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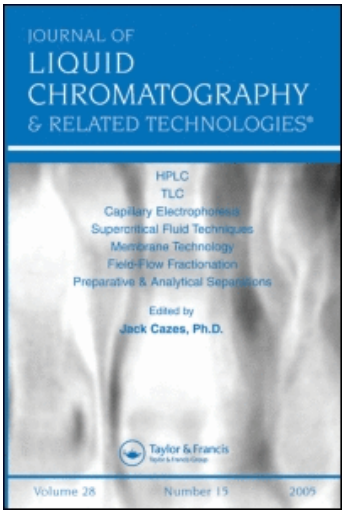

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENTAMIDINE IN PLASMA

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ABSTRACT

A high performance liquid chromatographic method for quantitating pentamidine in plasma has been developed. Sample clean-up involved precipitating plasma with acetonitrile containing the internal standard, hexamidine. The supernatant was passed through a C_8 Bond Elut column and eluted with a methanolic solution of sodium 1-heptanesulfonate. The eluate was then analyzed on an Altex C_8 column with a mobile phase consisting of 45% CH_3CN, 0.02% tetramethylammonium chloride and 0.1% H_3PO_4. Using fluorescence detection (EX: 275 nm and EM: 340 nm), the detection limit was 1.25 ng/ml for 0.5 ml of plasma. The coefficients of variation for interday and intraday were around 10%.

INTRODUCTION

Pentamidine [4,4'-diamidinodiphenoxypentane] (Fig. 1) has been extensively used outside the United States, mainly for treatment of specific tropical diseases. It is the drug of choice in the

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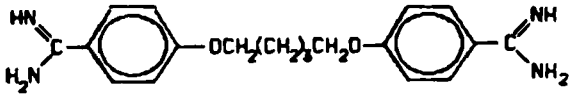
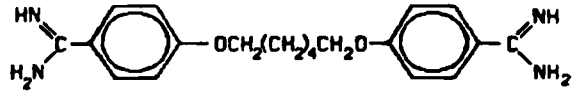
therapy of early African sleeping sickness[1] and is of value in the treatment of leishmanial resistance to sodium antimony tartrate and ethylstilbene.[2] Interest in pentamidine has been stimulated by its apparent effectiveness in the treatment of diffused interstitial pneumonia due to pneumocystis carinii.[3] A protozoal infection confined to the lung, pneumocystis carinii pneumonia (PCP) is one of the most common and most lethal diseases in patients with Acquired Immune Deficiency Syndrome (AIDS) and with disseminated malignant neoplasms.[4] Pharmacokinetic studies of pentamidine in animals and humans have not produced clear results because of the lack of an accurate and sensitive method. Due to the toxicity of the drug,[5] it is important to have a good method for monitoring pentamidine levels in patients who have received the drug.

Pentamidine concentrations in plasma have been determined by spectrophotofluorometry following extraction and derivatization, with a detection limit of 200 ng/ml[6] and by high performance liquid chromatography using U.V. detection with one ml of plasma



Pentamidine



Hexamidine

FIGURE 1: Pentamidine and the internal standard, hexamidine

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extraction.[7] The HPLC detection limit is 12 ng/ml and extraction recovery is between 20 to 30%. However, these methods are not very selective, nor sensitive and are relatively tedious. This paper describes a simple, specific and more sensitive HPLC method using solid-phase ion-pairing extraction and fluorescence detection for the quantitation of pentamidine in plasma. This method is suitable for both pharmacokinetic evaluation and drug level monitoring in patients.

MATERIALS AND METHODS

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REAGENTS AND CHEMICALS:

Pentamidine, as the isethionate salt, was supplied by May & Baker Co. (Dagenham, England), while hexamidine was a gift from Dr. John Conte of UCSF. Tetramethylammonium chloride (Fluka Chem. Co., Hauppauge, NY) and sodium 1-heptanesulfonate (Sigma Chem. Co., St. Louis, MO) were commercially obtained. Methanol and acetonitrile were of 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:

The HPLC system consisted of a solvent delivery pump (Model 110A, Beckman Inc., San Jose, CA), an automatic sample processor (WISP 710B, Waters Associates, Milford, MA), a Shimadzu RF-530 fluorescence detector set at 275 *nm for excitation and at 340 nm* for emission (Shimadzu Instru Spec, Inc., Concord, CA), an Altex Ultrasphere Octyl 5μ, 4.6 x 250 mm column (Beckman Inc., San Jose, CA) and an integrator (Model 3390A, Hewlett Packard, Santa Clara,

CA). The C_8 Bond Eluts were manufactured by Analytichem International (Harbor City, CA).

The mobile phase was 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 mixture was degassed and filtered before use. The mobile phase was pumped at a rate of 1.0 ml/min.

PROCEDURES:

Preparation of Standards: Pentamidine standard stock solution was 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 had a concentration of 0.574 mg/ml pentamidine free base. A working solution of 0.574 ng/μl was made by a 1000x dilution of the stock solution. These solutions were stable for three months when stored at 4°C. The internal standard, hexamidine, was prepared in a stock solution of 10 mg/ml in a method similar to that for the preparation of pentamidine. A working solution of internal standard was prepared by adding 5 mcg of this stock solution to 100 ml of acetonitrile.

Treatment of Plasma Samples: To 0.5 ml of plasma, 1.0 ml of internal standard working solution was added. The mixture was vortexed for one minute and centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to a 200 mg C_8 Bond Elut and washed with 1 ml of water, followed by 1 ml of methanol to remove indigenous impurities. The Bond Elut, which still contained the drug and the internal standard, is then placed over a silanized tube. The drugs are eluted with 1 ml of a solution consisting of 0.5% sodium 1-heptanesulfonate, 0.02% tetramethylammonium chloride and 0.1% H_3PO_4 in 97.5% methanol. The eluate is allowed to drip through the Bond Elut without the application of

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pressure. The collected eluate is evaporated to a volume of about 200 μl under nitrogen in a 30°C water bath and a 40° to 100 μl aliquot of this solution is then injected onto the column.

RESULTS

CALIBRATION CURVE:

A calibration curve using eleven different concentrations ranging from 1.15 to 344 ng/ml was obtained by calculating the ratio of the peak height of pentamidine to that of the internal standard versus their respective concentrations. The standard curve was linear with r^2 = 0.9997, the y-intercept = 0.0218 and the slope = 0.0136 (Table 1). However, this calculated concentration curve shows a significant deviation at the low end of the curve. When separate standard curves were calculated for the ranges of 1.15 - 34.4 ng/ml and 34.4 - 344 ng/ml, the accuracy for the lower concentration were greatly improved, as shown in Table 1. Figure 2 illustrates typical chromatograms for blank plasma, plasma spiked with pentamidine and a clinical plasma sample collected at 24 hours after an I.V. dose of 4 mg/kg. The retention times for pentamidine and hexamidine were 10 and 13 min, respectively.

PRECISION AND ACCURACY:

Precision of the method over the entire working concentration range was determined with the analysis of spiked samples. In Tables 2 and 3, the inter- and intra-day precision and coefficients of variation (CV) for five different concentrations are presented. The average CVs for the method were around 10%.

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TABLE 1. LINEARITY OF A REPRESENTATIVE CALIBRATION CURVE

PLASMA

SPIKED CONC. (NG/ML)	PEAK HEIGHT RATIO	CALCULATED CONCENTRATION (NG/ML)		
		0 - 344	FROM 0 - 34.4	FROM 34.4 - 344
0	0	0	0	
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
r^2:		0.9997	0.9996	0.9996

RECOVERY:

Recovery was determined by comparing the peak height ratio of the eluate passed through the Bond Elut to the peak height ratio of the aqueous solution not passed through the Bond Elut. Spiked plasma samples were treated as described previously except that the internal standard was not added until the evaporation step. The recoveries at four different concentrations appear in Table 4. The average recovery was 78%.

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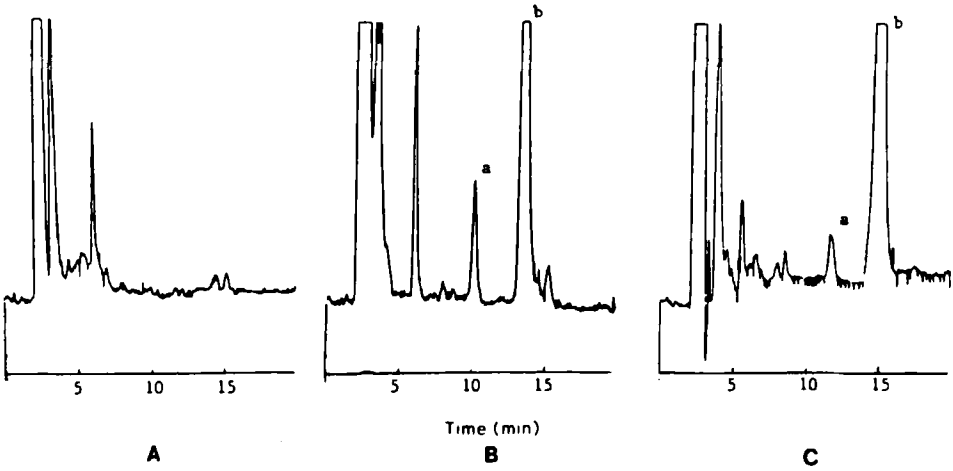

FIGURE 2: HPLC chromatograms of (A) blank plasma, (B) plasma fortified with 10 ng/ml pentamidine, and (C) an AIDS patient's plasma sample collected at 24 hrs, after an i.v. infusion dose of 4 mg/kg (concentration 3.82 ng/ml). (a) pentamidine and (b) internal standard.

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TABLE 2: 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 3: 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: 29.7 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 4: RECOVERY OF EXTRACTION (N=3)

SPIKED CONC. (NG/ML)	PEAK HEIGHT RATIO OF PLASMA SAMPLE	PEAK HEIGHT RATIO OF WATER SAMPLE	RECOVERY
2.30	0.051	0.069	73.9
5.74	0.110	0.141	78.0
23.0	0.410	0.524	78.2
57.4	1.138	1.423	80.0

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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 (Table 6). 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, as shown in Table 5.

DISCUSSION

Pentamidine exhibits a high affinity for the stationary phase of the C_8 Bond Elut. It can not be eluted by pure water or by water containing variable fractions of organic solvents such as tetrahydrofuran, methanol or acetonitrile. It can, however, be easily eluted in the presence of ion-pairing reagents (such as, 1-heptanesulfonate). This characteristic may be due to its unique, strongly basic diamidine group which is tightly bound to the residual silanol group in the stationary phase. A structurally similar compound, diminazene, also shows this property.[8] A methanolic solution containing heptanesulfonate and tetramethylammonium chloride eluted pentamidine with a recovery of 78%. Organic solvents, such as acetonitrile or tetrahydrofuran, and other types of Bond Elut columns (C_2 and C_{18}) were also tested in order to achieve higher recovery, but no improvement was obtained. By making use the unique property of pentamidine in this solid-phase ion-pairing extraction, the plasma can be thoroughly cleaned up. The endogenous compounds in the plasma can be selectively washed out from the Bond Elut by the organic solvents and the drug left in the cartridge. The drugs can later be selectively eluted out from the Bond Elut. Since fluorescence detection is quite specific and the detection will take advantage of the native

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TABLE 5: STABILITY OF PENTAMIDINE ISETHIONATE IN PLASMA AT −20°C

	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

TABLE 6: STABILITY OF PENTAMIDINE ISETHIONATE IN PLASMA AT −80°C

	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

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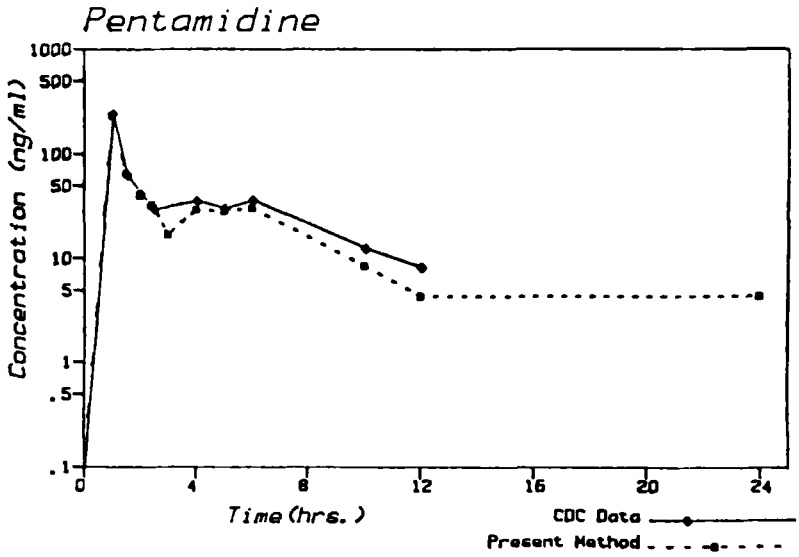

FIGURE 3: Pentamidine plasma concentration - time profile of an AIDS patient after an IV dose of 4 mg/kg.

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fluorescence of pentamidine, the assay described here is sufficiently specific and sensitive.

Drugs which might be taken in conjunction with pentamidine by patients infected with PCP were checked for interference. These include trimethoprim, sulfamethoxazole, aminoglycosides, cephalosporins, acyclovir, amphotericin, flucytosin, isoniazid, diphenhydramine, aspirin and acetaminophen. None of the drugs tested were found to interfere with pentamidine or the internal standard.

The method described here was compared with the UV assay published by the Center For Disease Control (CDC) on pentamidine plasma levels in an AIDS patient following a one hour I.V. infusion dose of 4 mg/kg (Fig. 3). In that study, the concentration of pentamidine in the patient's plasma was measured for 24 hours.

The peak concentration was 363 ng/ml and the trough level was 4.80 ng/ml. The coefficient of correlation was 0.9983, with a slope of 1.020 and a y-intercept of 1.410. Thus, the present assay has both a higher sensitivity and a better correlation than the previous published assay.

In conclusion, the sample clean-up procedure described here involves only a protein precipitation and a column elution step prior to separation by HPLC. A relatively small amount (0.5 ml) of sample is needed for this method, which has a sensitive detection limit of 1.15 ng/ml in plasma. The method is currently being used in the pharmacokinetic studies of pentamidine in AIDS patients.

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