Title

Paired ion liquid chromatographic method for the analysis of pentamidine in human biological fluids and mouse tissues

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PAIRED ION LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PENTAMIDINE IN HUMAN BIOLOGICAL FLUIDS AND MOUSE TISSUES

by

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THESIS

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ABSTRACT

A high performance liquid chromatographic method was developed for the analysis of pentamidine in human plasma, urine and mouse tissues. Sample clean-up involves precipitating plasma or homogenized tissues with acetonitrile containing hexamidine as an internal standard. The resulting supernatant of the plasma, homogenized mouse tissues, and urine spiked with internal standard were passed through a C_8 Bond Elut column and eluted with a solution containing 0.5% sodium 1-heptanesulfonate, 0.02% tetramethylammonium chloride and 0.1% phosphoric acid in 97.5% methanol. Using fluorescence detection (EX: 275 nm and EM: 340 nm), the detection limits were 1.15 ng/ml for plasma, 5.75 ng/ml for urine and 15 ng/gm for mouse tissues. The coefficients of variation for inter-day and intra-day were approximately 10%, while the average recoveries were 78%, 78%, 68%, 67%, 67% for plasma, urine, lung, kidney, and liver, respectively. The assay was not interfered from any drugs that might be taken in conjunction with pentamidine by patients infected with pneumocystis carinii pneumonia.

INTRODUCTION

GENERAL

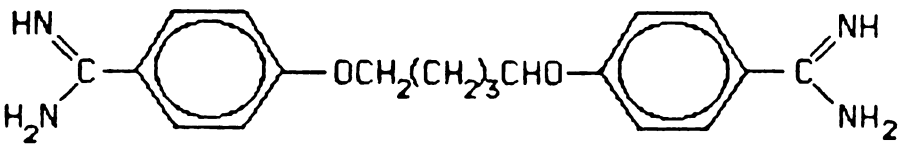
Pentamidine or 4,4'-diamidinophenoxypentane, is a member of a series of aromatic diamidine compounds synthesized in the 1930's and extensively evaluated for antiprotozoan activity (Figure 1).

MECHANISM OF ACTION

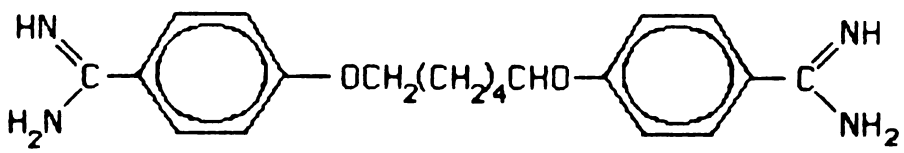
Pentamidine has some biological properties which may contribute to its pharmacologic and toxicologic effects. In 1944, Blaschko et al.[1-2] found that pentamidine inhibited liver histaminase. Gemmill[3] indicated that pentamidine, as well as other amidines, inhibited anaerobic glycolysis of glycogen to lactate in muscle extracts. The susceptibility of different species of trypanosomes to pentamidine appear to be related to the relative importance of aerobic and anaerobic glycolysis in their metabolism[4].

In vitro, pentamidine was found to react with isolated nucleic acids to form water-insoluble precipitates at neutral pH. This effect was observed with all types of DNA and RNA of bacterial, fungal and mammalian origin. The nucleotide diphosphate molecule is the minimum nucleic acid component necessary for the interaction with aromatic diamidines to occur[5].

Pentamidine may be effective against pneumocystis carinii by virtue of its ability to inhibit both dihydrofolate reductase (DHFR)[6-7] and thymidylate synthetase[8]. These two enzymes are essential for the conversion of dUMP to dTMP. The doses required to inhibit nucleic acid and protein synthesis in human are far in excess of the doses required in protozoans.



Pentamidine



Hexamidine

Figure 1. Chemical Structures of pentamidine and hexamidine.

Theoretically, such differences in drug sensitivity between parasite and host offer an opportunity for exploitation by chemotherapy[9-10].

CLINICAL USE

Since 1941, pentamidine has been extensively used outside the United States, primarily for the treatment of specific tropical diseases. It is the drug of choice in the therapy of early African sleeping sickness[11-12] and is used prophylactically against Trypanosoma gambiense[13-14] and is of value in the treatment of leishmania resistent to sodium antimony tartrate and ethyl stilbene[15]. Pentamidine also has antibacterial and fungicidal properties[16] it exhibits activity against specific types of malaria in animals[17]. Of particular significance are the excellent results obtained with pentamidine in the treatment of pneumonia caused by the pneumocystis carinii[18].

Pneumocystis carinii pneumonia (PCP) is a protozoal infection confined to the lung, and is most commonly seen in patients with disseminated malignant neoplasms, in patients with congenital or acquired immunologic defects, as well as in malnourished, premature infants[19-21]. If left untreated, it is lethal. Of the 743 Acquired Immune Deficiency syndrome (AIDS) patients with PCP reported to the Center for Disease Control, 45% were known to have died, usually of PCP, with or without other serious infections[22].

PHARMACOKINETIC STUDIES

Pentamidine isethionate is fairly well absorbed from parenteral sites of administration. Clinical pharmacokinetic-pharmacodynamic studies are limi-

ted[23-25] because of the lack of a precise, sensitive analytical method and because the drug is too toxic to give to volunteers. A spectrophotometric assay was used to measure pentamidine concentrations in plasma urine and various tissues[23-25]. Peak plasma concentration of 0.3 to 1.4 mcg/ml were present in 7 patients after receiving intramuscular injection of pentamidine 4 mg/kg/day. According to our study, following a single IV dose of 4 mg/kg, the peak concentration of pentamidine in human plasma is 228 ng/ml and the trough level is 2.37 ng/ml in an AIDS patient. Pentamidine is highly bound to nucleic acid in tissue and is gradually released[5]. After intravenous or intraperitoneal injection in mice, the highest concentration of pentamidine was found in the kidney, followed by the liver[24-25]. For the patient studied, pentamidine blood levels during therapy remained low and the amount excreted in urine was approximately 20% or less of the daily dose. It was found to be excreted intact in urine and feces with no evidence of any metabolic breakdown products in Launoy's C^{14} studies[26-27]. The TLC results of Wealkes and Devita support these findings[23].

ADMINISTRATION AND DOSAGE

Because of the high incidence of immediate undesirable effects when given intravenously, pentamidine is best given by intramuscular administration with a dose of 4 mg/kg of body weight[28]. The drug is more effective for the treatment of T. gambiense infections than in those casued by T. rhodesiense. The use of pentamidine is restricted to the treatment of early sleeping sickness without central nervous system involvement because the drug fails to penetrate the blood-brain barrier[29-30].

areas[28]. Intramuscular injections falling within the range of 3 to 5 mg/kg should be given at intervals of no longer than 6 months.

In the treatment of visceral leishmaniasis, pentamidine has been used successfully by administrating a daily dose of 4 mg/kg for 12–15 days. A second series of 12 to 15 doses may be necessary in areas where the infection is known to be less responsive to treatment. The drug is particularly useful in cases in which patients have failed to respond to antimonials.

Cases of PCP should be treated daily with 4 mg/kg pentamidine intramuscularly for 12 - 14 days. In severe cases the drug may be infused slowly over a 5 - 10 minute period. Clinical effects are usually apparent 4 - 6 days after the first injection, but radiographic improvement is often unapparent for several weeks[31]. A high percentage of cures can be expected, depending on supportive therapy. However, elimination of predisposing conditions is still essential.

TOXICITY AND SIDE EFFECTS

In mice, the LD_{50} for intravenous administration of pentamidine is equivalent to 28 mg/kg compared to 64 mg/kg for the subcutaneous route[32]. Death resulted from the generalized central nervous system depression followed by respiratory failure.

Numerous side effects were reported following intravenous administration of the drug. Dermarch estimated that existence of side effects from intravenous pentamidine may range as high as 75%, while with intramuscular pentamidine the range is 0.8 - 5%[33].

The immediate reactions to intravenous pentamidine include breathlessness, tachycardia, dizziness, sweating, headache and vomitting[34–35]. These

reactions are probably connected with the sharp fall in blood pressure that follows an intravenous administration of the drug which is too rapid. The signs and symptoms of these immediate reactions have been ascribed to "vago-sympathetic disequilibrium." These toxic manifestations invariably disappear after 10 - 30 minutes[35].

Other reported systemic adverse effects include skin rash, transient azotemia, hypoglycemia, followed by hyperglycemia, hypocalcemia, elevated liver enzyme levels and netropenia[36-39].

Renal lesions are the most common side effects reported to the Parasitic Disease Drug Service of Center for disease control. Animal studies have shown that pentamidine achieves its highest concentration in the kidney and is released from this organ slowly[23]. The interaction of pentamidine with nucleic acids and its inhibition of DHFR might play roles in its renal toxicity.

PRECAUTIONS

Because of its toxicity, pentamidine should be used carefully in patients with hypertension, diabetes mellitus, malnutrition, hepatic dysfunction, renal disease, or megaloblastic anemia. If the drug has to be used by a pregnant woman, the dosage should be reduced. In most instances, injection is limited to women who are more than five months pregnant.

Blood pressure and laboratory tests (e.g. CBC, platelets, urinalysis, renal function tests, liver function tests, serum calcium and phosphorus) should be performed before and during pentamidine therapy.

SIGNIFICANCE OF THE ASSAY

Knowledge of the pharmacodynamics and pharmacokinetic profiles of a drug is useful not only for the development of a better understanding of the drug's mechanism, but also for the assessment of a more rational dosing regimen. Although pentamidine has been used for more than forty years, knowledge of its clinical pharmacology is presently limited. The relationship of blood, urine and tissue concentrations of pentamidine to its efficacy and toxicity has not been studied. Furthermore, in patients with impaired renal function, the effect of repeated doses of pentamidine on the drug's pharmacokinetics is also unknown. Due to the drug's toxicity to the kidney, attempts are underway to use liposomes to deliver pentamidine to the target organ in PCP patients.

Liposomes are small, enclosed vesicles composed of phospholipid membrane, which can encapsulate a variety of macromolecules, including drugs. They can be used to transport drugs to various target sites throughout the body. The delivery of liposomes to the lungs depends upon the liposome's size as well as its phospholipid composition[40]. Larger liposome vesicles accumulate in the lung particularly well. Possible explanations for this phenomenon are that the largeer liposomes form pulmonary microembolisms[41], or that there is a binding affinity between liposomes and lung capillary endothelial cells. The area available for endothelial cell contact increases with liposome size. Phosphatidyl serine-containing liposomes tend to accumulate in the lungs more so than other negatively charged liposomes (the mechanism is still unknown). Thus the efficacy of the drug could be enhanced and its toxicity reduced.

In order to study pentamidine's pharmacokinetics and test the effectiveness of the liposome delivery system, a simple and precise analytical method is necessary. The CDC LC method is presently preferred over the spectrofluorometric method, but it is neither simple nor precise. The procedure is time-

consuming, involving three seperate extractions; the assay utilizes U.V. detection which is less specific than fluorescence detection; and the assay sensitivity is limited by its low (20 - 35%) recovery. Furthermore, its reproducibility is questionable due to the large number of sample repeats which must be run. For this reason, we developed the described HPLC method.

REVIEW OF ANALYTICAL METHODS

1. Spectrofluorometric method - Waalkes and Devita (23)

A spectrofluorometric method was developed by Waalkes and Devita to measure pentamidine concentration in human plasma, human urine and mouse tissues(23). Pentamidine is extracted under basic conditions from biological samples into butanol. Following back extraction into acid, the fluorescent glyxalidone derivative formed by reacting pentamidine with glyoxal, and benzaldehyde was measured with a spectrophoto-fluorometer. The procedure is quite tedious and the detection limit is only about 200 ng/ml.

2. High Performance Liquid Chrometography

A reverse phase HPLC assay for pentamidine using hexamidine as an internal standard was recently reported by the Centers for Disease Control (CDC)(42). After precipitating the protein with sodium hydroxide (NaOH) and neutralization with HCl, pentamidine is extracted into chloroform in the prescence of diethylhexyl phosphoric acid. After concentraction into the acid and back extraction into the organic phase (CH_2Cl_2), the sample is separated

by an Alltech C_{18} column in a mobile phase of 60% methanol, 0.05M sodium heptanesulfonate, and 0.014M triethylamine with the pH adjusted to 3.0 with phosphoric acid. Using UV detection set at 280 nm, the detection limit is about 12 ng/ml with a 20-35% recovery.

EXPERIMENTAL

MATERIALS AND CHEMICALS

Pentamidine, as isethionate salt, was supplied by May & Baker Co. (Dagenham, England), while the hexamidine was a gift from Dr. John Conte of UCSF. Quinine sulfate dihydrate was purchased from Aldrich Chemical Co. (Milwaukee, Wis). Tetramethylammonium chloride (Fluka Chem. Co., Hauppauge, NY) and sodium 1-heptanesulfonate(sigma chem. Co., St. Louise, MO) were obtained commercially. Methanol and acetonitrile were HPLC grade (J.T. Baker Chem. Co., Phillipsburg, NJ) while other reagents were of analytical grade. Water was obtained through a nanopure apparatus (Barnstead Co., Boston, MA).

APPARATUS

A solvent delivery pump (Model 110, Beckman Inc., San Jose, CA), an automatic sample processor (WISP 710B, Waters Associates, Milford, MA), a fluorescence detector RF-530 (Shimadzu Instru - Spec, Inc., Concord, CA), an Altex Ultrasphere Octyl 5 u, 4.6 mm x 25 cm column (Beckman Inc., San Jose,

CA) and an integrator (Model 3390A, Hewlett Packard, San Clara, CA) were used. The C_8 Bond Elut extraction columns were obtained from Analytichem International (Harbor City, CA.).

BIOLOGICAL SAMPLES

Human plasma was purchased from Irwin Memorial Blood Bank (S.F., CA.), while all mouse tissues were supplied by Dr. Bob Debs in the Department of Pharmacology at UCSF. Blank urine samples were collected from healthy adult volunteers.

HUMAN STUDY

G.H., an AIDS patient infected with PCP, was treated with pentamidine by single I.V. dose. A total dose of 284 mg (4mg/kg) was given. Blood samples were taken at 0, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 10, 12 and 24 hours. Heparinized plasma was stored at -80 C before being assayed. Pentamidine were determined by both CDC method and our method by the technician in the Infectious Diseases Research Unit.

ANIMAL STUDY

Except for the HPLC assay for pentamidine , the following animal studies were performed by Dr. Debs and Dr. Straubinger in the Department of Pharmacology at UCSF.

Liposomes, composed of phosphatidylserine, phosphatidylcholine and cholesterol (8:32:20) were prepared by modifying a method developed by Olson

et al.[43]. After the lipids in chloroform were dried down on a rotatory evaporator, 1 ml of 100 mg/ml pentamidine solution in sterile water was added to the dried lipid film and the mixture was slowly rotated for 48 hours under argon. Subsequently, the liposomes were dialyzed for 72 hours against 10 mM 2-[N-morpholino]ethanesufonic acid (MES) buffer (pH 6.2, 290 mOsm) with frequent dialyzing solution changes to remove unencapsulated drug. The dialysate was then spun at 20,000g for 40 minutes after which liposome pellets were resuspended in MES buffer. Liposomes were lyzed in MeOH/MES (9.5:1) and liposome encapsulated pentamidine was then measured by optical density at 262 nm. Lipid recovery was determined by a standard phosphate assay (44).

Mice (Balb/C, average 20 gm) received 5 mg/kg of either free or encapsulated pentamidine by tail vein injection. 20 mmoles buffer loaded with non-drug containing liposomes was injected into groups of three mice 1 hour prior to injection of liposome pentamidine (predose). The 3 mice from each group were sacrificed at 1, 4 and 24 hours post injection. Mice were anesthetized with ether, the chest cavity exposed, several drops of a 1:1000 dilution of pure heparin instilled and the heart cut. Blood was collected and lungs, liver and kidneys removed. After weighing, 0.5 CC PBS was added to each organ, and the organs were homogenized in a homogenizer. Samples were then frozen at -80 C and defrosted prior to being assayed.

Aerosolized pentamidine loaded liposomes were prepared as described by Szoka and Papahadjopoulous[45] and extruded through 0.4 micron polycarbonate filters. Groups of 9 mice were placed in a 1 liter container and received 2 mg of pentamidine in and aerosol of 1 um droplet size over 30 minutes. Mice were subsequently processed as in the I.V. study above.

PROCEDURES

FLUORESCENCE QUANTUM EFFICIENCY ESTIMATION

Quantum efficiency of pentamidine and hexamidine are estimated by the comparative method of Chen[46] using quinine bisulfate as a reference standard[47]. Peak areas of quinine, pentamidine or hexamidine all at the same molar concentration, are compared under their maximum spectra. The intensity of the excitation beam is maintained at a constant level for the detector used. Optical densities were measured at the excitation wavelength on a Perkin-Elmer spectrophometer.

PREPARATION OF SILANIZED TUBES.

A 5% of dichloromethylsilane (DCMS) was made by diluting 200 ml of stock solution DCMS with 3800 ml of toluene. Place clean and dry tubes in a large beaker and soak with the 5% DCMS solution for 20 minutes. Discard the DCMS solution and replace with toluene. Soak for 5 minutes in the toluene, then rinse once with methanol. The tubes were dried in an oven and cooled down to room temperature before use.

PREPARATION OF STANDARD SOLUTIONS.

Pentamidine standard stock solution is prepared by weighing 10 mg of pentamidine isethionate and diluting it with 50% CH_3CN and 0.1% H_3PO_4 to a volume of 10 ml in a volumetric flask. This stock solution has a concentration of 0.574 mg/ml pentamidine free base. A working solution of 0.574

ng/ul is made by diluting the stock solution 1000 times with the same solvent. These solutions are stable for three months when stored at 4°C. The internal standard, hexamidine, is prepared in a stock solution of 10 mg/ml as is pentamidine. A 5 ug/100 ml CH_3CN working solution of internal standard was prepared from this stock solution. The hexamidine stock solution is diluted 100 times to create a urine internal standard.

TREATMENT OF PLASMA.

To 0.5 ml of plasma, 1.0 ml of internal standard working solution is added. The mixture is mixed for one minute and centrifuged at 3000 rpm for 10 minutes. The supernatant is transfered to a 500 mg C_8 Bond Elut, and washed with 1 ml each of water and methanol. The Bond Elute containing the drug and internal standard is then placed over a silanized tube. The drugs are then eluted with 1 ml of a solution containing 0.5 % sodium 1-hepantensulfonate, 0.02% tetramethylammonium chloride and 0.1% H_3PO_4 in 97.5% methanol. The eluent is allowed to drip through the Bond Elut without applying pressure thereby avoiding the inconsistencies caused by vaccum elution. The collected eluate is evaporated to a volume of about 200 ul under nitrogen in a 30°C water bath. An aliquot of 40 to 100 ul of this solution is then injected onto the column.

TREATMENT OF URINE

A 25 ul aliquot of internal standard working solution (10 ng/ul) is spiked into 0.2 ml of urine. After vortexing, the sample is passed through the Bond Elut and the remainder of the procedure is the same as that for plasma.

TREATMENT OF TISSUES

Tissues are weighed (ranged from 0.08–1.0 g) and homogenized in 0.5 ml of phosphate buffer saline. For assaying pentamidine in mouse tissues, 200 ul of homogenate is pipetted into a test tube. 400 ul of nanopure water is added to tubes and the tubes are votexed 2 minutes. 0.8 ml of acetonitrile containing internal standard is added in order to precipitate tissue impurities. Tubes are vortexed for 1 minute and the mixture is then centrifuged at 3000 rpm for 10 minutes. If pentamidine concentration is expected to be high (depending on the dose and the route of administration), direct injection is possible, otherwise the remaining procedures are the same as that for plasma except the Bond Elut is washed with 2 ml each of water and 50% of methanol, and the drugs are eluted with 2 ml of the eluent.

CHROMATOGRAPHIC CONDITIONS

The mobile phase is prepared by mixing 1800 ml of acetonitrile, 2200 ml of water, 8 ml of 10% aqueous tetramethylammonium chloride and 4 ml of concentrated phosphoric acid. The solvent is degassed and filtered before use. The mobile phase is pumped at a flow rate of 1.0 ml/min.

The fluorescence detector is set at 275 nm for excitation and at 340 nm for emission with the slit widths of 18 nm for excitation and 22 nm for emission monochromators. The retention times for pentamidine and hexamidine are 10 and 13 minutes, respectively (Figure 2).

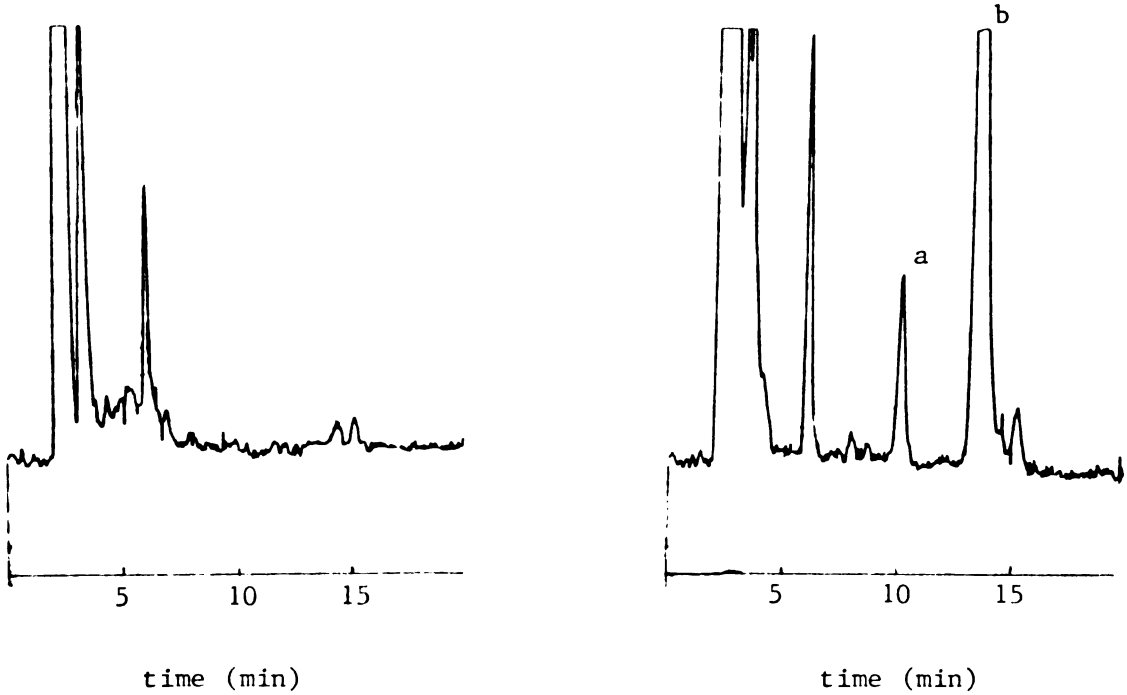


Figure 2. HPLC Chrmatograms of (A) Blank Plasma (B) Plasma fortified with a. Pentamidine (10 ng/ml) and b. Hexamidine .

RESULTS

QUANTUM EFFICIENCY

Relative quantum yields were calculated from the relation:

$$Q_x = Q_{st} \times \frac{F_x}{F_{st}} \times \frac{q_{st}}{q_x} \times \frac{OD_{st}}{OD_x}$$

where the subscripts st and x refer to standard and unknown solutions, Q is the quantum yield, F is the relative fluorescence (determined by the peak area), q is the relative photon output of the source (at the wavelength employed to excite a given solution and is manitained at a constant level here), and OD is the optical density. In Table 1 , the quantum yields of pentamidine and hexamidine are given and found to be 0.050 and 0.057 respectively, when quinine is used as a reference standard.

OPTIMIZATION OF EXTRACTION

The optimal ratio of acetonitrile to water for protein precipitation and removal of tissue impurities was found to be 2 to 1. By using 1-heptanesulfonate containing 0.02% tetramethylammonium chloride and 0.1% of H_3PO_4 as eluent rather than just 1-heptanesulfonate, less volume is required to elute the drugs.

OPTIMIZATION OF CHROMATOGRAPHY

A C_8 column was initially used, and the resultant peak shapes were sharp, so no other column was tested. Mobile phase consisting of 45% CH_3CN, 0.1% of

TABLE 1: FLUROESCENE QUANTUM YIELD AND SPECTRAL PROPERTIES FOR PENTAMIDINE, HEXAMIDINE AND QUININE

SUBSTANCES	EXCITATION MAXIMUM (nm)	EMISSION MAXIMUM (nm)	PEAK AREA	O.D.	QUANTUM YIELD
Pentamidine Isethionate	275	340	1984800	0.214	0.050
Hexamidine Isethionate	275	340	1916500	0.183	0.057
Quinine Bisulfate	312	450	5864700	0.058	0.55

H_3PO_4 and 0.02% tetramethylammonium chloride was found to optimize peak shapes and retention times on this system. The acetonitrile concentration is not as critical as the concentration of tetramethylammonium chloride. Acetonitrile concentrations of 30 to 60% only slightly changed retention times and peak shapes, whereas small differences in tetramethylammonium chloride concentrations gave significant changes.

DETECTION AND SENSITIVITY

0.3 ng of pure pentamidine can be detected under fluorescence detection with excitation at 275 nm and emission at 340 nm (signal/noise = 3.6). The lower detection limit is 0.574 ng when 0.5 ml of plasma is extracted. The accurate sensitivity of this method allows quantitation of 2.87 ng/ml of pentamidine in 0.5 ml of plasma with 10.2% C.V. Sensitivity in urine is at least as good as that in plasma.

CALIBRATION CURVES

Standard curves for plasma, urine and various tissues were obtained by calculating the ratio of the peak height of pentamidine to that of the internal standard and plotting that ratio versus the spiked concentration as shown in Figures 3 through 7. All standard curves are linear with $r^2 = 0.999$. However, these standard curves show a significant deviation at the lower concentrations. If the standard curve is divided into lower and higher concentrations, and the two curves are calculated separately as shown in Tables 2 through 6, the accuracy at the lower concentration is greatly improved. This is due to the fact that actual uncertainty for each sample is equivalent but the percentage of error increases with lower concentration.

For this method, the range of values is too great for one standard curve. The low concentration are far less accurate than the high conccentrations. Two curves for this range are required to produce accurate values along the whole range.

PRECISION

The precision of the method over the entire working range was determined as follows: Repeated assays were performed on plasma and urine samples containing different concentrations of the drug. Those analyses performed on the same day were used to validate intra-day precision, while those samples done on 6 consecutive days were used to validate inter-day precision. The means, standard deviations and coefficients of variation were determined. The results of this study appear in Tables 7 through and 13. Except the extra low concentration in plasma (2.87 ng/ml, CV 10.2%), all the intra-day and inter-day precision data were within 10%.

RECOVERY

Recovery was determined by comparing the peak height ratio of the eluent passed through the Bond Elut to the peak height ratio of the aqueous solution not passed through the Bond Elut. Spiked plasma, various tissues and urine samples were treated as described above except that the internal standard is not added until the evaporation step. The recoveries at different concentrations for plasma, urine and tissues appear in Tables 14, 15, and 16. The average absolute recoveries are 78%, 78% and 67% for plasma, urine and tissus, repectively.

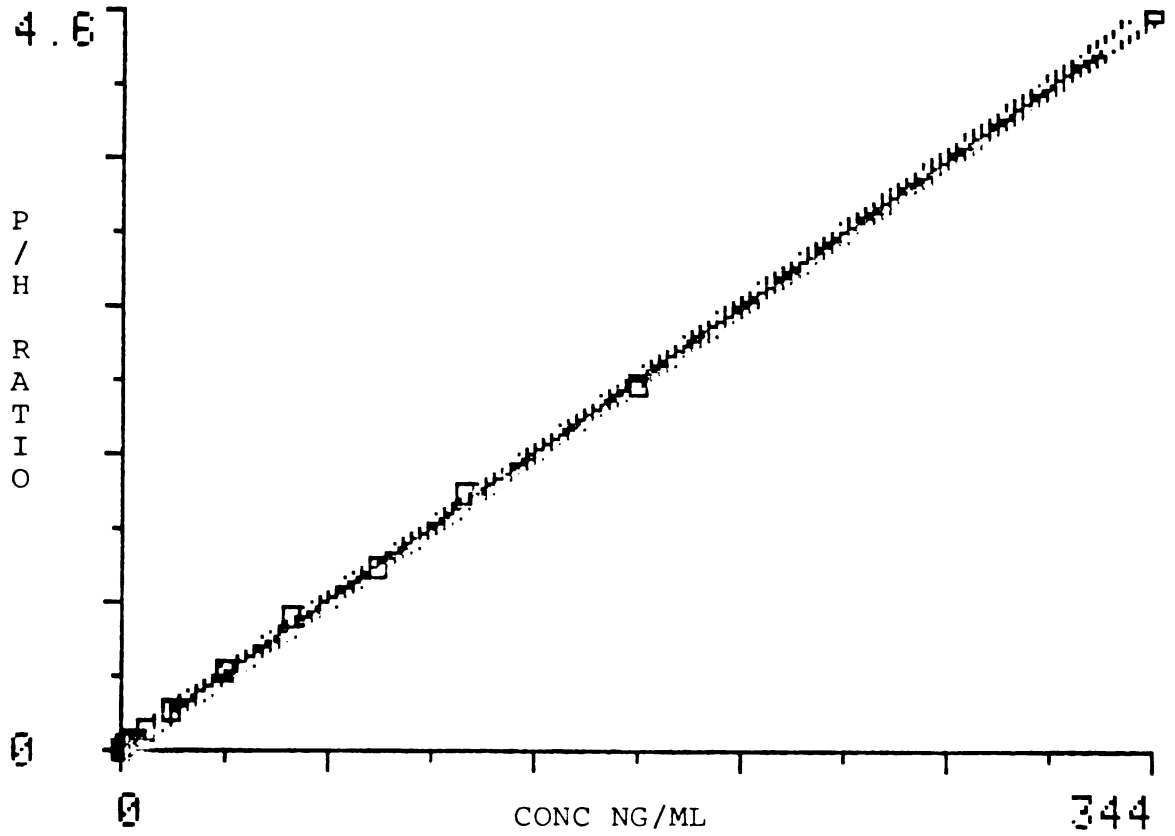


Figure 3. Linearity of a plasma calibration curve.

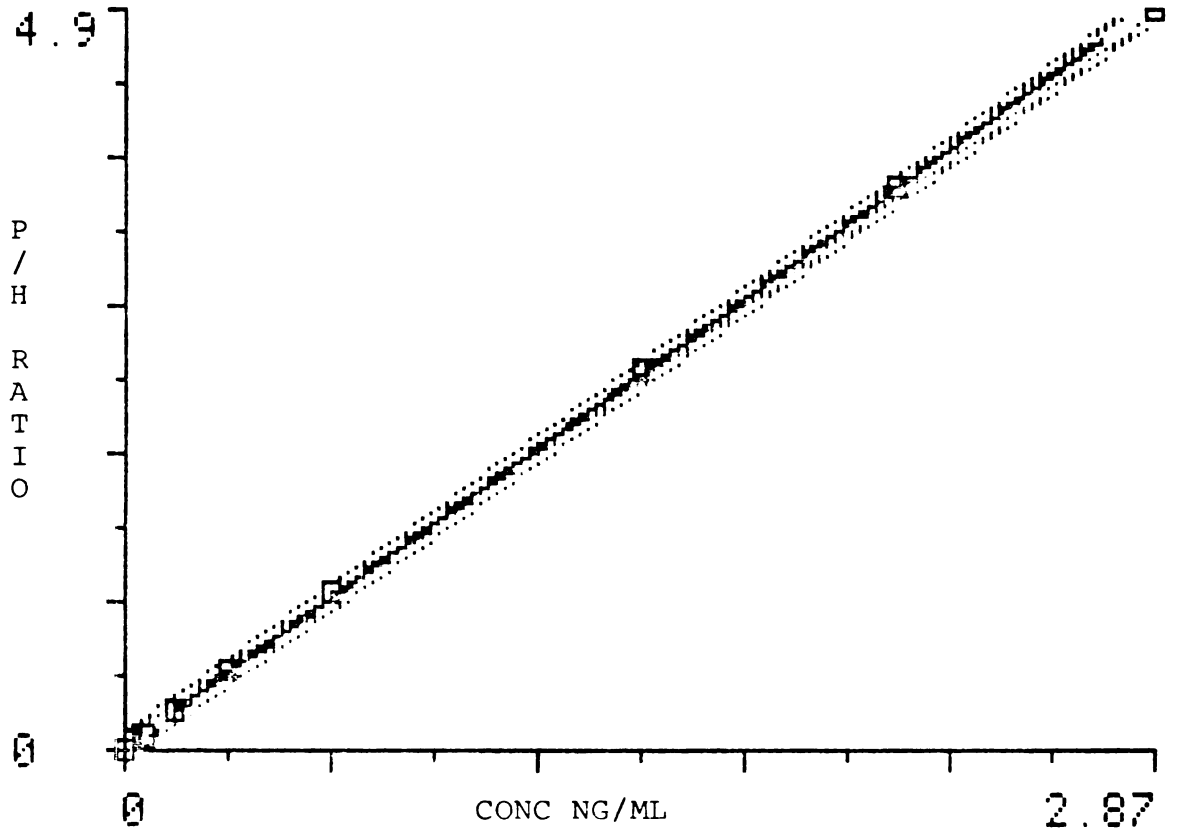


Figure 4. Linearity of a urine calibration curve.

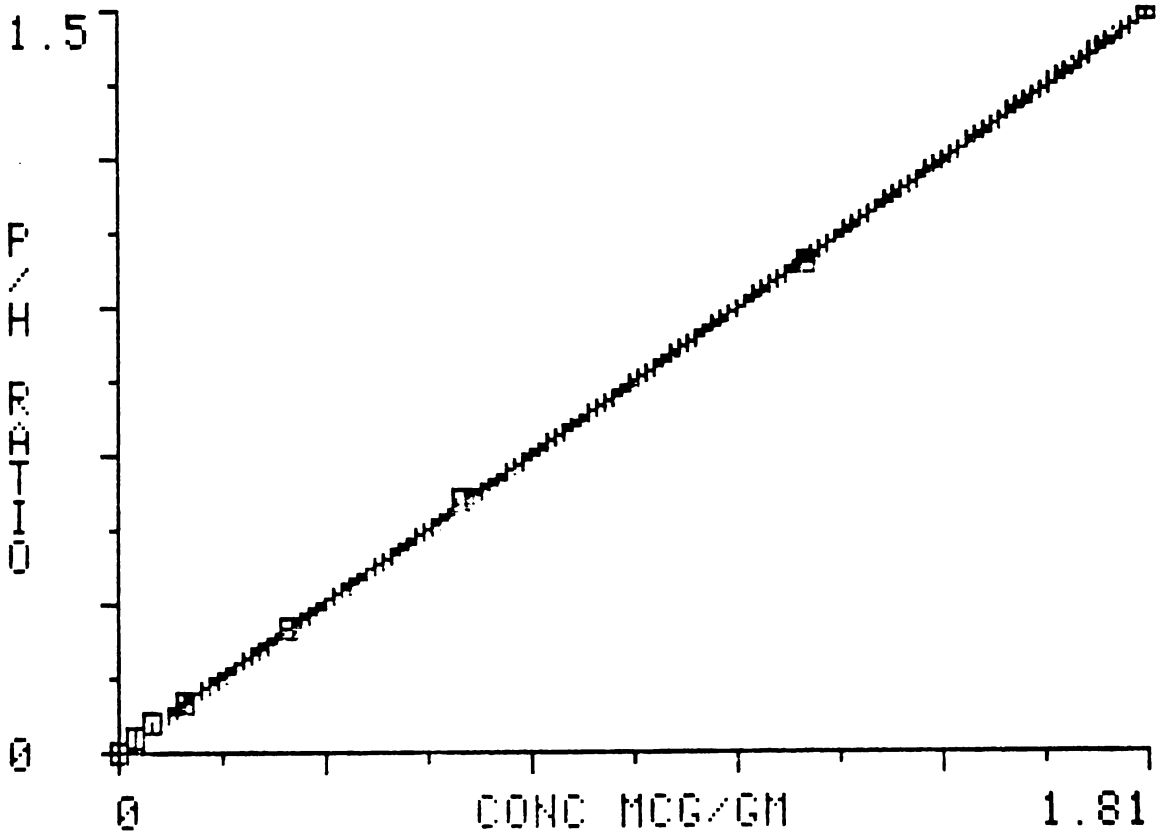


Figure 5. Linearity of a liver calibration curve.

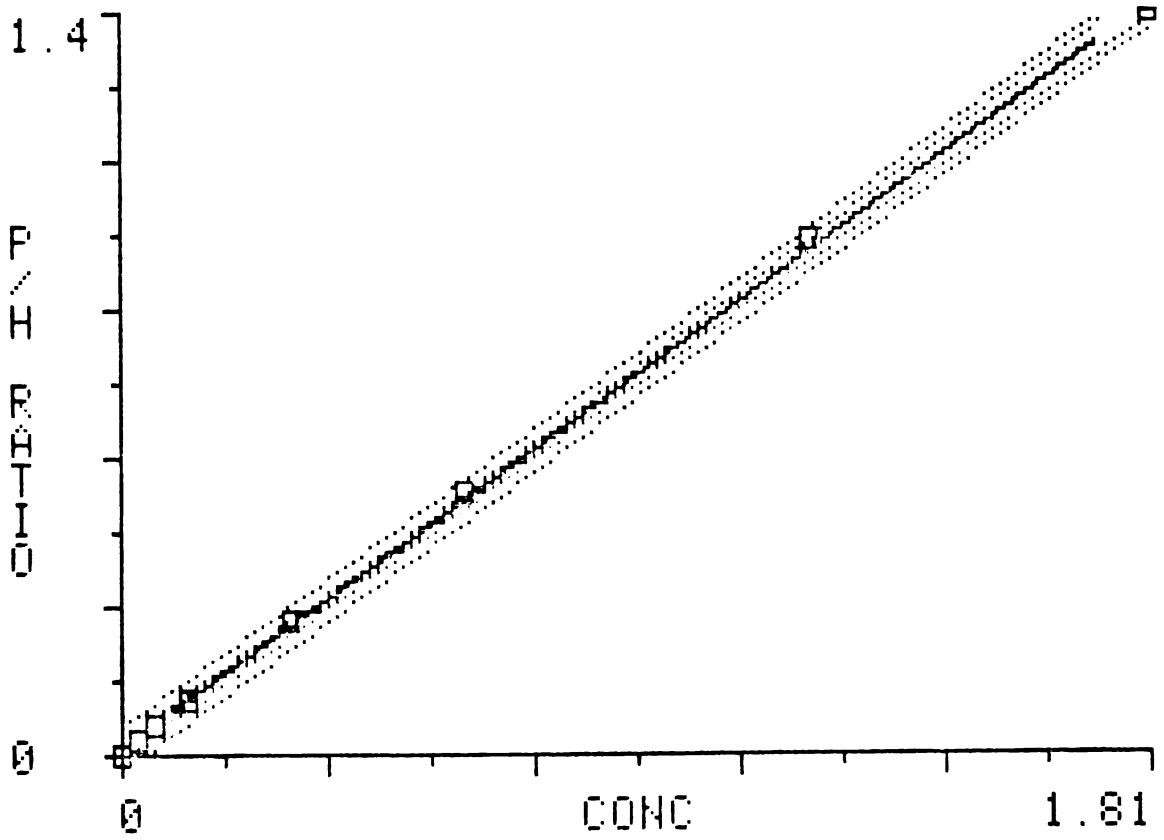


Figure 6. Linearity of a lung calibration curve.

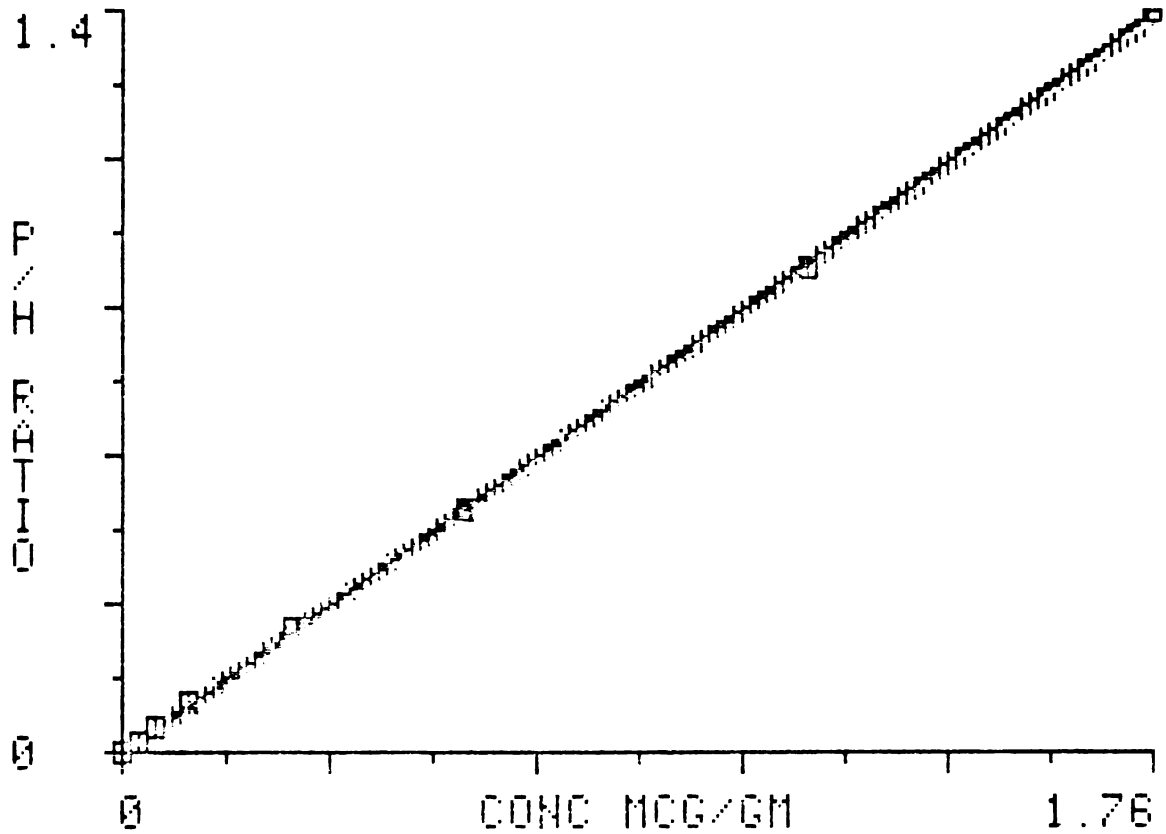


Figure 7. Linearity of a kidney calibration curve.

TABLE 2. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE: PLASMA

SPIKED CONC. (NG/ML)	PEAK HEIGHT	CALCULATED CONCENTRATION (NG/ML)		
		0 - 344	FROM 0 - 34.4	FROM 34.4-344
1.15	0.021	-0.404	0.760	
2.30	0.045	1.36	2.44	
4.60	0.080	3.94	4.89	
9.20	0.140	8.37	9.09	
17.2	0.258	17.1	17.4	
34.4	0.500	34.9	34.3	33.4
57.4	0.846	60.4		59.1
86.1	1.185	85.4		84.3
115	1.639	119		118
172	2.335	170		170
344	4.681	343		344
Intercept:		0.0268	0.0102	0.0602
Slope:		0.0136	0.0143	0.0134
r2:		0.9997	0.9996	0.9996

TABLE 3. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE: URINE

SPIKED CONC. (MCG/ML)	PEAK HEIGHT	CALCULATED CONCENTRATION (MCG/ML)		
		0 - 2.87	FROM 0 - 0.574	FROM 0.574 - 2.87
0.0287	0.068	0.0095	0.0258	
0.0574	0.128	0.0444	0.0581	
0.144	0.293	0.140	0.147	
0.287	0.554	0.282	0.287	
0.574	1.086	0.600	0.573	0.570
1.44	2.551	1.45		1.44
2.15	3.791	2.17		2.17
2.87	4.948	2.84		2.86
Intercept:		0.0521	0.0201	0.1249
Slope:		1.7219	1.8593	1.6885
r2:		0.9998	1.0000	0.9998

TABLE 4. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE: MOUSE LIVER

SPIKED CONC. (MCG/GM)	PEAK HEIGHT	CALCULATED CONCENTRATION (MCG/GM)		
		0 - 1.81	FROM 0 - 0.302	FROM 0.302 - 1.81
0.0302	0.0345	0.0136	0.0291	
0.0604	0.0595	0.0511	0.0586	
0.121	0.116	0.126	0.125	
0.302	0.265	0.305	0.301	0.280
0.604	0.520	0.621		0.615
1.21	1.017	1.24		1.25
1.81	1.458	1.78		1.78
Intercept:		0.0195	0.0098	0.0401
Slope:		0.8056	0.8482	0.7853
r2:		0.9993	0.9995	0.9980

TABLE 5. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE: MOUSE LUNG

SPIKED CONC. (MCG/GM)	PEAK HEIGHT	CALCULATED CONCENTRATION (MCG/GM)		
		0 - 1.81	FROM 0 - 0.302	FROM 0.302 - 1.81
0.0302	0.036	0.0310	0.0312	
0.0604	0.061	0.0610	0.0614	
0.121	0.108	0.118	0.118	
0.302	0.261	0.303	0.303	0.302
0.604	0.515	0.609		0.609
1.21	1.006	1.20		1.20
1.81	1.512	1.81		1.81
Intercept:		0.0104	0.0101	0.0116
Slope:		0.8281	0.8283	0.8272
r2:		1.0000	0.9998	1.000

TABLE 6. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE: MOUSE KIDNEY

SPIKED CONC. (MCG/GM)	PEAK HEIGHT	CALCULATED CONCENTRATION (MCG/GM)		
		0 - 1.76	FROM 0 - 0.293	FROM 0.293 - 1.76
0.0293	0.029	0.0318	0.0286	
0.0586	0.053	0.0611	0.0582	
0.117	0.102	0.121	0.119	
0.293	0.243	0.293	0.292	0.300
0.586	0.477	0.579		0.584
1.17	0.950	1.16		1.16
1.76	1.451	1.77		1.77
Intercept:		0.0031	0.0058	-0.0034
Slope:		0.8180	0.8109	0.8229
r2:		0.9999	1.0000	0.9998

TABLE 7. INTER-DAY PRECISION FOR PENTAMIDINE IN PLASMA

1	2	3	4	5	6	MEAN	SD	% CV
X-HIGH CONCENTRATION (SPIKED: 144 ng/ml)								
148	146	142	144	146	145	145	2.04	1.40
HIGH CONCENTRATION (SPIKED: 86.1 ng/ml)								
87.8	85.5	85.5	88.4	83.2	83.2	85.6	2.20	2.57
MEDIUM CONCENTRATION (SPIKED: 28.7 ng/ml)								
28.6	29.7	30.4	28.6	29.0	27.5	29.0	1.00	3.45
LOW CONCENTRATION (SPIKED: 8.61 ng/ml)								
8.78	8.50	8.90	8.03	8.72	8.15	8.51	0.35	4.2
X-LOW CONCENTRATION (SPIKED: 2.87 ng/ml)								
2.51	2.85	3.25	2.74	3.07	3.27	2.95	0.30	10.2

TABLE 8. INTRA-DAY PRECISION FOR PENTAMIDINE IN PLASMA

1	2	3	4	5	6	MEAN	SD	% CV
X-HIGH CONCENTRATION (SPIKED: 144 ng/ml)								
142	139	148	141	139	146	142	3.73	2.63
HIGH CONCENTRATION (SPIKED: 86.1 ng/ml)								
86.7	87.2	82.1	86.7	85.5	82.6	85.1	2.23	2.62
MEDIUM CONCENTRATION (SPIKED: 28.7 ng/ml)								
28.9	29.7	27.9	28.4	30.0	29.4	29.0	0.80	2.77
LOW CONCENTRATION (SPIKED: 8.61 ng/ml)								
8.67	8.72	8.32	9.01	8.50	8.38	8.60	0.255	2.96
X-LOW CONCENTRATION (SPIKED: 2.87 ng/ml)								
2.64	2.57	2.43	2.72	2.92	2.64	2.65	0.163	6.15

TABLE 9. INTER-DAY PRECISION FOR PENTAMIDINE IN URINE

1	2	3	4	5	6	MEAN	SD	% CV
X-HIGH CONCENTRATION (SPIKED: 1.29 mcg/ml)								
1.30	1.34	1.31	1.34	1.28	1.32	1.32	0.0235	1.78
HIGH CONCENTRATION (SPIKED: 0.430 mcg/ml)								
0.432	0.451	0.459	0.416	0.456	0.437	0.442	0.0165	3.74
MEDIUM CONCENTRATION (SPIKED: 0.215 mcg/ml)								
0.213	0.218	0.232	0.218	0.208	0.224	0.219	0.0084	3.84
LOW CONCENTRATION (SPIKED: 0.0781 mcg/ml)								
0.0712	0.0672	0.0804	0.0689	0.0735	0.0695	0.0718	0.0047	6.59
X-LOW CONCENTRATION (SPIKED: 0.0431 mcg/ml)								
0.0413	0.0465	0.0422	0.0433	0.0393	0.0449	0.429	0.0026	6.00

TABLE 10. INTRA-DAY PRECISION FOR PENTAMIDINE IN URINE

1	2	3	4	5	6	MEAN	SD	% CV
X-HIGH CONCENTRATION (SPIKED: 1.29 mcg/ml)								
1.31	1.33	1.34	1.29	1.31	1.35	1.32	0.0223	1.69
HIGH CONCENTRATION (SPIKED: 0.430 mcg/ml)								
0.460	0.439	0.462	0.465	0.454	0.449	0.455	0.0097	2.12
MEDIUM CONCENTRATION (SPIKED: 0.215 mcg/ml)								
0.215	0.208	0.218	0.212	0.219	0.213	0.214	0.0041	1.90
LOW CONCENTRATION (SPIKED: 0.0781 mcg/ml)								
0.0695	0.0672	0.0712	0.0654	0.0723	0.0654	0.0685	0.0030	4.31
X-LOW CONCENTRATION (SPIKED: 0.0431 mcg/ml)								
0.0448	0.0413	0.0419	0.0402	0.0436	0.0453	0.0429	0.0020	4.74

TABLE 11. INTRA-DAY PRECISION FOR PENTAMIDINE IN TISSUE LIVER

1	2	3	4	5	6	MEAN	SD	% CV
HIGH CONCENTRATION (SPIKED: 0.907 mcg/gm)								
0.895	0.872	0.878	0.907	0.884	0.901	0.890	0.0137	1.54
MEDIUM CONCENTRATION (SPIKED: 0.453 mcg/gm)								
0.447	0.477	0.447	0.461	0.461	0.468	0.460	0.0118	2.56
LOW CONCENTRATION (SPIKED: 0.120 mcg/gm)								
0.124	0.115	0.129	0.122	0.130	0.117	0.123	0.0061	4.96

TABLE 12. INTRA-DAY PRECISION FOR PENTAMIDINE IN LUNG

1	2	3	4	5	6	MEAN	SD	% CV
HIGH CONCENTRATION (SPIKED: 0.907 mcg/gm)								
0.895	0.867	0.895	0.913	0.947	0.918	0.906	0.0270	2.98
MEDIUM CONCENTRATION (SPIKED: 0.453 mcg/gm)								
0.459	0.449	0.447	0.456	0.457	0.452	0.453	0.0048	1.05
LOW CONCENTRATION (SPIKED: 0.120 mcg/gm)								
0.119	0.125	0.120	0.117	0.119	0.122	0.120	0.0028	2.34

TABLE 13. INTRA-DAY PRECISION FOR PENTAMIDINE IN KIDNEY

1	2	3	4	5	6	MEAN	SD	% CV
HIGH CONCENTRATION (SPIKED: 1.27 mcg/gm)								
1.26	1.27	1.27	1.28	1.28	1.30	1.28	0.0137	1.10
MEDIUM CONCENTRATION (SPIKED: 0.439 mcg/gm)								
0.449	0.417	0.442	0.443	0.452	0.433	0.439	0.0128	2.91
LOW CONCENTRATION (SPIKED: 0.120 mcg/gm)								
0.117	0.123	0.118	0.119	0.109	0.106	0.115	0.0065	5.65

TABLE 14. RECOVERY OF EXTRACTION IN PLASMA (N=3)

SPIKED CONC. NG/ML	PEAK HEIGHT RATIO OF PLASMA SAMPLE	PEAK HEIGHT RATIO OF WATER SAMPLE	AMOUNT RECOVERED NG/ML	S.D. NG/ML	RECOVERY
2.30	0.051	0.069	1.71	0.108	73.91
5.74	0.110	0.141	4.49	0.863	78.01
23.0	0.410	0.524	18.0	0.569	78.24
57.4	1.138	1.423	45.9	2.97	79.97

TABLE 15. RECOVERY OF EXTRACTION IN URINE (N=3)

SPIKED CONC. (MCG/ML)	PEAK HEIGHT RATIO OF PLASMA SAMPLE	PEAK HEIGHT RATIO OF WATER SAMPLE	AMOUNT RECOVERY MCG/ML	S.D. MCG/ML	RECOVERY
0.0574	0.181	0.232	0.448	0.00242	78.02
0.215	0.375	0.490	0.164	0.00607	76.65
0.359	0.626	0.791	0.284	0.00852	79.14
0.718	1.213	1.508	0.578	0.0282	80.07

TABLE 16. RECOVERY OF EXTRACTION IN DIFFERENT MOUSE TISSUES (N=3)

SPIKED CONC. (MCG/GM)	PEAK HEIGHT RATIO OF TISSUE SAMPLE	PEAK HEIGHT RATIO OF WATER SAMPLE	AMOUNT RECOVERED MCG/GM	S.D. MCG/GM	RECOVERY
LIVER					
0.120	0.0723	0.107	0.0811	0.0014	67.6
0.453	0.258	0.388	0.301	0.0057	66.5
0.907	0.501	0.762	0.600	0.0040	65.8
LUNG					
0.120	0.0703	0.107	0.0788	0.0028	65.7
0.453	0.262	0.388	0.306	0.0006	67.5
0.907	5.34	0.762	0.690	0.0052	70.1
KIDNEY					
0.120	0.0697	0.107	0.0782	0.0044	65.1
0.453	0.263	0.388	0.307	0.0025	67.7
0.878	0.521	0.762	0.581	0.036	68.4

STABILITY

Pooled plasma samples spiked with various concentrations of pentamidine were divided into 0.5 ml aliquots and stored in -20°C and -80°C freezers until assayed. No appreciable degradation of pentamidine in frozen plasma was seen at -80°C during the two months of study. When stored at -20°C, samples remained stable for the first two weeks. Following this period, there was a degradation of approximately 10% over a two month period. Results are shown in Table 17.

INTERFERENCE

Drugs which might be taken in conjunction with pentamidine by patients infected with PCP were checked for interferences. These include the drugs which are antibacterial (e.g. aminoglyiosides, cephalosporins), antiviral (e.g. acyclovir), antifungal (e.g. isoniazid, rifampin, ethanbutol), antihisteminic (e.g.diphenhydramine) and analgesics (e.g. aspirin, acetaminophen). None of these drugs tested were found to interfere with pentamidine or the internal standard.

HUMAN STUDY

The pentamidine plasma levels in the AIDS patient were measured by both the CDC method and by this method. The plasma concentration-time curves are superimposable for both methods as shown in Figure 8. The coefficient of correlation was 0.998, with a slope of 1.02 and an intercept of 1.41. However, the method developed at CDC is not sensitive enough to detect the plasma concentration 24 hours post injection.

TABLE 17. STABILITY OF PENTAMIDINE ISETHIONATE IN PLASMA

	CONCENTRATIONS AT -20 C (NG/ML)				
DAY	5.74	23.0	57.4	115	172
0	5.45	22.1	54.9	113	176
1	4.86	18.78	54.0	113	171
3	5.73	22.0	52.6	113	174
7	5.77	23.4	57.9	111	170
14	5.24	23.7	58.5	111	175
30	5.37	19.0	51.6	100	154
63	4.79	18.4	49.0	99.0	156

	CONCENTRATIONS AT -80 C (NG/ML)				
DAY	5.74	23.0	57.4	115	172
0	6.56	23.8	56.9	116	172
1	5.17	22.5	52.0	109	168
3	6.88	23.5	54.2	116	171
7	6.27	21.4	53.2	112	168
14	5.61	22.1	58.8	118	175
33	5.56	20.4	53.7	109	164
63	5.12	20.7	54.4	110	168

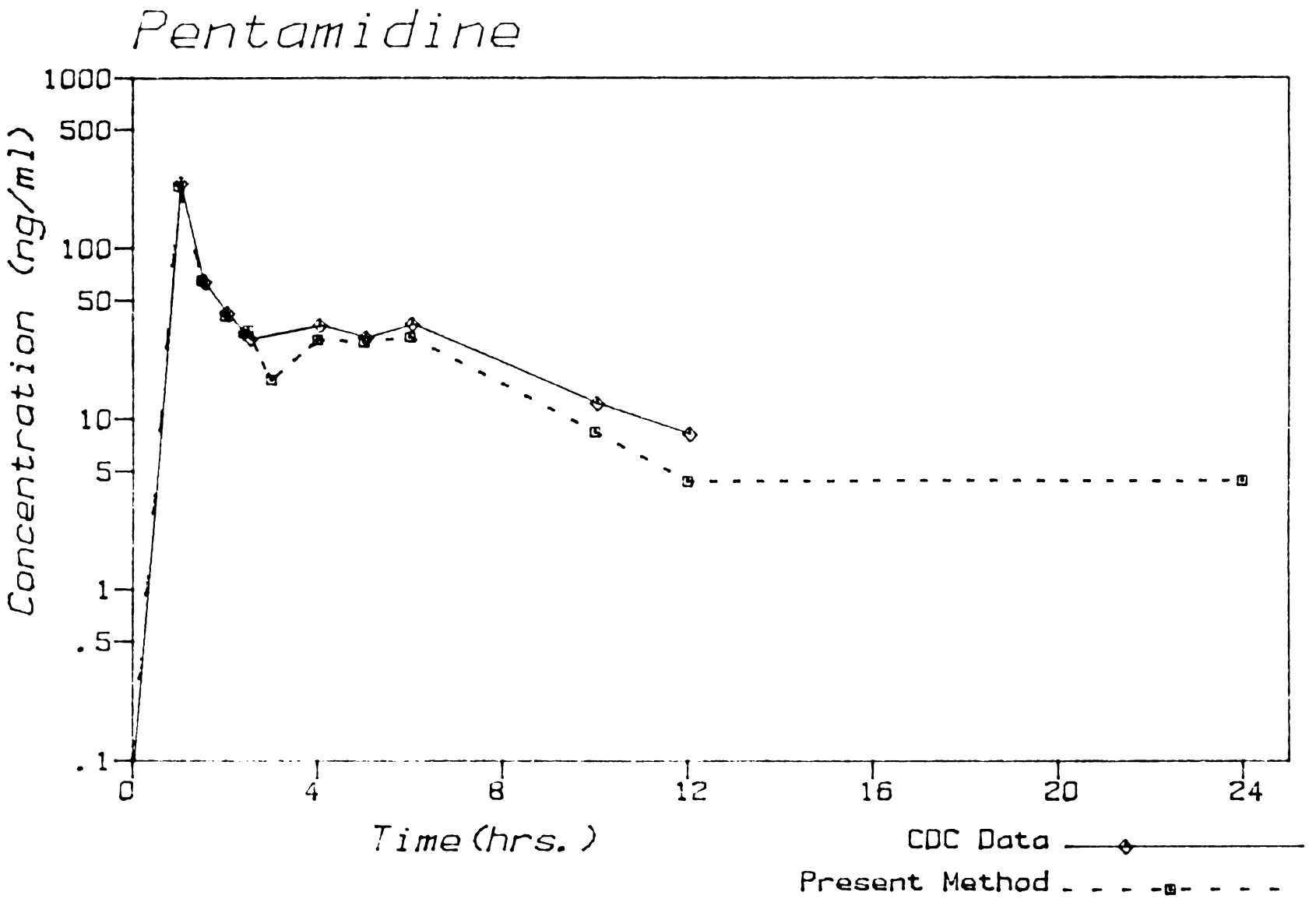


Figure 8. Pentamidine pharmacokinetic profile after an I.V. dose of 4mg/kg in an AIDS patient.

ANIMAL STUDY

In this study, both direct precipitation and Bond Elut extraction methods were used. These chromatograms can be seen in Figures 9 and 10. The sensitivity of the direct precipitation method (0.0625 mcg/gm) was limited by its less clean background, thus less than 50 ul of the supernatant could be injected using this method.

Administration of pentamidine via liposomes compared to administration of the free drug increased drug levels almost forty times in the mouse lung while concurrently reducing kidney levels 3 times during 4 hour period post injection. However, liver uptake of encapsulated pentamidine also increased. Figures 11 to 13 and Tables 18 and 19 demonstrate these results.

Pentamidine delivered by aerosolization in either free or liposome encapsulated forms produced drug levels 40 times higher in mouse lung than in mouse kindney or liver. This is in contrast to free pentamidine administered intravenously, which concentrated most in the kidney, followed by the liver, and finally the lung. Still, administration of aerosolized encapsulated pentamidine reduced drug uptake by the kidney and liver 50% and 33% less compared to aerosolized free drug.

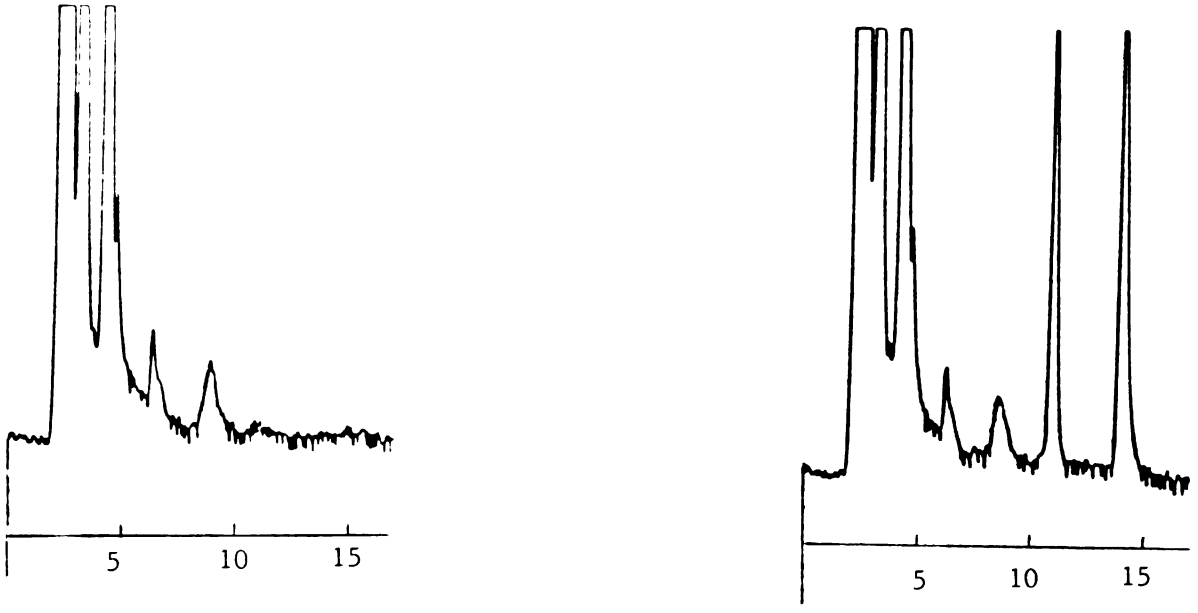
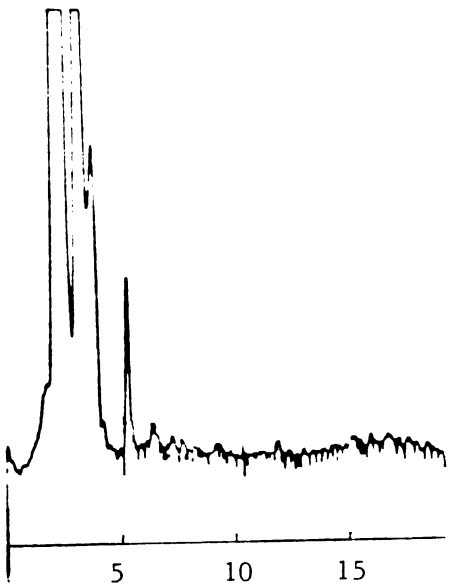
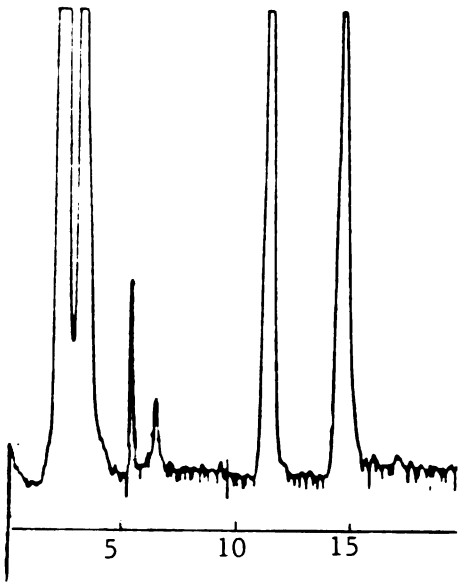


Figure 9 HPLC Chromatograms of mouse kidney tissues: direct injections after acetonitrile precipitation (A) Blank kidney sample (B) Kidney sample after an I.V. dose of 5 mg/Kg .



A



B

Figure 10 HPLC Chromatograms of mouse kidney tissues: injections after acetonitrile precipitation and solid phase extraction (A) Blank kidney (B) Kidney sample after an I.V. dose of 200 mcg/mouse injection.

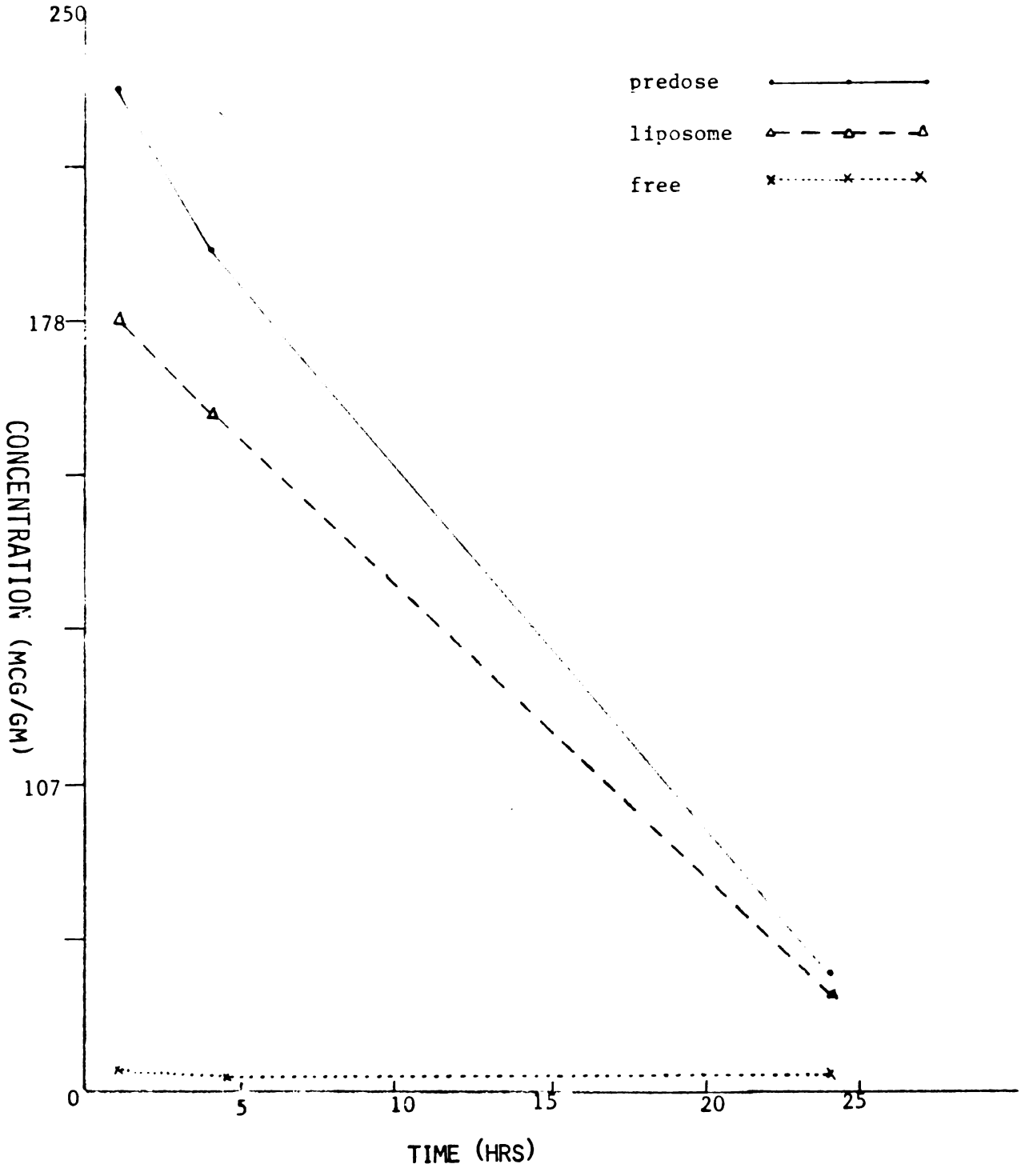


FIGURE 11 PENTAMIDINE LOCALIZATION IN LUNG FOLLOWING I.V. ADMINISTRATION OF 5 MG/KG.

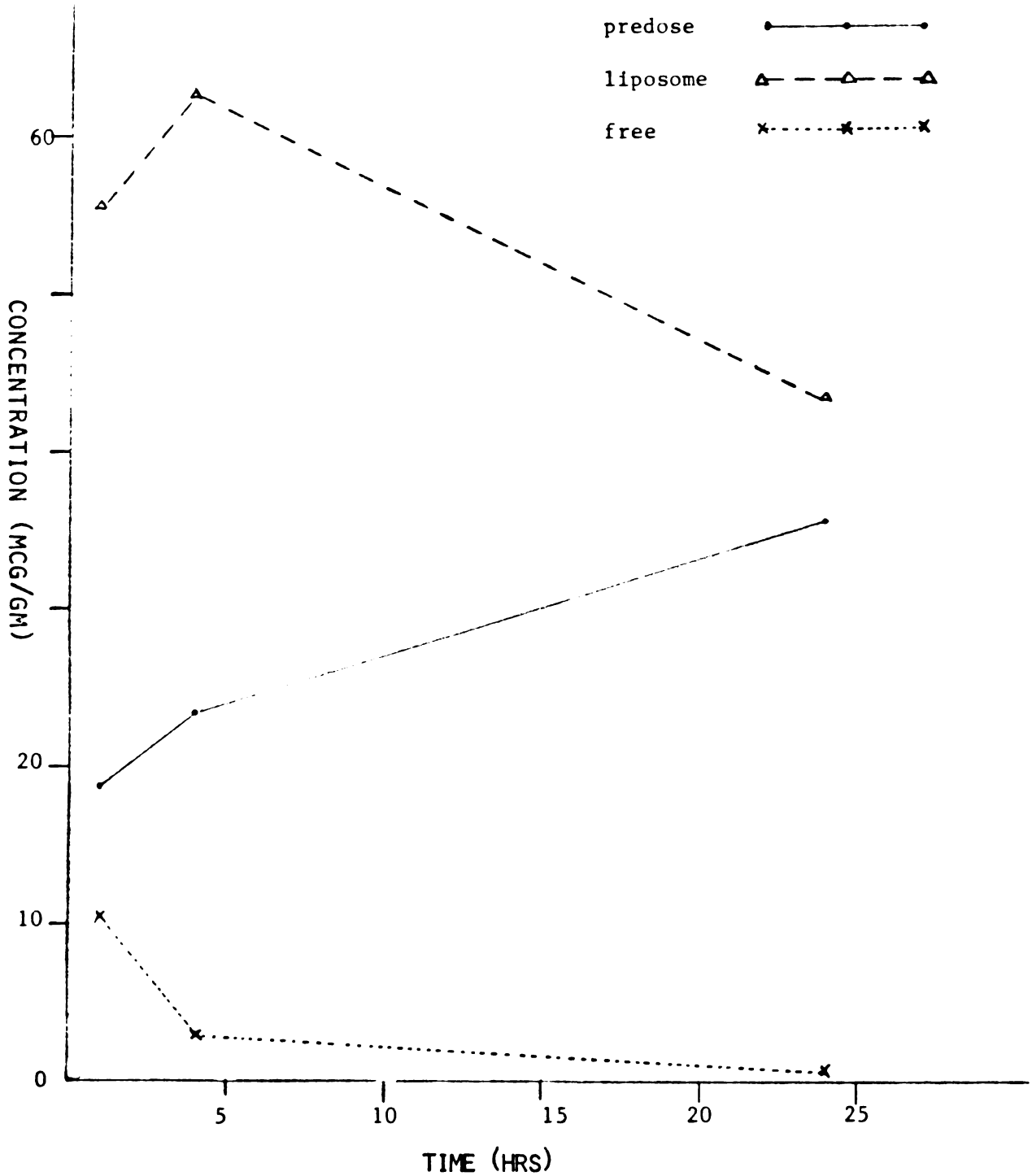


FIGURE 12. PENTAMIDINE LOCALIZATION IN LIVER FOLLOWING I.V. ADMINISTRATION OF 5 MG/KG.

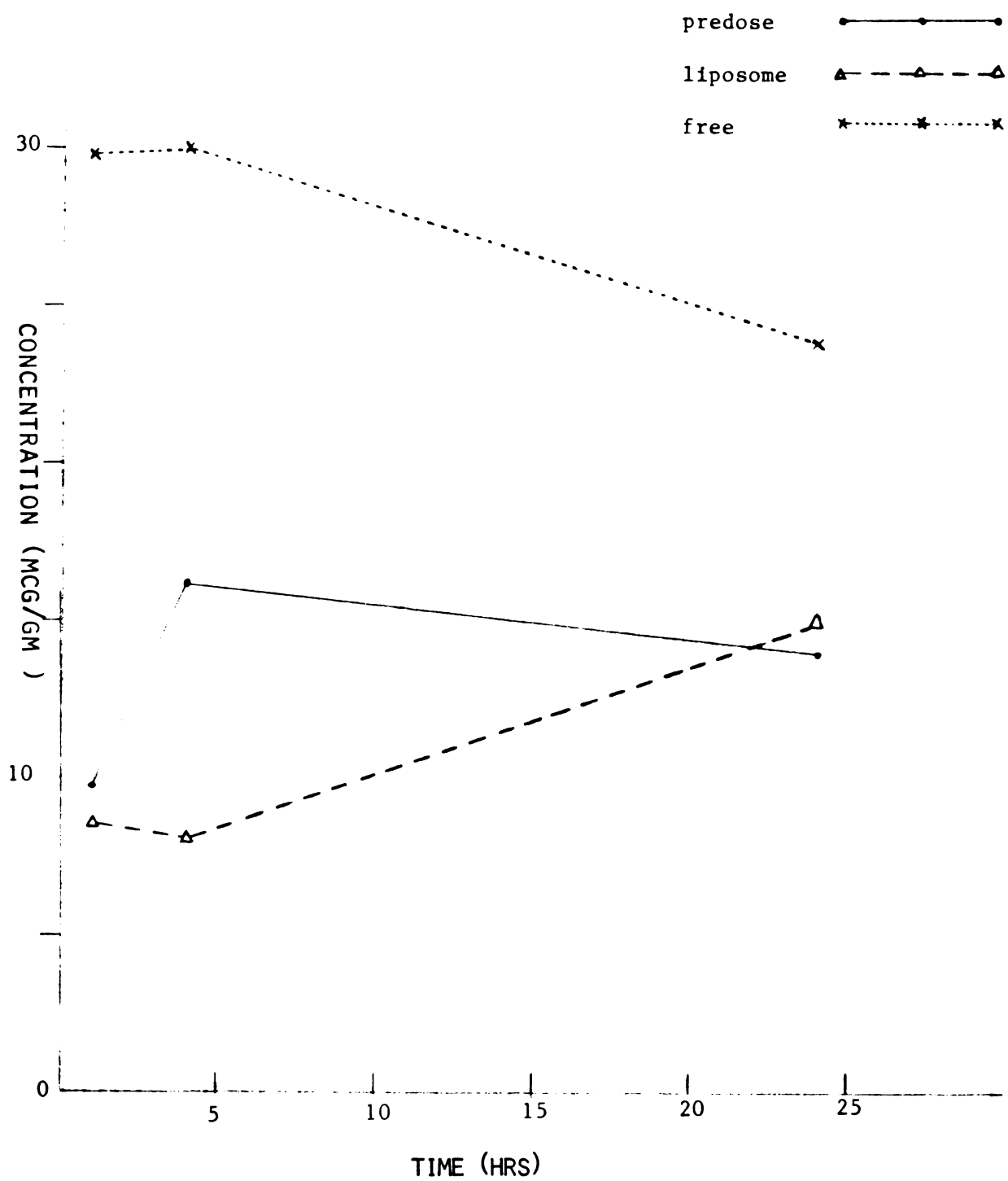


FIGURE 13. PENTAMIDINE LOCALIZATION IN KIDNEY FOLLOWING I.V. ADMINISTRATION OF 5 MG/KG.

TABLE 18. PENTAMIDINE LOCALIZATION FOLLOWING I.V. ADMINISTRATION OF 5 MG/KG

TISSUE	PREPARATION	1 HR	4 HR	24 HR
		LUNG		
	Predose[a]	232[b]	195	27.7
	Liposomes	177	157	22.29
	Free	4.56	3.34	3.73
		LIVER		
	Predose	18.8	23.3	35.7
	Liposomes	55.6	62.7	43.3
	Free	10.5	3.93	0.590
		KIDNEY		
	Predose	9.82	16.1	14.0
	Liposomes	8.65	8.24	15.0
	Free	29.7	28.0	23.8

(a) Animals were predosed with 20 umoles of butter-loaded liposomes 1 hour before injection of pentamidine containing liposomes.

(b) All units represent mcg pentamidine per gram wet tissue. Each data point represents the mean value from three animals.

TABLE 19. PENTAMIDINE LOCALIZATION FOLLOWING AEROSOLIZATION OF 2 MG/30 MIN

TISSUE	PREPARATION	1 HR	4 HR	24 HR
		LUNG		
	Liposomes	42.8	47.3	32.6
	Free	45.8	41.8	48.4
		LIVER		
	Liposomes	0.657	0.771	0.247
	Free	0.992	1.24	0.373
		KIDNEY		
	Liposomes	0.914	1.29	1.18
	Free	1.59	2.15	3.88

DISCUSSION

Pharmacokinetic-pharmacodynamic studies in patients provide information on the therapeutic plasma concentration range of a drug that should be maintained in order to yield maximum benifits. Due to the renal toxicity of pentamidine and its frequent usage in immunocompromised patients, such information is especially important for pentamidine. All these studies need a simple, sensitive and precise analytical assay to quantitate pentamidine concentration in plasma, urine and various tissues. The HPLC procedure presented here is suitable for this purpose.

Pentamidine exihibits a high affinity for the stationary phase of the C_8 Bond Elut. It can not be eluted by pure water or by pure organic solvents but can be eluted only in the presence of ion-pairing reagents (1-heptanesulfonate). This characteristic could be due to its unique, very basic diamidine group.

During the development of the present assay, various parameters were evaluated to assess the optimum conditions:

First, silanized tubes were used to collect the eluate because pentamidine could be adsorbed by glassware. The Bond Elut columns in this experiments were reused. After elution, the Bond Elut columns were washed first with 3 ml of eluent (to make sure that they were free of both drug and internal standard), followed by water. Before the experiment, the columns were conditioned with methanol and water.

Second, the proportion of acetonitrile to water in precipitating plasma proteins or tissue impurities is critical. If the concentration of acetonitrile is higher than 50%, pentamidine and hexamidine will elute from the Bond

Elut when supernatant is passed through the column. However, too low a concentration will result in incomplete precipitation. The ratio is always set at 2:1.

Third, the concentration of tetramethylammonium chloride in the mobile phase is also important. If the concentration is too low, the drug will be retained on the HPLC column too long. On the other hand, if the concentration is too high, the drug will merge with the solvent front. 0.02% of this solution was found to be the optimal concentration. At this concentration, using a mobile phase of 45% CH_3CN and 0.1% H_3PO_4, the retention times of pentamidine and hexamidine are 10 minutes and 13 minutes, respectively.

Finally, the eluent originally consisted only of 0.5% sodium 1-heptane-sulfonate in 97.5% methanol. By adding 0.02% tetramethylammonium chloride and 0.1% phosphoric acid to this eluent, 1 ml of eluent produced the same plasma recovery as 3 ml of the first eluent. However, if the pentamidine concentration is over 3 mcg per sample, 2 to 3 ml of eluent is still required to elute the drugs completely. Pentamidine concentration is always low in plasma while high in tissues after a 4 mg/kg injection, so in tissue assays 2 ml of eluent was used instead of 1 ml.

In order to improve the recovery, organic solvents (such as acetonitrile or tetrahydrafuran) and other types of Bond Elut columns (like C_2 or C_{18}) were tested, but no improvement was observed

Propranolol was used as a reference standard before hexamidine was available. A validation of this pentamidine assay using propranolol as the reference standard was performed, and good precision was obtained. Because propranolol can be eluted by methanol, it was added to the eluent instead of being added at the beginning of the test.

In the human study, the patient was taking penicillin, acetaminophen and

heparin simultaneously, and none of these were found to interfere with the assay. In this study which utilized the pentamidine assay developed by the CDC, no pentamidine was detectable in the plasma sample drawn 24 hours post injection.

If the biological concentration is high, pentamidine can be quantitated directly after acetonitrile precipitation and the Bond Elut column may be bypassed. Because fewer steps are involved, higher precision could be expected.

Direct precipitation was used in one of the pentamidine-liposome drug delivery studies used to quantitate pentamidine in various mouse tissues. Using this HPLC assay, it was discovered that pentamidine levels in lung could be increased up to 50 fold, while concurrently reducing renal uptake 2-3 fold via intravenous administration of liposome encapsulated pentamidine. Though liver uptake increased at the same time, this could be countered by predosing the mice with non-drug containing liposomes.

In another experiment, pentamidine was delivered by aerosolization in both free and liposome encapsulated forms. Both techniques produced significantly higher drug levels in mouse lung, but much lower levels in kidney and liver. However, administration of aerosolized liposomes contening pentamidine reduced drug uptake by the kidney (50% less) and liver (33% less) compared to aerosolized free drug. Thus, treatment of PCP by pentamidine can be enhanced while renal toxicity can be reduced by choosing the proper route of administration.

SUMMARY

Since pentamidine is mostly used in the treatment of PCP in AIDS or immunocompromised patients in the United States, it is important that there exists a simple method that minimizes the technician's contact time with the sample, while maintaining the sensitivity and precision of the method.

The method developed by Waalkes and DeVita is tedious and not sufficiently sensitive. The method reported by the CDC, though more sensitive (12 ng/ml) requires three extractions, and the sensitivity is limited by its low recovery (20-35%).

These two methods are not practical for clinical pharmacokinetic studies and drug level monitoring. The present method avoids all the problems mentioned above by utilizing solid phase ion-pairing extraction and the native fluorescence of pentamidine. The assay requires only 0.5 ml of sample, and the detection limit is 1.15 ng/ml.

This assay will be used in a clinical pharmacokinetic study in AIDS patients by the Department of Infectious Diseases, and it has been applied to a liposome-pentamidine drug delivery study in the Department of Pharmacology at UCSF.

REFERENCES

(1) Blaschko, H., Fastier, F.N., Wajda, I., The inhibition of histamine by amidines. Biochem. J., 49; 250-253, 1951.

(2) Blaschko, H., Himms, J.M., The inhibition of amine oxidase and spermine oxidase by amidines. Br. J. Pharmacol., 10; 451-453, 1955.

(3) Germmill, C.L., The effects of amidines and related compounds on glycolysis in muscle extracts. J. Pharmacol. Exp. Ther., 96; 173-178, 1949.

(4) Goodman, L.S., Gilman, A., The Pharmacological Basis of Therapeutics 4th ed. New York, Macmillan, 1970, p. 1146.

(5) Makulu, D.R., Waalkes, T.P., Interaction between aromatic diamidines and nucleic acids: Possible implications for chemotherapy. J. Natl. Cancer Inst. 54; 305-310, 1975.

(6) Waalkes, T.P. and Makulu, D.R., Pharmacologic aspects of pentamidine. Natl. Cancer Inst. Monogr. 43; 171-176, 1976.

(7) Jaffe, J.J., McCormach, J.J., Gutteridge, W.E., Dihydrofolate reductase within the genus Trypanosoma. Exp. Parasitol. 25; 311-318, 1969.

(8) Kaplan, H.G. and Myers, C.E., Complex inhibition of thymidylate synthetase by aromatic diamidines: Evidence for both rapid, freely reversible and slowly progressive, nonequilibrium inhibition. J. Pharmacol. Exp. Ther. 201; 554-563, 1977.

(9) Burchall, J.J. and Hitchings, G.H., Inhibitor binding analysis of dihydrofolate reductase from various species. Mol. Pharmacol 1; 126-136, 1965.

(10) Bornstein, R.S. and Yarbro, J.W., An evaluation of the mechanism of action of pentamidine isethionate. J. Surg. Oncol. 2; 393-398, 1970.

(11) Sanders, G.F.T., Preliminary report on the treatment of sleeping sickness by 4,4'-diamidinodiphenoxypentane. Ann. Trop. Med. Parasitol., 35; 69, 1941.

(12) Lourie, E.M., Treatment of sleeping sickness in Sierra Leone. Ann. Trop. Med. Parasitol., 36; 113-13 1942.

(13) Jonchere, H., Chemoprophylaxis of human trypanosomiasis in French West Africa. Bull. Soc. Pathol. Exot., 44; 83-93, 1951.

(14) VanHoof, L., Lewillon, R., Henrad, G., et al., A field experiment on prophylaxic value of pentamidine in sleeping sickness. Trans. R. Soc. Trop. Med. Hyg., 39; 327-329, 1946.

(15) Manson-Bahr, P.E., Heish, R.B., Studies in leishmaniasis in East Africa, (3). Clinical features and treatment. Trans. R. Soc. Trop. Med. Hyg., 50; 465-471, 1956.

(16) Schoenbach, E.B., Greenspan, E.M., The pharmacology, mode of action and therapeutic potentialities of stilbamidine, pentamidine, propamidine and other aromatic diamidines - A review. Medicine, 27; 327-377, 1948.

(17) Fulton, J.D., The course of plasmodium relictum infection in canaries and the treatment of bird and monkey malaria with synthetic bases. Ann. Trop. Med. Parasitol., 34; 53-66, 1940.

(18) Vogel, C.L., Gooddell, B., Levine, A., and Waalkes, T.P., Successful treatment of diffuse interstitial pneumonia caused by Pneumocystis Carinii in patients with malignant disease, San Francisco, 1969, Clinical Oncology Society Meeting (Abst.).

(19) Hughes, W.T., Price, R.A., Kim, H.K., et al., Pneumocystis carinii pneumonitis in children with malignancies. J. Pediatr., 82; 404-415, 1973.

(20) Vogel, C.L., Cohen, M.H., Powell, R.D., et al., Pneumocystis carinii pneumonia. Ann. Intern. Med., 68; 97-108, 1968.

(21) Burke, B.A., Good, R.A., Pneumocystis carinii infection. Medicine, 52; 23- 51, 1973.

(22) Haverkos, H.W., Assessment of therapy for pneumocystis carinii pneumonia PCP therapy project group. Am. J. Med., 76; 501-508, 1984.

(23) Waalkes, T.P., DeVita, V.T., The determination of pentamidine (4,4'-diamidinophenoxypentane) in plasma, urine and tissues. J. Lab. Clin. Med. 75; 871-878, 1970.

(24) Waalkes, T.P., Denham, C., DeVita, V.T., Pentamidine: Clinical pharmacologic correlations in man and mice. Clin. Pharm. Ther. 11; 505-512, 1970.

(25) Waldman, R.H., Pearce, D.E., Martin, R.A., Pentamidine isethionate levels in lungs, livers and kidneys of rats after aerosol or intramuscular administration. Amer. Rev. Resp. Dis. 108; 1004-1006, 1973.

(26) Lannoy, L., Guillot, M., and Joncher, H., Etude du stockage et de l'elimination de la pentamidine chez la Souris et le Rat blanc. Ann. Pharm. Franc. 18; 273-284 , 1960.

(27) Launoy, L., Guillot, M., and Jonchere, H., Etude du stockage et de l'elimination de la pentamidine chez la Souris et le Rat blanc. Ann. Pharm. Franc. 18; 424-439 , 1960.

(28) VanHoof, L., Henrard, C., and Peel, E., Pentamidine in the prevention and treatmentof trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 37; 271, 1944.

[29] Gilbert, F. W., Preliminary report on pentamidine in the treatment of late cases of sleeping sickness. Trans. Roy. Soc. Trop. Med. Hyg., 36; 353, 1943.

[30] Jonchere, H., Treatment of the blood-lymph stage of humen trypanosomiasis with diamidine in French West Africa. Bull. Soc. Path. Exot. 44; 603, 1951.

[31] Ivady, G., Paldy, L., Koltay, M., Toth, G., and Kovaks, Z., Pneumocystis carinii pneumonia. Lancet, 1; 616-617, 1967.

[32] Wein, R., The pharmacological activity of certain aromatic diamidines possessing trypanocidal activity. Ann. Trop. Med. Parasit., 37; 1, 1943.

[33] Demarchi, J., Rapport sur la chiminoprophylaxic de la trypanosomiase T. gambiense Dansi Comite Scientifique International de Recherches surles Trypanosomiase. Pruxelles, Commossion de Cooperation technique en Afrique au Sud du Sahara. Publication No. 41, 1958.

[34] Schoenbach, E.B., and Greenspan, E.M., The pharmacology, mode of action and therapeutic potentialities of stilbamidine, pentamidine, propamidine and other aromatic diamidines. A review. Medicine, 27; 327, 1948.

[35] Schnesider, J., Treatment of human African trypanosomiasis. Bull WHO, 28; 763, 1963.

[36] Western, K.A., Perera, D.R., Schultz, M.G., Pentamidine isethionate in treatment of Pneumocystis carinii pneumonia. Ann. Intern. Med., 92, 762-769, 1980.

[37] Polsky, B., Dryjanski, J., Whimbey, E., Wong, B., Gold, J.W.M., and Armstrong, D., Severe Neutropenia during pentamidine treatment of Pneumocystis carinii pneumonia in patients with AIDS. JAMA, 251; 1253-1254, 1984.

[38] Sharpe, S.M., Pentamidine and hypoglycemia (letter). Ann. Intern. Med., 99; 128, 1983.

[39] Bouchard, P., Sai, P., Reach, G., Caubarrere I., Ganeval D., and Assan R., Diabetes mellitus following pentamidine-induced hypoglycemia in humans. Diabetes. 31; 40-45, 1982.

[40] Hunt, C.A., Rustum, Y.M., Meyhew, E. and Papahadjopoulos D., Retention of cytocine arabinoside in mouse lung following intravenous administration in liposomes of different size. Drug Metab. Dispos. 7; 124-128, 1979.

[41] Abra, R.M., Hunt, C.A., and Lau, D.T., Liposome Disposition in vivo: Delivery to the lung. J. Pharm. Sci., 73; 203-206, 1984.

[42] Unpublished. Method developed at the Parasitic Drug Service (PDDS) of the Center for Disease Control (CDC).

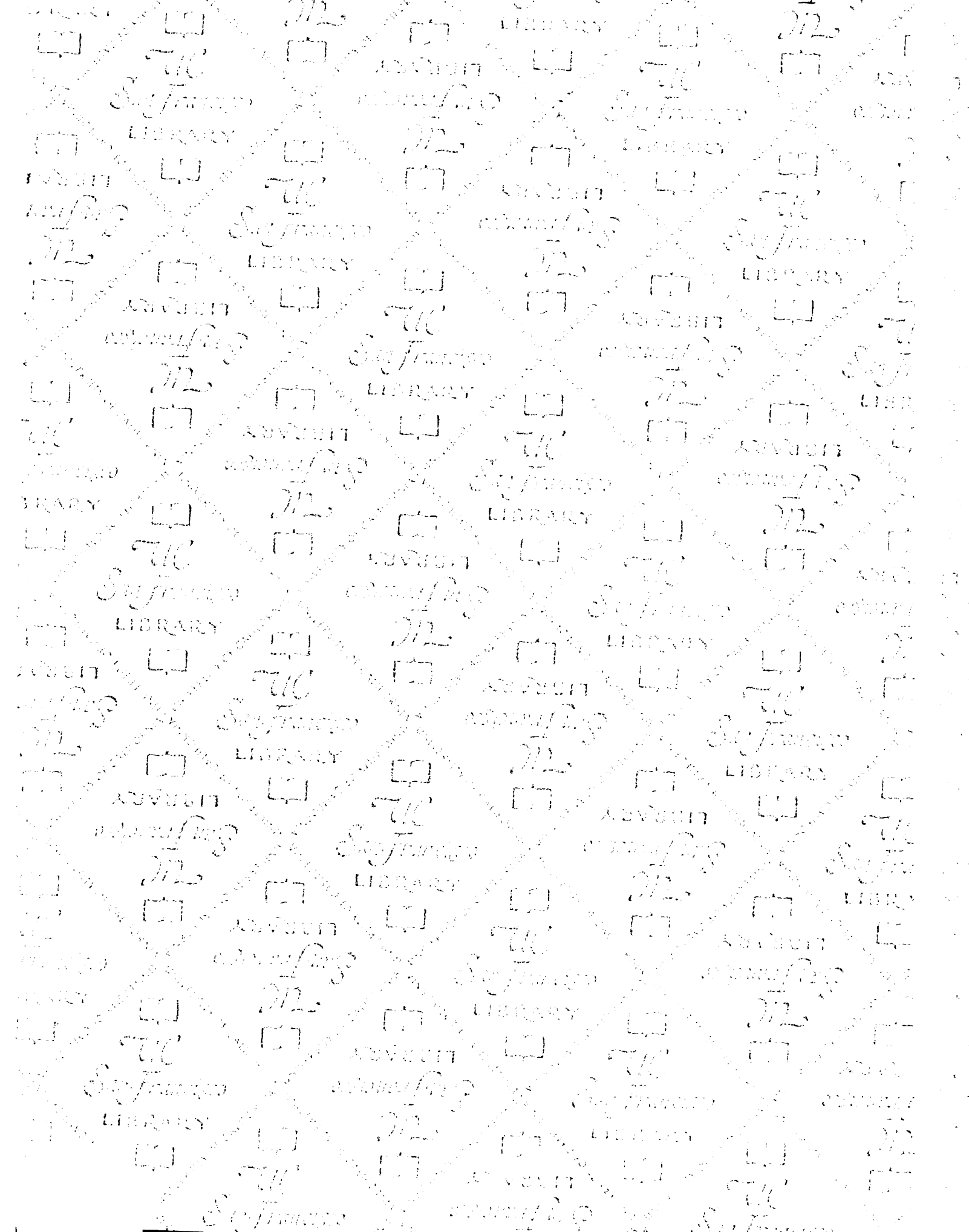
[43] Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D., Preparation of liposomes of defined size distribution by extrution through polycarbonate membranes. Biochimica et Biophysica Acta., 557; 9-23, 1979.

[44] Bartlett, G.R., Phosphorus assay in column chromatography. J. Biol. Chem. 234; 466-468, 1959.

[45] Szoka, F. Jr., and Papahadjopoulous, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75; 145-149, 1978.

[46] Chen, R.F., Fluorescence quantum yield measurements: Vitamin B_6 compounds. Science, 150; 1593-1595, 1965.

[47] Melhuish, W.H., Quantum efficiencies of fluorescence of organic substances: Effect of solvent and concentration of the fluorescent solute. J. Phys. Chem., 65; 229-235, 1961.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