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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF SEVERAL DIAMIDINE COMPOUNDS WITH POTENTIAL CHEMOTHERAPEUTIC VALUE

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SUMMARY

A high-performance liquid chromatographic method has been developed for the detection and quantification of pentamidine and pentamidine analogues of chemotherapeutic value in order to measure their concentration in physiological fluids The compounds were extracted from urine over octadecyl solid-phase extraction columns, followed by chromatographic separation with an octadecyl reversed-phase column For the mobile phase, a gradient of 31 5–37 5% acetonitrile in water, with sodium heptanesulfonate and tetramethylammonium chloride as ion modifiers, was used This method was used to reliably detect levels as low as 341 ng/ml without concentration of the compounds during the solid-phase extraction The assay was used to determine the effectiveness of several solid-phase extraction columns for isolating the compounds of interest and to quantify the amount of pentamidine and its analogues contained in the urine of dosed rats

INTRODUCTION

Pentamidine isethionate, an aromatic diamidine first synthesized in the late 1930s, has been utilized in African trypanosome prophylaxis since the 1950s and was first shown to have activity against *Pneumocystis carinii* in 1957 [1]

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Recently, the high rate of *P. carinii* pneumonia (PCP) associated with AIDS (acquired immunodeficiency syndrome) has renewed interest in the chemotherapeutic use of pentamidine [2,3]. For many years, the use of pentamidine was hindered by the lack of a sensitive assay for detection and determination of pharmacokinetics. However, recent advances in high-performance liquid chromatography (HPLC) [4–6] for the detection of pentamidine have led to preliminary studies of the drug's distribution and pharmacokinetics [7,8].

The prophylactic effectiveness of pentamidine is restricted by its toxicity, as nearly 50% of all patients treated with the drug show adverse reactions [9]. For this reason, pentamidine analogues are being synthesized in this laboratory and tested for anti-*P. carinii* activity and toxicity. Several compounds have been synthesized which show anti-*P. carinii* activity with less of the toxicity associated with pentamidine [10]. The pharmacokinetics of these new compounds must be determined before human trials take place, and the beginning of such a study is the development of a sensitive assay for the drugs. None of the published HPLC assays gave satisfactory results for all of the diamidines of interest. Peak shape was consistently poor, and several of the compounds eluted too close to the solvent front for adequate resolution. Therefore, a protocol was devised, using increased amounts of ion modifiers and an acetonitrile gradient for the mobile phase, which gave sensitive and reproducible detection, adequate separation of the various compounds, and excellent peak shape.

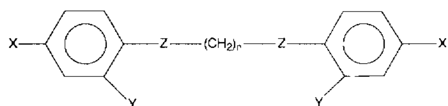
EXPERIMENTAL

Chemicals

HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.), and all water was filtered and deionized through a Darco water purification system (Durham, NC, U.S.A.). The ion-pairing agent, 1-heptanesulfonic acid sodium salt, was obtained from Sigma (St. Louis, MO, U.S.A.) and tetramethylammonium chloride (TMAC) was acquired from Fluka (Zurich, Switzerland). All diamidine compounds (see Fig. 1) were synthesized as hydrochloride salt in the laboratories of Dr. R. Tidwell [10,11]. Routine assays by elemental analysis, infrared and nuclear magnetic resonance spectroscopy, and thin-layer chromatography and HPLC were performed to confirm purity.

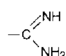
Apparatus

A Hewlett-Packard Model 1084B liquid chromatograph equipped with a Model 79841A autoinjector and a Model 79850B reporting integrator (Boblingen, F.R.G.) was used for all analyses. The column used was a Zorbax ODS 250 mm×4.6 mm I.D. reversed-phase column with 5 μm particle size from DuPont (Wilmington, DE, U.S.A.), kept at 40°C. The compounds were detected by a Hewlett-Packard Model 79875A variable-wavelength ultraviolet spectrophotometer or a Kratos Model FS970 fluorimeter (Ramsey, NJ, U.S.A.).



COMPOUND	n	X	Y	Z
Pentamidine	5	Am *	H	O
Hexamidine	6	Am	H	O
Butamidine	4	Am	H	O
Diaminopentamidine	5	Am	NH_2	O
Dianilinopentamidine	5	Am	H	NH
Dimethoxypentamidine	5	Am	OCH_3	O
Dimethoxypropamidine	3	Am	OCH_3	O
Imidazolinopentamidine	5	Im **	H	O

*Am =



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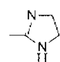

Fig 1 Structural formulae of the compounds used in this study All the compounds were manufactured as the hydrochloride salts

TABLE I

ULTRAVIOLET ABSORBANCE AND FLUORESCENCE WAVELENGTHS USED FOR DETECTION

Compound	Absorbance[a]/excitation[b] wavelength (nm)
Pentamidine	265
Hexamidine	265
Butamidine	265
Diaminopentamidine	235
Dianilinopentamidine	220
Dimethoxypentamidine	265
Dimethoxypropamidine	265
Imidazolinopentamidine	265

[a]540 nm was used for the ultraviolet reference wavelength
[b]A 340-nm cutoff filter was used for the emission wavelength

equipped with a 340-nm cutoff filter. The wavelengths used for each compound are given in Table I.

Sample preparation

For determining the relative retention times of the various compounds, 1 0

m*M* stock solutions of each were made in 45% acetonitrile (v/v in water) containing 10 m*M* TMAC, 10 m*M* heptanesulfonate, and 4 2 m*M* phosphoric acid Small aliquots (10 μl) from each stock solution were then diluted ten-fold in the same buffer for HPLC analysis.

For standard addition curves, stock solutions of 1 0 m*M* were made for each compound by dissolving the appropriate amount in 45% acetonitrile (v/v in water) containing 4.2 m*M* phosphoric acid. Rat urine was spiked by adding 100 μl of 1.0 m*M* hexamidine stock solution, to act as an internal standard, and various amounts of the test compounds to the urine, giving a final volume of 1 0 ml. Concentrations of 200, 100, 50, 20, 10, 5, and 1 μ*M* for each compound were made, allowing the generation of standard curves The entire 1 0 ml of spiked urine was transferred to an activated 300-mg Prep-Sep C_{18} column (Fisher Chemical Co., Fairlawn, NJ, U S.A.) and washed with 1 0 ml of water, followed by 1.0 ml of acetonitrile. The compounds of interest were then eluted with 1.0 ml of 10 m*M* heptanesulfonate, 10 m*M* TMAC, and 4.2 m*M* phosphoric acid in 95% acetonitrile, under gentle vacuum conditions An aliquot of this eluate was then analyzed by HPLC. Eight solid-phase C_{18} extraction columns (see Table II) were compared for their relative ability to extract pentamidine and hexamidine. Each solid-phase extraction column was conditioned by passaging 1.0 ml distilled water and then 1 0 ml acetonitrile through the column matrix. To each column was added a 1 0-ml solution of rat urine containing 100 μ*M* pentamidine and 100 μ*M* hexamidine. The urine was extracted as described for the standard addition curves, and the peak areas for both pentamidine and hexamidine were combined to give a single value This value was then used to determine the relative effectiveness of each column for extracting diamidines.

Chromatographic conditions

The mobile phase consisted of 10 m*M* heptanesulfonate, 10 m*M* TMAC, and 4 2 m*M* phosphoric acid in water for pump A and of 75% acetonitrile, 10 m*M* heptanesulfonate, 10 m*M* TMAC, and 4 2 m*M* phosphoric acid in water for pump B. The high concentration of TMAC was necessary to prevent the highly charged drugs from binding to storage containers, HPLC lines, and column packings. Using a flow-rate of 1 5 ml/min, runs were initiated at 31 5% acetonitrile and increased to 37 5% acetonitrile in a linear gradient over 7 0 min The latter concentration was held for 0 5 min before being dropped to 31 5% acetonitrile over 1 0 min The concentration of acetonitrile was then held at 31 5% for 1 0 min Due to the small gradient range utilized, a 1-min equilibration time was sufficient to prepare the HPLC system for immediate use

Analyzing urine from dosed rats

The ability of the assay to detect pentamidine and its analogues was tested in an immunosuppressed rat model of PCP, simulating the conditions under

which the drugs would be used in practice. Twelve male, barrier-raised Sprague–Dawley rats (Hilltop Labs , Scottdale, PA, U S A) were kept in metabolic chambers for a period of eight weeks The animals were kept on a low (8%) protein diet (ICN Biomedicals, Cincinnati, OH, U.S A) and drinking water containing 0 5 mg/ml tetracycline and 1 0 μg/ml dexamethasone This treatment was continued for eight weeks to induce immunosuppression [12] After six weeks, the three rats each were injected with either saline (0 3–0 4 ml per day), pentamidine (10 mg/kg), diaminopentamidine (10 mg/kg), or dimethoxypentamidine (5 mg/kg), once daily for fourteen days Urine was collected for a 24-h period after each injection The urine was mixed with 25 ml of 0 1 *M* hydrochloric acid to prevent microbial growth

A 100-μl volume of 1 0 m*M* hexamidine was added to 9 90 ml of each dilute urine sample, before the entire 10 ml were added to a Prep-Sep C_{18} solid-phase extraction column. The column was then thoroughly washed with distilled water and acetonitrile, before elution with 1 0 ml of the 95% acetonitrile elution buffer The eluate was then analyzed by HPLC

RESULTS AND DISCUSSION

Detection and recovery of the diamidines

To determine the relative retention times of the diamidines, a single test compound and hexamidine were made up to 100 μM in 45% acetonitrile containing 10 m*M* heptanesulfonate, 10 m*M* TMAC, and 4 2 m*M* phosphoric acid Each compound was then assayed by HPLC using identical run conditions (see Experimental) Fluorescence detection was used for each, with the exception of diaminopentamidine and dianilinopentamidine which exhibited very little fluorescence and had to be detected by ultraviolet absorption The wavelengths used for fluorescence and ultraviolet detection are listed in Table I All of the compounds were found to be adequately separated from both the solvent front and the hexamidine peak (Fig 2) While earlier experiments indicated that isocratic conditions existed that would resolve a single compound from the solvent front and the internal standard, only the gradient conditions allowed the possibility of analyzing more than one compound at a time The resolution provided by this assay has been of importance in determining the purity of the drugs synthesized for this project Several of the compounds were found to contain impurities The hexamidine internal standard was found to contain a small peak with a retention time of approximately 7 min, which was apparently due to C_7 contamination of the C_6 starting material in the synthesis The HPLC assay allowed us to monitor the progress of purification and aided in the identification of the contaminant This application of the assay is of importance in a laboratory where purity of test compounds must be assured

Several commercially available C_{18} solid-phase extraction columns were evaluated to determine their effectiveness in recovering diamidines Pentam-

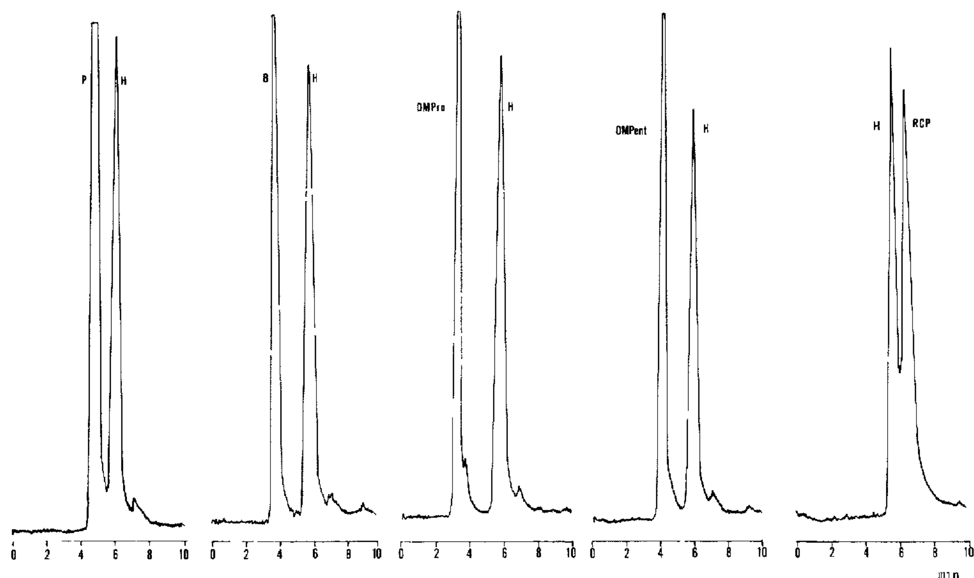

Fig 2 Representative chromatograms of several of the compounds tested in this study, with 100 μM hexamidine as internal standard Each of the compounds, with hexamidine, was diluted to a concentration of 100 μM, and 25 μl were injected Fluorescence detection was used for all, with an excitation wavelength of 265 nm and a 340-nm cutoff filter for emission Peaks H = hexamidine, P = pentamidine, B = butamidine, DMPro = dimethoxypropamidine, DMPent = dimethoxypentamidine, RCP = imidazolinopentamidine The retention times of diaminopentamidine and dianilinopentamidine (not shown) are approximately 3 7 and 4 4 min, respectively (using ultraviolet absorbance at 235 nm for diaminopentamidine and 220 nm for dianilinopentamidine)

idine and hexamidine were added to rat urine to give a final concentration of 100 μM each and then added to the C_{18} column for extraction The peak areas of pentamidine and hexamidine were combined, and this value from each column was compared Since, in a previous experiment, the Prep-Sep columns were found to recover over 90% of 100 μM pentamidine and hexamidine when compared to control chromatograms of 100 μM unextracted drug, they were arbitrarily given a relative recovery value of 100% for comparison with the other columns An extremely wide range of recoveries was found (as shown in Table II), with the best recovery found with the Bond Elut C_{18} columns (100 48%) and the worst with the Bond Elut LRC C_{18} OH columns (17 50%) It was soon obvious that not all solid-phase extraction columns were ideally suited for our protocol Several of the columns, such as Sep-Pak, HHP Octadecyl, Extract Clean, and Bond Elut LRC, gave recoveries of less than 65% of the value for the Prep-Sep columns In fact, those columns which had been end-capped to remove free OH groups in the silica (the HHP Octadecyl and Bond Elut LRC columns) gave consistently poor results The end-capping pro-

TABLE II

RELATIVE RECOVERIES OF PENTAMIDINE AND HEXAMIDINE BY VARIOUS C_{18} SOLID-PHASE EXTRACTION COLUMNS

Column	Company	Relative recovery (%)
Prep-Sep C_{18}	Fisher (Fairlawn, NJ, U S A)	100[a]
Sep-Pak C_{18}	Waters (Milford, MA, U S A)	60 58
Supelclean LC-18	Supelco (Bellefonte, PA, U S A)	95 89
Octadecyl	Baker (Phillipsburg, NJ, U S A)	65 84
HHP Octadecyl	Baker (Phillipsburg, NJ, U S A)	41 24
Extract Clean C_{18}	Alltech (Deerfield, IL, U S A)	21 79
Bond Elut C_{18}	Bond Elut (Harbor City, CA, U S A)	100 48
Bond Elut LRC C_{18} OH	Bond Elut (Harbor City, CA, U S A)	17 50

[a]Prep-Sep C_{18} was given a value of 100% recovery (see Results and discussion)

cedure increases the cost of the columns, but rendered them ineffective for our uses Since the only other diamidine assay using solid-phase extraction columns [6] involved C_8 columns, there are no published values of C_{18} recovery for comparison However, several of the columns (Prep-Sep, Supelclean, Bond Elut) consistently gave us extremely high recoveries For cost and recovery optima, it was decided to proceed with the Prep-Sep C_{18} solid-phase columns for the remainder of the study

Standard addition curves

Calibration curves were generated for each of the compounds by using rat urines spiked with diamidine to give concentrations of 200–1 μM test compound and 100 μM hexamidine The peak-area ratio of the test compound and hexamidine were compared with the corresponding concentration of test compound (Table III) The curves for all of the compounds were found to be linear, with the lowest r^2-value being 0 98 for dimethoxypropamidine The lowest values tested were 341 ng/ml for pentamidine, 8 1 μg/ml for butamidine, 5 1 μg/ml for dimethoxypentamidine, 8 9 μg/ml for dimethoxypropamidine, 25 3 μg/ml for imidazolinopentamidine, 4 35 μg/ml for dianilinopentamidine, and 5 4 μg/ml for diaminopentamidine The assay was found to be quite accurate and precise, with accuracy of pentamidine measurements at 1 0 and 10 μM being 98 and 86%, respectively Day-to-day precision was also calculated for 1 0 and 10 μM pentamidine, with the coefficient of variation being 12 3 and 17 8%, respectively (based on measurements made on five separate days) In recent experiments, the limit of detection was greatly improved by eluting the test compounds off the solid-phase extraction columns in a volume less than that of the applied urine, effectively concentrating the analyte Spiked samples of 0 1 μM pentamidine (34 1 ng/ml) and 0 5 μM dimethoxypropamidine (4 45 μg/ml) have been easily detected in urine

TABLE III

STANDARD CURVES FOR THE DETECTION OF DIAMIDINES

Compound	Concentration tested (μM)		b^a	m^a	r^2
	Highest	Lowest			
Pentamidine	100	1 0	0 0876	0 0083	1 00
Butamidine	200	20	0 0911	0 0033	0 99
Dimethoxypropamidine	100	20	0 3276	0 0151	0 98
Dimethoxypentamidine	200	10	−0 1209	0 0129	1 00
Dianilinopentamidine	200	10	0 1007	0 0054	1 00
Diaminopentamidine	200	10	−0 0367	0 0423	0 99
Imidazolinopentamidine	200	50	0 0700	0 0097	1 00

[a]These values are from equation $y = mx + b$ for the standard curves

Detection of compound in urine from dosed rats

Since the purpose of designing a procedure for detecting these diamidine compounds is to determine their pharmacokinetic properties in the treatment of *P carinii*, the ability to detect the compounds in physiological fluids from dosed patients is essential To this end, immunosuppressed Sprague–Dawley rats were treated with either saline or chemotherapeutic concentrations of one of three diamidines· pentamidine (10 mg/kg), diaminopentamidine (10 mg/kg), or dimethoxypentamidine (5 mg/kg) The rats were injected once a day for fourteen days and kept in metabolic chambers to collect urine for 24-h periods The results of HPLC analysis of this urine are summarized in Table IV and representative chromatograms shown in Fig 3 Although fluorescence detection provided greater sensitivity to the test compounds, ultraviolet absorbance gave a better signal-to-noise ratio Therefore, ultraviolet detection was used on all samples where minimal concentrations were expected For all the compounds, it was found that urine concentrations were extremely low Dimethoxypentamidine, given intravenously at a dose of 5 0 mg/kg, was found in concentrations ranging from 1 46 to 3 07 μg/ml in urine, and diaminopentamidine, given intravenously at 10 μg/ml, was 8 27–10 21 μg/ml Pentamidine was found to be excreted in even lower concentrations, with urine levels being in the range 0 15–0.21 μg/ml. However, of greater interest is the amount of compound excreted in the urine over a 24-h period Dimethoxypentamidine is excreted in larger amounts than the other two drugs, with 122.52–363 88 μg being excreted over 24 h (Table IV). Diaminopentamidine showed the most variability, with the range being 5 44–67 54 μg excreted over a single day. The amount of pentamidine detected in the urine over this interval was found to be 1 60–3 90 μg

The renal excretion of inhaled or intravenously injected pentamidine has been measured in humans by Conte and Golden [8] Patients treated with 3

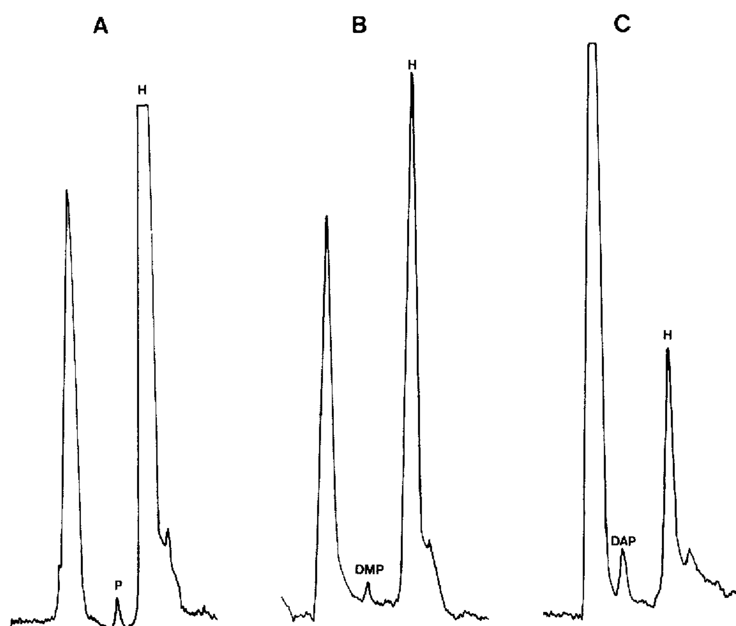

Fig 3 Detection of aromatic diamidines in urine from dosed rats Immunocompromised rats were given daily intravenous injections for fourteen days with (A) 10 mg/kg pentamidine, (B) 5 mg/kg dimethoxypentamidine, and (C) 10 mg/kg diaminopentamidine Urine was collected and analyzed by HPLC as described in the Experimental section Representative chromatograms are shown for each drug Pentamidine (P) and dimethoxypentamidine (DMP) were detected by ultraviolet absorbance at 265 nm, while diaminopentamidine (DAP) was detected by ultraviolet absorbance at 235 nm Hexamidine (H) (100 μM) was used as the internal standard

TABLE IV

CONCENTRATION OF DIAMIDINE IN THE URINE OF DOSED RATS

Compound	Animal	Dosage (mg/kg)	Concentration in urine (mean ± S D) (μg/ml)	Amount excreted in 24 h (mean ± S D) (μg)
Pentamidine	1	10 0	0 16 ± 0 15	1 60 ± 1 50
	2	10 0	0 21 ± 0 02	1 68 ± 0 16
	3	10 0	0 15 ± 0 01	3 90 ± 0 26
Dimethoxypentamidine	1	5 0	1 46 ± 0 91	122 52 ± 74 28
	2	5 0	3 07 ± 1 15	187 00 ± 127 60
	3	5 0	2 72 ± 0 54	363 88 ± 234 08
Diaminopentamidine	1	10 0	10 21 ± 6 19	11 68 ± 7 28
	2	10 0	8 50 ± 5 80	67 54 ± 25 30
	3	10 0	8 27 ± 5 32	5 44 ± 1 08

or 4 mg/kg ınhaled pentamıdine excreted 0 131–0 083 μg/ml of urıne, with a total of 0 214 ± 0 156 μg beıng excreted over the first 24 h after treatment Patıents gıven 3 or 4 mg/kg pentamıdıne ıntravenously excreted 2 10 μg/ml of urıne, wıth 4 23 ± 1 60 μg excreted over the first 24 h Our findıngs are almost ıdentıcal to the values for ınhaled pentamıdıne (0 15–0 21 μg/ml versus 0 131 μg/ml) Why excretıon rates in ıntravenously dosed rats should approxımate humans treated wıth ınhaled pentamıdıne ıs a poınt for speculatıon The fact that two dıfferent specıes were used for these studıes could well be the source of thıs correlatıon As well, the humans treated ıntravenously ın the Conte and Golden study [8] were gıven the pentamidıne usıng a 2-h ınfusıon, whıch would ıncrease the urıne output due to the large volume of fluıd ıntroduced ınto the patıent. The rats ın our study were gıven the drugs ın a sıngle ınjectıon, so there was no major change ın the amount of fluıd ın the plasma However, further study on the tıme course of urıne excretıon of pentamıdıne ın ımmunosuppressed rats ıs necessary before accurate comparıson can be made wıth human clearance

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