism of pentamidine and phenacetin was studied simultaneously in rat liver homogenate experiments. Incubation of pentamidine with rat liver homogenates for 4 h showed a <2% decrease in the HPLC peak corresponding to pentamidine. This is similar to the extent of nonenzymatic degradation in the pre-boiled liver homogenates (<3%). In comparison, the same liver enzyme preparation metabolized phen-

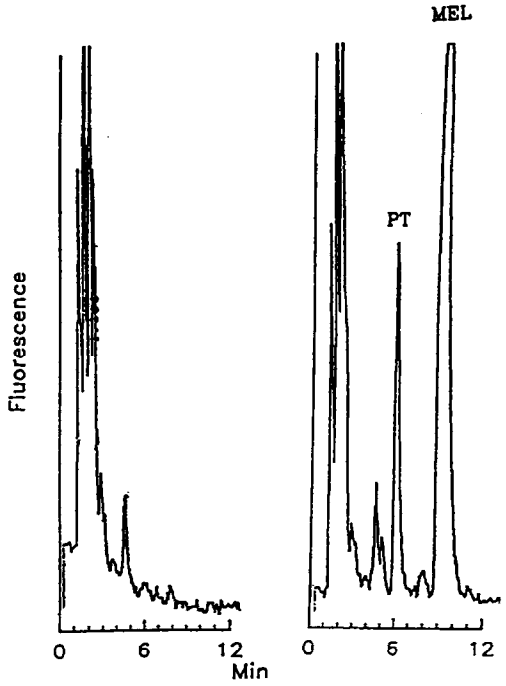

Fig. 1. Chromatograms of plasma extracts. Extracts of rat plasma samples were analyzed using a C_{18} column. The mobile phase consisted of 18% acetonitrile, 2% methanol, 0.5% triethylamine and 0.2 *M* ammonium acetate buffer (pH adjusted with acetic acid to 3.8). Blank plasma (left panel); plasma spiked with 28.7 ng/ml pentamidine (PT) and 400 ng/ml melphalan (MEL) (right panel).

TABLE I

ASSAY PRECISION

Concentration (ng/ml)	Pentamidine/melphalan peak-height ratio		
	Mean	S.D.	C.V. (%)
A. Intra-day variation ($n = 3$)			
17.2	0.096	0.0010	1.0
28.7	0.156	0.0127	8.1
57.4	0.313	0.0078	2.5
114.8	0.647	0.0190	2.9
229.6	1.148	0.1168	10.2
287.0	1.573	0.0381	2.4
B. Inter-day variation ($n = 3$)			
17.2	0.102	0.0056	5.5
28.7	0.162	0.0067	4.1
57.4	0.342	0.0318	9.3
114.8	0.645	0.0021	0.3
229.6	1.179	0.0670	5.7
287.0	1.466	0.0910	6.2

acetin to acetaminophen with 19% ± 3.5% (n = 3) of phenacetin degraded after 3 h. There was no nonenzymatic degradation of phenacetin by the pre-boiled enzymes after 4 h incubation. The phenacetin metabolism confirmed the metabolic activity of the rat liver enzymes. These data indicated no pentamidine metabolism under the same conditions. To rule out the coelution of pentamidine with its metabolites, additional experiments examined the UV spectra of the eluting peaks. Fig. 2 shows the UV spectra of the pentamidine peaks obtained from pure pentamidine and from mixtures of pentamidine with liver homogenates before and after 4 h of incubation. These UV spectra were identical with UV_{max} at 267 nm and UV_{min} at 235 nm and 300 nm. These data rule out coelution of pentamidine metabolites with pentamidine. Similarly, the UV spectra of the pentamidine peaks obtained from plasma samples either extracted or not extracted by the SPE columns were identical to that from the pure pentamidine (data not shown).

Several groups of investigators examined the metabolism of pentamidine under *in vitro* and *in vivo* conditions [10,17–20,23–29]. All except one group reported minimal or undetectable pentamidine metabolism [10,23–29]. This is in agreement with our results showing a lack of pentamidine metabolism under *in vitro* conditions by metabolically active rat liver homogenates and under *in vivo* conditions in rats.

Application to pharmacokinetic study

Fig. 3 shows the plasma concentration–time profile of pentamidine in a rat given an intravenous infusion of 7.3 mg/kg. Plasma pentamidine concentrations increased during infusion and reached a peak at the end of the 3 h infusion. After termination of infusion, the concentrations declined biexponentially, with a terminal half-life of 9.4 h.

CONCLUSIONS

This report describes an analytical method for pentamidine using solid-phase extraction and reversed-phase HPLC. This method offers several advantages over existing bioassays and ion-

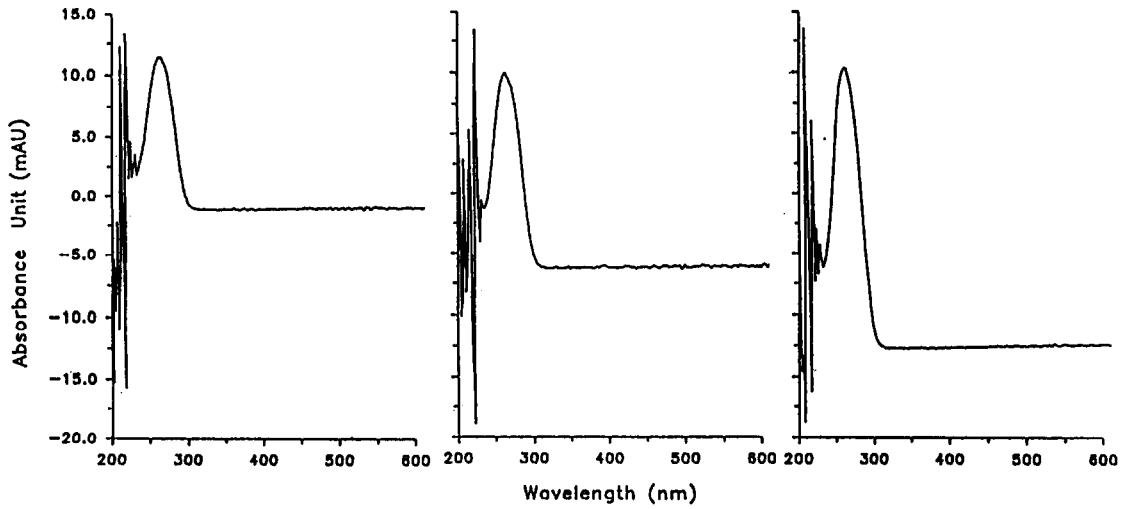

Fig. 2. UV spectra of pentamidine. UV spectra of pentamidine peaks obtained from pure pentamidine (left panel) and from mixtures of pentamidine with liver homogenates before (center panel) and after 4 h incubation (right panel).

pairing HPLC assays [10–15,17–21]. This method does not require ion-pairing reagents in the mobile phase nor in the eluting buffer, offering the advantage of a shorter column equilibration time compared to ion-pairing HPLC. Furthermore, this assay has a high sensitivity with a lower detection limit of 8.6 ng/ml using a 100-μl sample, and uses a commercially available compound, melphalan, as the external standard. As shown in this report, this assay can be used to study the pharmacokinetics of pentamidine in small rodents.

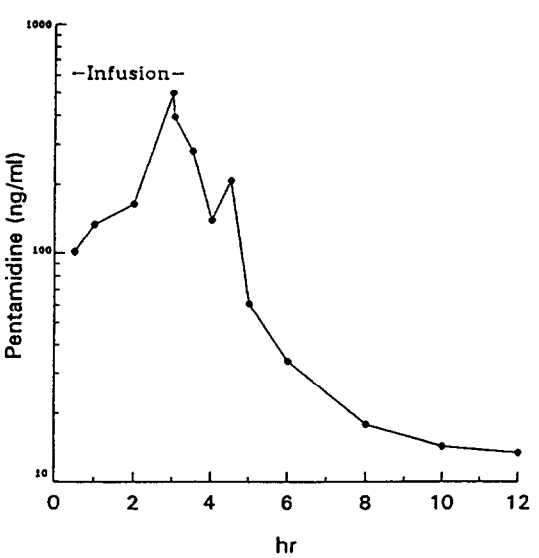

Fig. 3. Concentration–time profile of pentamidine in a rat. A rat was given an intravenous infusion of pentamidine (7.3 mg/kg) over 3 h. The rate of infusion was 0.0206 ml/min.

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