Immunoassays for Pentamidine and Related Compounds: Development of a Facile Inhibitory ELISA Suitable for Clinical Use

Howard M. Reisner,* Danny R. Gray, Susan K. Jones, Beate G. Rose, and Richard R. Tidwell

Department of Pathology and Laboratory Medicine, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Aromatic dicationic drugs have a broad spectrum of activity against protozoal and fungal pathogens including *Pneumocystis carinii*, *Leishmania mexicana amazonensis*, *Cryptosporidium parvum* and *Cryptococcus neoformans*. Pentamidine serves as the exemplar for an extensive collection of newly synthesized related compounds, which have reduced toxicity and a wider range of target organisms. Assays of pentamidine and related compounds have depended on HPLC-tandem mass spectrometry (HPLC-TMS) for the quantitation and identification of drug and metabolites. Immunoassays for pentamidine would have many advantages over the HPLC methods including relative simplicity of assay format and required equipment, convenience in sample preparation and reduction in time and cost of assays.

In this report we describe a simple ELISA

based immunoassay for pentamidine and pentamidine-like drugs with requisite sensitivity and specificity for use as a clinical assay (EC₅₀ value of about 50 nanomolar). Immunogen was synthesized by coupling the hapten aminopentamidine to ovalbumin (chemically modified to provide an optimal number of –SH groups) using sulfo-MBS. Maleic-anhydride activated ELISA plates were covalently sensitized using the aminopentamidine hapten and used in an inhibitory ELISA assay format whereby the ability of analyte to suppress antibody binding to sensitized plate was measured. The assay detects primarily the phenolic amidine of pentamidine when in a para position and hence can also detect structurally related derivatives of pentamidine of potential interest as new therapeutic agents. J. Clin. Lab. Anal. 14:73–82, 2000. © 2000 Wiley-Liss, Inc.

Key words: immunoassay; ELISA; pentamidine; dicationic molecules; *Pneumocystis carinii*; *Leishmania mexicana amazonensis*

INTRODUCTION

Aromatic dicationic drugs have a broad spectrum of activity against protozoal and fungal pathogens including *Pneumocystis carinii*, *Leishmania mexicana amazonensis*, *Cryptosporidium parvum* and *Cryptococcus neoformans* (1–4). Pentamidine (the sole example in current clinical use) was synthesized more than fifty years ago, has been the subject of pharmacologic investigations since the 1940s and has been recognized as an anti-*Pneumocystis* agent since 1957 (5–9). Today, pentamidine remains a significant second-line therapeutic agent in AIDS associated *Pneumocystis carinii* pneumonia (PCP), and is also used to treat drug-resistant leishmaniasis (10–13). Widespread use of pentamidine is inhibited by its lack of oral activity and dose-dependent nephro-, hepato-, and pancreatic toxicity (14–15). Although the exact mode of action of pentamidine-like drugs remains uncertain, structure-function studies suggest that DNA binding, particularly high-affinity binding to the minor groove of

DNA, is a likely prerequisite for therapeutic activity (16–17). Studies by Tidwell and coworkers indicate that pentamidine is converted to a number of derivatives by a cytochrome P-450 dependent metabolic transformation (8,17–18). Such drug metabolites are likely to be of significance both in determining drug activity and toxicity (19–20).

Recent pharmacologic studies of pentamidine and related compounds have depended on HPLC-tandem mass spectrom-

Grant sponsor: North Carolina Biotechnology Center. Grant sponsor: NIH-STTR; grant number: R41-AI 42460 to Immtech Inc. and Dr. Reisner. In addition the continued financial and scientific support of Immtech is gratefully acknowledged.

*Correspondence to: Howard M. Reisner, CB# 7525, BBB228-H, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7525. E-mail: reisner@med.unc.edu

Received 2 September 1999; Accepted 22 November 1999

etry (HPLC-TMS) for the quantitation and identification of drug and metabolite although simpler HPLC systems may suffice when identification of metabolites is not a primary concern (21–22). While such techniques are well suited for experimental studies of drug metabolism, they have significant drawbacks for use in a clinical setting. Immunoassays for pentamidine would have many potential advantages over the HPLC methods including relative simplicity of assay format and required equipment, convenience in sample preparation and reduction in time and cost of assays.

In this report we describe a simple ELISA based immunoassay for pentamidine and pentamidine-like drugs with requisite sensitivity and specificity for use as a clinical assay to determine levels in blood (23–24). The assay appears to primarily detect the aromatic amidine of pentamidine when in a para position relative to the ether bond and can also detect certain structurally related derivatives of pentamidine.

MATERIALS AND METHODS

Preparation of Reactive Hapten

1-(4'-Amidinophenoxy)-5-(4'-Aminophenoxy)pentane (trivial name, aminopentamidine)

A monoamino derivative of pentamidine [1-(4'-amidinophenoxy)-5-(4'-aminophenoxy)pentane (trivial name, aminopentamidine)] was prepared by alkylating 4-nitrophenol with 1, 5-dibromopentane to produce 1-(4-nitrophenoxy)-5-bromopentane (Fig. 1, **1**, **2** and **3**, respectively) (25–27). The product (**3**) was reacted with *p*-phenolnitrile (**4**) and sodium to produce 1,5-diphenoxypentane (**5**). The nitrile group on **5**

was subsequently converted to an amidine group by reaction with a methanol/hydrochloric acid solution followed by treatment with ammonia in ethanol (**6**). Thereafter, the nitro group was reduced to the amine by catalytic dehydrogenation to yield aminopentamidine (**7**) (28). The structure of the product was confirmed by 300 MHz NMR analysis. Analysis using HPLC indicated a purity of greater than or equal to 98% for three independent (21).

Ovalbumin (Carrier) Modification

The amino groups of various proteins, including ovalbumin, were modified using Traut's reagent (Fig. 2, **9**) to increase the molar ratio of free sulfhydryl groups available for haptenylation on the protein (Fig. 2, Step I) (29). Traut's reagent (2-Iminoethiolane-HCl) (Pierce, Rockford, IL) was prepared as a 7.5 mM solution in reaction buffer (RB, 0.05 M triethanolamine/triethanolamine HCl, 7 mM potassium phosphate, 0.1 M NaCl, 1mM disodium EDTA pH 8.0) immediately prior to use. Ovalbumin (grade III, Sigma Chemical Company, St. Louis, MO) was dissolved RB (1mL, 0.75mM) and mixed with an equal volume of Traut's reagent solution. The ten-fold molar excess of Traut's reagent provided optimal addition of free sulfhydryl groups (Fig. 2, **10**) and satisfactory overall reaction yields (as discussed in Results). The mixture was allowed to react at room temperature for 2 hours and then subjected to gel filtration on a column (2.5 × 20 cm) of Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) using borate buffer (0.01 M, pH 8.0). The absorbance at 280 nm was used to identify the void volume (protein containing) peak, which was collected in a volume of about 3 mL for subsequent analysis and haptenylation.

Fig. 1. Synthesis of 1-(4' amidinophenoxy)-5-(4' aminophenoxy)pentane.

Fig. 2. Synthesis of hapten-carrier conjugate.

Hapten Activation and Conjugation

m-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sulfo-MBS) (Pierce), an NHS ester-maleimide heterobifunctional crosslinker, was reacted with aminopentamidine so as to form a sulfhydryl reactive intermediate (Fig. 2, Step II) (30). A three-fold molar excess of Sulfo-MBS to the calculated number of available sulfhydryl groups on the ovalbumin (see below) was dissolved in borate buffer (generally 32 mM in 200 μ l). A two-fold excess of aminopentamidine to the Sulfo-MBS was dissolved in dimethyl sulfoxide (DMSO) (generally 64 mM total in 200 μ l). The aminopentamidine solution was slowly combined with the Sulfo-MBS solution and allowed to react at room temperature for 2 hours.

The activated hapten was then coupled to the modified protein to yield conjugate that was used for immunizations (Fig. 2, Step III). The aminopentamidine-Sulfo-MBS solution was added drop-wise with constant agitation to the modified ovalbumin solution and allowed to react for 2 hours at room temperature. The haptenylated protein solution was clarified by centrifugation followed by filtration through a Whatman GF/

C glass fiber pad and then subjected to gel filtration on a column (2.5 $\times$ 20 cm) of Sephadex G-25 (Pharmacia) using 0.01 M Borate Buffer, pH 8.0. The absorbance at 280 nm was used to identify the derivatized ovalbumin peak as above.

Determination of Sulfhydryl Groups

Ellman's reagent was used to measure the generation of free sulfhydryl groups on the ovalbumin (Fig. 2, Step I) and also to monitor subsequent loss of such groups as a result of reaction with the activated hapten (Fig. 2, Step III). A microwell assay using Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid) (Pierce) was performed following the manufacturer's instructions using L-cysteine standards (3.8 to 0.2 mM in RB). Untreated ovalbumin (0.8 mM) was included as a negative control as no free-SH groups were detected on the unreduced protein. All assay wells contained 200 μ l PBS/EDTA (1 $\times$ Phosphate Buffered Saline (PBS) pH 7.4 (Sigma) with 1 mM EDTA) to retard spontaneous oxidation. All wells were then treated with 20 μ l of Ellman's reagent (1mg/ml). The plate was allowed to react for 15 minutes at room temperature in

the dark. The absorbance was read at 405 nm in a DigiScan plate reader (ASYS Hitech, Eugendorf, Austria) and the average values calculated using BioSwift software (Columbia Bioscience, Inc., MD). Concentration curves were plotted and number of substituted groups were calculated using the cysteine standard. Values observed were between 5 and 15-SH groups on modified ovalbumin. No free-SH groups were found in ovalbumin preparations after reaction with the activated hapten. Control Traut's reagent treated ovalbumin preparations (not reacted with activated hapten) demonstrated no loss of free-SH groups after a 24-hour incubation period. Column chromatography to remove low-molecular-weight interfering substances from reaction mixtures was necessary prior to the use of Ellman's reagent.

Total Protein Quantification Using a BCA Assay

BCA protein analysis was used to determine total protein recovered following purification procedures. Assays were done in microwells following the manufacturer's instructions (Pierce). Bovine serum albumin standards (Pierce) were used in all assays. Plates were read at 540 nm as above.

Conjugation Analysis by Western Blot

SDS PAGE Western Blot analysis was used to characterize the degree of ovalbumin haptenylation by measuring the apparent gain in molecular weight of ovalbumin subsequent to reaction with the activated pentamidine derivative. Conjugated and control ovalbumin was diluted 1:2 in Laemmli sample buffer containing 0.05% β -mercaptoethanol. Samples were heated to 100°C for 5 min and spun briefly. Fifteen μ l of each sample were loaded onto a 4–15% (w/v) SDS polyacrylamide mini gradient gel (Bio-Rad Laboratories, Hercules, CA). Electrophoretic separation was accomplished at 200 constant V over 45 minutes. The separated proteins were then transferred to a nitrocellulose membrane using a Multiphor II semi-dry transfer unit (Pharmacia Biotech, Uppsala, Sweden). The immobilized proteins were incubated with blocking buffer (TBST [25 mM Tris + 50mM NaCl + 0.1% Tween-20] + 5% nonfat dry milk + 10% normal goat serum) for 1 hour then incubated with rabbit anti-chicken ovalbumin (Sigma Chemical Co., St. Louis, MO) diluted to 1:2000 in TBST for 14 hours at 4°C. Bound antibody was detected by incubation with an alkaline phosphatase-conjugated mouse anti-rabbit IgG (Sigma). The secondary antibody was used at a dilution of 1:10,000 in TBST, and the resultant signal was disclosed by incubation in nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (BioRad Life Sciences, Melville, NY). Apparent molecular weights were determined by comparison to known standards using linear regression (31).

Production of Antisera

The aminopentamidine-ovalbumin conjugate (0.2 mg/mL protein, hapten-carrier ratio of 23) was used to immunize rab-

bits (New Zealand White, 2.5–3.0 Kg). Rabbits were injected both subcutaneously and intramuscularly using a total of 250 μ l of protein mixed with an equal volume of Titermax™ adjuvant (CytRX Corporation, Norcross, GA) at six sites. Rabbits were boosted at two-week intervals using an identical protocol and bled after four and seven weeks. Bleeds were pooled and purified using a ImmunoPure (A/G) IgG Purification Kit (Pierce). Protein concentration was quantified using the BCA Assay protocol above and found to be 1.9 mg/mL.

Additionally, aminopentamidine-ovalbumin conjugate (0.8 mg/mL hapten-carrier ratio of 9) was used to immunize Balb/C mice. Mice were immunized with 100 μ g of antigen in Titermax subcutaneously (first injection) and intraperitoneally (subsequent injections) for a total of six times biweekly. All animal experimentation was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

ELISA Method for Detection of Anti-Pentamidine Antibodies

In this assay maleic anhydride activated polystyrene microtiter plates were reacted with aminopentamidine to form a covalent link between antigen and plastic substrate. Reacti-Bind™ maleic anhydride Activated Polystyrene Strip 96 well plates (Pierce) were sensitized with 100 μ l of aminopentamidine diluted to an optimal concentration in PBS pH 7.4 (Sigma) immediately prior to dispensing. Aminopentamidine stock was kept as a 10 mg/mL solution in DMSO at 4°C protected from light. Optimal concentration of aminopentamidine for sensitization was generally 0.01 μ g/mL (see Results). Plates were incubated for 2 hours at 37°C and tapped out until dry. Reactive sites were blocked with 250 μ l of Blotto per well [Blotto 5% nonfat dry milk (Carnation) and 10% normal goat serum (Biodesign International, Kennebunk, ME) in Tris Buffered Saline pH 8.0 (TBS, Sigma)]. The plate was then washed four times with Wash Solution [PBS pH 7.4 (Sigma) with 0.05% Polyxyethylene Sorbitan Monolaurate (Tween 20) (Sigma)], and tapped out until dry. One hundred μ l of appropriate dilutions of animal sera or purified immunoglobulin preparations were made in PBS/Blotto [(PBS with 2% Blotto)] and incubated in sensitized and blocked wells for 2 hours at 37°C. The plate was then washed four times with Wash Solution, and tapped out until dry. Reactions were generally done in triplicate and included normal animal sera or Ig preparations as negative controls. One hundred μ l per well of goat anti-mouse or anti-rabbit IgG (H&L) horseradish peroxidase labeled (Jackson ImmunoResearch Laboratories Inc.) diluted 1:20,000 in PBS/Blotto was used as secondary antibody. The plate was incubated for 1 hour at 37°C, washed four times with wash solution, and tapped out until dry. The ABTS Microwell Peroxidase Substrate System Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used according to the product information sheet with the plate

being allowed to develop for 45 minutes at room temperature. The absorbance was read at 405 nm in a DigiScan plate reader (ASYS Hitech) and the averages values calculated using BioSwift (Columbia Bioscience).

Inhibitory ELISA for Detection of Pentamidine and Immunoreactivity of Pentamidine-Like Drugs

The inhibitory ELISA assay was carried out as above with the following modifications. Fifty μ l of analyte, buffer, or pentamidine standard were mixed with 50 μ l of an appropriate dilution of anti-pentamidine reagent (generally about 10 μ g/mL of purified rabbit Ig or a 1/200 dilution of mouse serum) in sensitized blocked well and incubated for 2 hours at 37°C followed by washing and addition of secondary antibody to wells (as described above). The use of purified rabbit anti-pentamidine Ig preparations significantly reduced assay background. Pentamidine standards of between 100 and 0.8 ng/mL in PBS/Blotto and a zero dose control for maximum binding were included. Inhibition curves were plotted and the EC₅₀ calculated using four parameter non-linear curve fitting (Prizm 2.01, GraphPad Software, Inc., San Diego, CA or AssayZap 2.22 Biosoft, Cambridge, UK), the latter for some murine-based immunoassays. Pentamidine-like drugs were assayed for immunoreactivity as above but using dilutions starting at 5 μ g/mL. Three drugs and one pentamidine standard were simultaneously assayed on a single plate. Drugs showing high immunoreactivity were re-assayed at a greater initial dilution. EC₅₀ values for drugs were determined using non-linear curve fitting but setting maximal and minimal binding parameters to the values derived from the pentamidine standard curve.

RESULTS

Production of Hapten-Carrier Conjugate and Animal Immunizations

The monoamino derivative of pentamidine (Fig. 1, 7) was chosen as a hapten based on ease of synthesis and a desire to orient one amidinophenoxy moiety away from the site of crosslinking to protein (via the amino group on the aminophenoxy moiety). The use of a heterobifunctional crosslinker (Sulfo-MBS) required the addition of free-SH groups to common carrier proteins in order to achieve a sufficient degree of protein haptenylation. Bovine serum albumin proved to have insufficient solubility after modification with Traut's reagent and addition of activated hapten solutions in DMSO (data not shown). Ovalbumin could be modified to obtain acceptable levels of free-SH groups for haptenization while maintaining reasonable solubility (Table 1). Runs 3 and 4 Pool and 5 were done using an excess of Traut's reagent to potential modifiable (epsilon amino) groups on the protein (50:1 excess in terms of ovalbumin). Under such conditions essentially all available groups were modified. [There are 20 lysine residues and four cysteine residues in chicken ovalbu-

TABLE 1. Number of substituted -SH groups, total protein and percent yield

Conjugation run	Groups	Total protein (mg)	% Yield
3 & 4 Pooled	23	1.0	3.3
5	22	1.8	6.0
6	9	3.9	13
7	13	5.4	18
8	5	6.0	20

min (32)]. The low yield was the result of protein precipitation on addition of the activated hapten. Subsequent adjustment of the amount of Traut's reagent so that available amino groups became limiting (Table 1 runs 6–8; conditions as described in Methods) reduced the degree of modification (mean of 9 for the three runs) and more than doubled the yield of hapten carrier conjugate. It should be noted that degree of haptenylation was estimated under the assumption that all available-SH groups conjugated with the activated hapten. To more directly measure crosslinking of hapten, the apparent gain in molecular weight of the haptenylated protein was measured using SDS gradient gel electrophoresis coupled with immunodetection of the modified ovalbumin (Fig. 3, runs 3 and 4, 5 and 6). The observed shift in molecular weight was roughly consistent with values of modification reported in Table 1 although the broad nature of the ovalbumin band made exact determination of weight shifts impossible. Run 5 (having an average of 22-SH groups per molecule) demonstrates a greater and more uniform shift in apparent molecular weight than run 6 (average of 9-SH groups per molecule). Run 6 also demon-

Fig. 3. Western immunoblot of haptenylated ovalbumin. Arrow (→) and line (superimposed on image) indicate position of native ovalbumin.

strates considerable trailing into the area of the gel where unmodified ovalbumin migrates. The higher molecular weight bands (in control and at shifted positions in the modified proteins) are likely to be ovalbumin polymers known to be present in the preparation used.

Comparison of titers of mice immunized with the early conjugate runs (high hapten carrier ratio) versus mice immunized with the later runs (lower hapten carrier ratio) suggests that the less haptenylated material is optimal. In addition, the use of Freund's Complete Adjuvant in initial immunizations followed by Freund's Incomplete in subsequent boosts was no better than the described Titermax protocol (data not shown).

Detection of Anti-Pentamidine Activity

Initial attempts to detect anti-pentamidine activity in immunized animal sera used several ELISA formats. A variety of microtiter plates proved unsuitable for direct sensitization with pentamidine because of low drug binding and/or high background. Pentamidine-BSA or KLH conjugates required alternative coupling chemistry (using EDC) for synthesis to prevent detection of neoantigens related to the MBS moiety. Such alternative chemistries were inconvenient and produced very low yields of conjugate. The use of such conjugates in ELISA assays did demonstrate that immunizations had produced anti-pentamidine activity.

The availability of aminopentamidine suggested that covalent coupling of this drug via the aminophenoxy moiety to a plastic substrate should serve to orient the amidinophenoxy portion of the molecule (presumed to contain the epitope of interest) away from the surface (and hence available for antibody binding). Reacti-Bind™ maleic anhydride activated plastic microtiter strips were evaluated as an ELISA substrate (Pierce). Both blocking agent and concentration of aminopentamidine used to react with plates proved critical. Concentrations of aminopentamidine between 0.1 to 0.01 $\mu\text{g/mL}$ proved optimal for plate sensitization using substrate absorbance as an indicator (Fig. 4, rabbit data shown, results similar for mouse). Higher or lower levels of aminopentamidine reduced assay sensitivity. Unimmunized controls (or unsensitized plates) showed acceptable levels of background color development when using blotto to block plates. A number of additional blocking agents evaluated proved unsuitable. Using this ELISA format, titers of immunized rabbits and mice (defined as working dilutions for use in the immunodetection of pentamidine-see below) were found to be equivalent to a 1/400 dilution of immune rabbit serum, 1/200 for mice (high hapten carrier immunization) and equal to or greater than 1/1600 (low hapten carrier immunization).

Sensitivity and Specificity of Immunoassay for Pentamidine and Pentamidine-Like Drugs

Inhibitory ELISA assays for pentamidine immunoreactivity showed similar sensitivity using either murine or rabbit

Fig. 4. Effect of concentration of aminopentamidine used in sensitization of ELISA plates on assay signal. Assay as in Materials and Methods using 1/400 dilution of rabbit anti-pentamidine reagent. Concentrations of aminopentamidine used to coat plate as follows: (○) no aminopentamidine, (□) 0.1 ng/mL, (◆) 1.0 ng/mL, (▲) 10 ng/mL, (●) 100 ng/mL, (■) 1000 ng/mL.

antisera. Mean EC₅₀ values were 36.3 ± 10.6 and 21.1 ± 10.0 ng/mL (n of 9 and 21 assays) for murine- and rabbit-based ELISAs respectively. Sensitivity in both cases was between 1 and 2 ng/mL (0.1 to 0.2 ng/ assay well) of pentamidine dissolved in diluting buffer. Use of a 1/6 dilution of human serum as sample matrix was evaluated using rabbit reagents (Fig. 5A). Although the rate of substrate conversion was slower in the presence of human serum, overall assay sensitivity was somewhat increased in the presence of serum. For the assay illustrated in Fig. 5A, the EC₅₀ in the presence of serum was 9.7 ng/mL (95% CI 6.6–14.2 ng/mL) as compared to 24.6 ng/mL (95% CI 18.4–32.8 ng/mL) for the assay in buffer. Preparation of pentamidine standards in 25% heparinized rat plasma resulted in a slight (about one doubling dilution) loss in sensitivity for the murine reagent-based assay.

The nature of the epitopes recognized in the immune response to pentamidine was delineated by comparing the inhibitory activity of selected pentamidine-like compounds to pentamidine using the inhibitory ELISA assay (Table 2, Fig. 5B). Clearly the aromatic amidine plays a critical role in the response as compounds 2 and 3, which substitute imidazoline and methylamine groups, show a reduction in immunoreactivity of about 1000-fold (murine antibody) and at least 20-fold (rabbit antibody). The aromatic amidine group alone, however, is not sufficient for significant cross-reactivity. Benzamidine and meta-pentamidine (drug 1) (Table 2, Fig. 5B) demonstrated 100- to 1000-fold or greater reductions in

Fig. 5. (A) Immunoassay for pentamidine using pentamidine standard diluted in PBS/blotto (●) or a 1/6 dilution of human serum in PBS/blotto (▲). Vertical bars represent standard deviation. Lines represent best fit using non-linear regression as described in Material and Methods. The parameter representing maximal binding was estimated and fixed using "0-dose" controls either with or without serum. (B) Immunoassays for pentamidine and related drugs as follows; (●) pentamidine, (▼) drug 1 (see Table 2), (▲) butamidine, (■) drug 4 (see Table 2). Vertical bars represent standard deviation. Lines represent best fit using non-linear regression as described in Materials and Methods with the exception of drug 1 where the line is hand fit (see note to Table 2 for drug 1). The parameter representing maximal binding was fixed for all drugs to the same "0-dose" estimate so as to make curves comparable. The pentamidine is the average of two independent assays; the other drugs are single assays.

antigenicity suggesting that the position of the amidine on the ring and other ring substituents are also important. The length of the alkyl chain (see butamidine Table 2 and Fig. 5B) and substitution of nitrogen for ether oxygen so as to form an amidinoanilino derivative (Table 2, compound 4) had a more minor effect. The cross-reactivity of butamidine was reduced by about half (murine antibody) or to about 10% (rabbit reagent). Compound 4 showed a striking difference in re-

activity in the rabbit assay where it showed almost two times the reactivity of pentamidine and in the murine assay where it was 20% as active as the immunogen. With this exception, rabbit and murine reagents show a similar spectrum of specificity. The greatest differences were observed with compounds showing relatively high cross-reactivity such as the above mentioned butamidine and Table 2, compound 4.

DISCUSSION

Pentamidine has a long history as a therapeutic agent for use in treating African trypanosomiasis, Leishmania, and more recently, pneumocystis infections (particularly in association with AIDS). The toxicity of pentamidine and the development of better tolerated drugs has relegated pentamidine to the position of a second-line agent, reserved for cases resistant to other drugs. Nevertheless, pentamidine still sees significant use in a variety of clinical settings (10–13).

Recent insights into the potential mechanism of action and mode of toxicity of pentamidine has led to the design of new pentamidine-like drugs having efficacy against an extended range of organisms and reduced systemic toxicity for example, (see 3–4, 33–35). Hence, the ability to conveniently measure the level of pentamidine and pentamidine-like drugs in biological fluids has increasing clinical relevance. Immunological reagents of defined specificity might also detect epitopes that are correlated with either drug efficacy or toxicity and thus be a useful adjunct in the rational design of new agents. The latter is supported by the finding that binding of pentamidine-like compounds to the imidazoline 2 class of cellular receptors may help explain some aspects of drug toxicity (20).

Pentamidine and related compounds have been characterized and quantified in biological materials primarily by sophisticated HPLC technology sometimes coupled to mass spectroscopy. Such methods are capable of a high degree of specificity and sensitivity for a given compound, but with requirements for extensive instrumentation, tedious sample preparation and considerable effort and cost (9,22). Immunoassays, although not having the level of specificity of HPLC/MS-based analysis, can achieve similar levels of sensitivity with great savings in time, labor and cost. The relatively broad analytical specificity of immunoassays may, in fact, be advantageous should related compounds (such as metabolites) be detected.

Given the potential utility of immunoassays for pentamidine-like compounds it is surprising that no immunoassay for pentamidine has been reported. The development of such an assay is likely to have been inhibited by the lack of a suitable reactive hapten and conjugation procedure for production of an effective immunogen. The monoamino derivative of pentamidine described in this publication has proven to be both a convenient derivative for coupling to carrier through the use of heterobifunctional crosslinkers and also

TABLE 2. Inhibitory activity of “pentamidine-like” compounds

Drug ID							Antibody	
	X ₁	X ₂	X position	R	Y	N	Mouse	Rabbit
							EC ₅₀ ^a μM (%)	EC ₅₀ ^a μM (%)
Pentamidine			Para	H	O	5	0.08 (100)	0.05 (100)
1			Meta	H	O	5	120 (0.07)	Trace ^b
2			Para	H	O	5	9.9 (0.8)	1.0 (5.0)
3	CH ₃ NH ₂ —	CH ₃ NH ₂ —	Para	H	O	5	80 (0.1)	0.9 (5.3)
4			Para	H	N	5	0.4 (20)	0.025 (188)
Butamidine			Para	H	O	4	0.1 (56)	0.51 (9.8)
Benzamidine			Am				100 (0.08)	4.5 (1.1)

^aEC₅₀ determined using four parameter non-linear regression as described in Materials and Methods. Percent (%) is relative to EC₅₀ of the pentamidine (100%). Drugs were tested at concentrations of up to 100 μg/ml (mouse assay) and 5 μg/ml (rabbit assay).

^bThe compound inhibited at the greatest concentration tested but an EC₅₀ could not be determined. Inhibition is <1% of pentamidine.

for the production of sensitized ELISA plates for use in drug and antibody assays. The conjugation method adopted is based on technology used to produce drug immunoassays specific for amino-glycoside antibiotics and has the advantage that degree of carrier haptenylation can be conveniently altered by adjustment of the relative concentration of Traut's reagent to carrier protein (36). Unfortunately, it proved impossible to directly assay pentamidine groups coupled to carrier, necessitating an indirect procedure for establishing hapten-carrier ratios. The general agreement of SDS gradient gel molecular weight based estimates to those derived from quantitation of available protein sulfhydryl groups suggests that the reported degrees of protein haptenylation are reasonable estimates. Immunization studies suggest that a relatively low degree of haptenylation (Table 1 runs 6–8 and Fig. 3) results in higher titered murine reagents, a not unexpected finding (37). Because specificity studies reported herein used reagents produced using only high hapten-carrier ratio immunogen, the effect of hapten density on assay specificity is uncertain.

Assay EC₅₀ values of 20–140 ng/mL and sensitivity of at least 2 ng/mL appears more than sufficient for clinical applications. Plasma levels of pentamidine in patients undergoing standardized IV or inhalation therapy (resulting in very high lung levels of drug) have been established in clinical studies

using HPLC methods. The range of maximal plasma levels after IV therapy was 164–1360 ng/mL while the equivalent range after aerosol therapy was about 5% of post-IV levels (6.1–79 ng/mL). The described inhibitory ELISA format could be modified to use fluorescent or luminescent detection systems with a potential 10- to 100-fold gain in sensitivity should such be necessary.

Because a variety of dicationic, diamidine analogs of pentamidine have proven to be of potential clinical interest (3–4,17,33–35) an immunoassay with a relatively broad specificity towards such compounds was desired. Cross-reactivity studies summarized in Table 2 suggest that the presence of an aromatic amidine group in a para position on the phenol ring is necessary (but not sufficient) for efficient antibody binding. The low level of reactivity of benzamidine (1% or less than pentamidine) and the high reactivity of compound 4 (in rabbit-based assays) supports the importance of some additional substituent groups on the ring (such as a phenolic oxygen or anilino nitrogen) in epitope expression. Neither the substitution of nitrogen for the ether oxygen or changes in alkyl chain length appears critical (although both do change cross-reactivity significantly). Additional cross-reactivity studies currently in progress support the ability of the assay to recognize the aromatic amidine in a wide variety of dicationic pentamidine analogues. The ability of the

assay to recognize pentamidine metabolites, now known to be of importance in the toxicity of the drug will be of great interest. Based on current specificity data, chain hydroxylated metabolites are very likely to show a high degree of cross-reactivity (8,19,38).

In summary, this immunoassay for pentamidine appears to be a good candidate for a useful clinical test. The assay uses a convenient format, tolerates plasma or serum as a sample matrix and has more than sufficient sensitivity to detect clinically relevant (and certainly toxic) levels of pentamidine in blood. The specificity of the assay appears broad enough to detect at least some of the metabolites of pentamidine although more extensive cross-reactivity studies will be necessary to confirm this. The ability of the described protocols to generate murine-based assays suggests that production of monoclonal-based assays will be feasible. Such reagents would be of particular interest in use in structure function studies directed toward the rational design of pentamidine-like agents with an increased range of antiprotozoal effect and reduced toxicity.

ACKNOWLEDGMENTS

The authors would also like to thank Dr. Larry Potempa of Immtech, Jessica Hohrmann (Undergraduate Honors Student) and Karen Oyerly (Undergraduate SURE Fellow) for contributions to the manuscript.

REFERENCES

- Bell CA, Hall JE, Kyle DE, et al. Structure-activity relationships of analogs of pentamidine against *Plasmodium falciparum* and *Leishmania mexicana amazonensis*. *Antimicrob Agents Chemother* 1990;34:1381.
- Tidwell RR, Jones SK, Naiman NA, Berger LC, Brake WB, Dykstra CC, Hall JE. Activity of cationically substituted bis-benzimidazoles against experimental *Pneumocystis carinii* pneumonia. *Antimicrob Agents Chemother* 1993;37:1713.
- Hall JE, Kerrigan JE, Ramachandran K, et al. Anti-pneumocystis activities of aromatic diamidoxime prodrugs. *Antimicrob Agents Chemother* 1998;42:666.
- Del Poeta M, Schell WA, Dykstra CC, et al. Structure-in vitro activity relationships of pentamidine analogues and dicationic-substituted bis-benzimidazoles as new antifungal agents. *Antimicrob Agents Chemother* 1998a;42:2495.
- Schoenbach EB, Greenspan EM. The pharmacology, mode of action and therapeutic potentialities of stilbamidine, pentamidine, propamidine, and other aromatic diamidines—A review. *Medicine* 1948;27:327.
- Sands M, Kron MA, Brown RB. Pentamidine: a review. *Rev Infect Dis* 1985;7:625.
- Tidwell RR, Jones SK, Geratz JD, Ohemeng KA, Bell CA, Berger BJ, Hall JE. Development of pentamidine analogues as new agents for the treatment of *Pneumocystis carinii* pneumonia. *Ann NY Acad Sci* 1990b;616:421.
- Berger BJ, Naiman NA, Hall JE, Peggins J, Brewer TG, Tidwell RR. Primary and secondary metabolism of pentamidine by rats. *Antimicrob and Chemother* 1992b;36:1825.
- Bronner U, Ericsson O, Nordin J, et al. Metabolism is an important route of pentamidine elimination in the rat: disposition of ¹⁴C-pentamidine and identification of metabolites in urine using liquid chromatography-tandem mass spectrometry. *Pharmacol Toxicol* 1995;77:114.
- Ioannidis JPA, Capelleri JC, Skolnik PR, Lau J, Sacks HS. A meta-analysis of the relative efficacy and toxicity of *Pneumocystis carinii* prophylactic regimens. *Arch Intern Med* 1996;156:177.
- Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last ten years. *Clin Infect Dis* 1997;24:684.
- Machado CM, Macedo MCA, Medeiros RSS, et al. Primary *Pneumocystis carinii* prophylaxis with aerosolized pentamidine after bone marrow transplantation. *Acta Haematol* 1998;99:54.
- Jones JL, Hanson DL, Alderton DL, Fleming PL, Kaplan JE, Ward J. Surveillance for AIDS-Defining Opportunistic Illnesses, 1992–1997. In: *CDC Surveillance Summaries*. *MMWR* 1999;48:1.
- Assan R, Perronne C, Assan D, Chotard L, Mayaud C, Matheron S, Zucman D. Pentamidine-induced derangements of glucose homeostasis: Determinant roles of renal failure and drug accumulation—A study of 128 patients. *Diabetes Care* 1995;18:47.
- O'Brien JG, Dong BJ, Coleman RL, Gee L, Balano KB. A 5-year retrospective review of adverse drug reactions and their risk factors in human immunodeficiency virus-infected patients who were receiving intravenous pentamidine therapy for *Pneumocystis carinii* pneumonia. *Clin Infect Dis* 1997;24:854.
- Edwards KJ, Jenkins TC, Neidle S. Crystal structure of a pentamidine-oligonucleotide complex: implications for DNA-binding properties. *Biochemistry* 1992;31:7104.
- Tidwell RR, Bell CA. Pentamidine and Related Compounds in the Treatment of *Pneumocystis carinii* Infection. In: Walzer PD, editor, *Pneumocystis carinii* Pneumonia. 2nd ed., Revised and Expanded. New York: Marcel Dekker, Inc.; 1994. p 561.
- Tuttle RH, Hall JE, Tidwell RR. High-performance liquid chromatographic assay detects pentamidine metabolism by Fisher rat liver microsomes. *J Chromatogr B Biomed Sci App* 1997;688:319.
- Berger BJ, Reddy VV, Le ST, Lombardy RJ, Hall JE, Tidwell RR. Hydroxylation of pentamidine by rat liver microsomes. *J Pharm Exp Ther* 1991;256:883.
- Wood DH, Hall JE, Rose BG, Tidwell RR. 1,5-Bis(4-amidinophenoxy)pentane (pentamidine) is a potent inhibitor of [3H] idazoxan binding to imidazole I2 binding sites. *Eur J Pharmacol* 1998;353:97.
- Berger BJ, Naiman NA, Hall JE, Peggins J, Brewer TG, Tidwell RR. Problems and pitfalls in the assay of pentamidine. Implications for clinical use. *Clin Pharmacokinet* 1992a;22:163.
- Nordin J, Wikström I, Bronner U, Gustafsson LL, Ericsson O. Liquid chromatography tandem mass spectrometry applied to a study of the metabolism of pentamidine—Discussion of possibilities and problems. *J Chromatogr A* 1997;777:73.
- Lidman C, Bronner U, Gustafsson LL, Rombo L. Plasma pentamidine concentrations vary between individuals with *Pneumocystis carinii* pneumonia and the drug is actively secreted by the kidney. *J Antimicrob Chemother* 1994;33:803.
- Sattler FR, Jelliffe RW. Pharmacokinetic and Pharmacodynamic Considerations for Drug Dosing in the Treatment of *Pneumocystis carinii* Pneumonia. In: Walzer PD, editor, *Pneumocystis carinii* Pneumonia. 2nd ed., Revised and Expanded. New York: Marcel Dekker, Inc.; 1994. p 467.
- Tidwell RR, Jones SK, Geratz JD, Ohemeng KA, Cory M, Hall JE. Analogues of 1,5-bis(4-amidinophenoxy)pentane (pentamidine) in the treatment of experimental *Pneumocystis carinii* pneumonia. *J Med Chem* 1990a;33:1252.
- Ashley JN, Barber HJ, Ewins AJ, Newberry G, Self ADH. *J Chem Soc* 1942;103.
- Berg SS, Newberry G. *J Chem Soc* 1949.
- Parrish RF, Strauss JW, Paulson JD, Polakoski KL, Tidwell RR, Geratz JD, Stevens FM. Structure-activity relationships for the inhibition of acrosin by benzamidine derivatives. *J Med Chem* 1978;21:1132.
- Blättler WA, Kuenzi BS, Lambert JM, Senter PD. New heterobifunctional protein cross-linking reagent that forms an acid-labile link. *Biochem* 1985;24:1517.
- Aithal HN, Knigge KM, Kartha S, Czyzewski AE, Toback FG. An al-

- ternate method utilizing small quantities of ligand for affinity purification of monospecific antibodies. *J Immunol Methods* 1988;112:63.
31. Levin LG, Rudd A, Bletsa A, Reisner HM. Expression of IL-8 mRNA by cells of the odontoblast layer in-vivo. *Eur J Oral Sci* 1999;107:131.
 32. Hermanson GT. Types of Immunogen Carriers. In: *Bioconjugate Techniques*. San Diego: Academic Press; 1996. p 421.
 33. Patrick DA, Boykin DW, Wilson WD, et al. Anti-*Pneumocystis carinii* pneumonia activity of dicationic carbazoles. *Eur J Med Chem* 1997; 32:781.
 34. Del Poeta M, Dykstra CC, Jones SK, Tidwell RR, Kumar A, Boykin DW, Perfect JR. In vitro antifungal activities of a series of dication-substituted carbazoles, furans, and benzimidazoles. *Antimicrob Agents Chemother* 1998b;42:2503.
 35. Hopkins KT, Wilson DW, Bender BC, et al. Extended aromatic furan amidino derivatives as anti-*Pneumocystis carinii* agents. *J Med Chem* 1998;41:3872.
 36. Kitagawa T, Fujiwara K, Tomonoh S, Takahashi K, Koida M. Enzyme immunoassays of kanamycin group antibiotics with high sensitivities using anti-kanamycin as a common antiserum: reasoning and selection of a heterologous enzyme label. *J Biochem* 1983;94:1165.
 37. Erlanger BF. The Preparation of Antigenic Hapten-Carrier Conjugates: A Survey. In: Van Vunakis H. and Langone JL, editors. *Immunochemical Techniques Part A*. New York: Academic Press; 1980. p 85.
 38. Berger BJ, Lombardy RJ, Marbury DG, Bell CA, Dykstra CC, Hall JE, Tidwell RR. Metabolic N-hydroxylation of pentamidine in vitro. *Antimicrob Agents Chemother* 1990;34:1678.