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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTITATION OF PENTAMIDINE IN BLOOD SERUM

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the determination of pentamidine concentrations in serum samples. A microbore, reversed-phase column was used with a mobile phase consisting of methanol and water with sodium heptanesulfonate and triethylamine as modifiers. Pentamidine could be extracted from serum only by the addition of an ion-pairing agent, di(2-ethylhexyl) phosphoric acid, to the chloroform used for extraction. The method can be used to reliably detect levels as low as 5 ng/ml. The pentamidine concentration in the serum of eleven patients 24 h after their tenth daily dose of pentamidine averaged 60 ± 34 ng/ml.

INTRODUCTION

Pentamidine isethionate is used primarily to treat patients with *Pneumocystis carinii* pneumonia, a parasitic infection that attacks patients with severe immunosuppression. Although rare in the past, the incidence of *Pneumocystis carinii* pneumonia has increased dramatically since 1981 in parallel with the ongoing epidemic of acquired immunodeficiency syndrome (AIDS). As the use of pentamidine has increased, the need for a reliable method for quantitation of the drug's concentration in serum has become more apparent. An especially urgent problem is adjustment of dosages of the drug given to patients with decreased renal function.

A fluorometric method for pentamidine assay in plasma, urine and tissue published in 1970 [1] did not produce repeatable results in our laboratory. A recently published bioassay [2] is not as sensitive as the method reported here and may lack selectivity.

The method described here involves an ion-pair extraction of pentamidine

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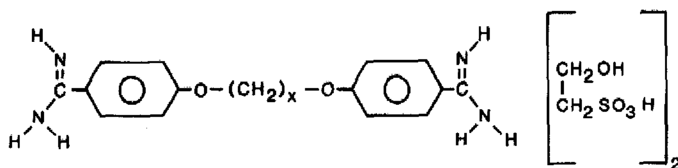

Pentamidine x=5 Hexamidine x=6 Propamidine x=3

Fig. 1. Structural formulae of pentamidine isethionate and potential internal standards.

and hexamidine (added as an internal standard) from the serum, followed by two extractive clean-up steps. The structures of the isethionates of pentamidine, hexamidine, and propamidine (the latter an internal standard candidate) are depicted in Fig. 1. The separation and quantitation are accomplished by high-performance liquid chromatography (HPLC) with a microbore, reversed-phase column and ultraviolet detection at 280 nm. The method permits precise determination of serum levels both in experimental animals and in human patients being treated for *Pneumocystis carinii* pneumonia by the recommended regimen.

EXPERIMENTAL*

Chemicals

Organic solvents, which were HPLC grade, were purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Purified water was obtained from a laboratory water purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, U.S.A.). The ion-pairing agent 1-heptanesulfonic acid, sodium salt, was purchased from Aldrich (Milwaukee, WI, U.S.A.). Di(2-ethylhexyl) phosphate [hereafter referred to as di(2-ethylhexyl)phosphoric acid, DEHPA] was obtained from Sigma (St. Louis, MO, U.S.A.). Triethylamine (TEA) from Fisher Scientific (Fair Lawn, NJ, U.S.A.) was distilled before use. Pentamidine isethionate was supplied by May and Baker (Dagenham, U.K.) and hexamidine isethionate by Vicks International (Surrey, U.K.).

Human serum samples

Human serum for preparation of spiked standards was obtained from healthy volunteers. Samples of serum from patients being treated with pentamidine isethionate for *Pneumocystis carinii* pneumonia were obtained from various hospitals and physicians in the United States.

Preparation of standard solutions

Pentamidine isethionate standard stock solution was prepared by weighing 55.10 mg of the dried drug standard (135°C for 8 h) and diluting with methanol to a volume of 50 ml in a volumetric flask. This stock solution had a concentration of 1.102 mg/ml pentamidine isethionate or 0.633 mg/ml as free base. A series of dilutions was carried out to produce a working solution with

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a concentration of 6.33 ng/μl free base, which was used to fortify serum for establishing the standard curve. A standard for injection on the HPLC system was prepared by diluting 1 ml of the working standard to a volume of 10 ml (with mobile phase as the solvent) to give a final concentration of 0.633 ng/μl as free base. A standard solution of hexamidine isethionate internal standard was prepared by dissolving 42.4 mg of the isethionate (24.6 mg as free base) in 100 ml of 0.01 *M* hydrochloric acid. A series of dilutions was carried out to prepare a solution with a concentration of 4.92 ng/μl (as free base). This solution was added to serum samples as the internal standard.

Equipment

A Varian Model 5060 liquid chromatograph equipped with a Rheodyne Model 7125 injector and a Micromeritics Model 788 variable-wavelength detector, set at 280 nm, were used in this analysis. The column used was a 150 mm × 2.1 mm I.D. Alltech solvent miser C_{18} with 5 μm particle size. The column was used at ambient temperature, approximately 22°C.

Analysis of serum samples

Serum (1 ml) was pipetted into a clean, glass 15-ml centrifuge tube and the calculated amount of pentamidine working standard solution was added with a 100-μl Hamilton syringe. Normally, a standard curve was prepared by using 10, 20, 30, and 40 μl of working standard solution to prepare samples containing 63.3, 127, 190, and 254 ng of pentamidine free base in each ml of serum. A 1-ml volume of serum was pipetted into a 15-ml centrifuge tube. A 50-μl volume of internal standard solution containing 246 ng of hexamidine isethionate was added and mixed by brief vortexing. The protein was precipitated by first adding 0.5 ml of 2 *M* sodium hydroxide, then neutralizing with 0.5 ml of 2 *M* hydrochloric acid, vortexing to mix after each addition. Carbonate buffer, pH 10, was added in the amount of 1 ml to ensure that the solution was slightly basic, between pH 7 and 8. The sample was vortexed to achieve thorough mixing. At this point, 4 ml of chloroform containing 0.02 *M* DEHPA were added, and the layers were mixed by vortexing for 1 min. The layers were separated by centrifuging at 700 *g* for 15 min. Three layers are seen in the centrifuge tube: the aqueous layer on top, a solid protein layer in the middle, and the chloroform layer containing the pentamidine and hexamidine on the bottom. A Pasteur pipet was carefully inserted past the solid layer and used to transfer the chloroform layer to a clean, dry 15-ml centrifuge tube.

The aqueous and solid layers were discarded. To the tube containing the chloroform layer 1 ml of 0.20 *M* hydrochloric acid was added. The tube was vortexed for 1 min. The layers can be most readily separated by drawing the interface into the Pasteur pipet, then slowly releasing the lower layer; complete separation can be achieved in the narrow portion of the pipet. The chloroform layer was discarded. The aqueous acid was brought to a pH of approximately 12 by the addition of 4 drops (from a Pasteur pipet) of 2 *M* sodium hydroxide. The pentamidine was then extracted by the addition of 2 ml of methylene chloride. After the tube was vortexed for 1 min, the separated aqueous layer was discarded. The methylene chloride was evaporated to dryness by warming the centrifuge tube in a water bath at 35°C while dry nitrogen was passed over the sample.

The sample was reconstituted by adding 100 μl of a solution prepared by mixing 50 ml of methanol and 50 ml of aqueous 0.05 *M* sodium heptanesulfonate containing 0.4% triethylamine, with the pH adjusted to 3.0 by phosphoric acid. The sample was vortexed for about 30 s to ensure that pentamidine and hexamidine dissolve. The injecting solvent contains more of the aqueous solution than the mobile phase, because complete solution of the pentamidine and hexamidine requires the additional solubility.

The mobile phase was prepared by pre-mixing and filtering 300 ml of methanol and 200 ml of an aqueous solution that is 0.05 *M* in sodium heptanesulfonate and 0.014 *M* in TEA, with the pH of the aqueous solution adjusted to 3.0 with phosphoric acid. The flow-rate was 0.3 ml/min. A 20-μl sample loop was used in the analysis. Repeated injections of a standard pentamidine solution were made until both the peak height and retention time of pentamidine were constant, thereby ensuring that the column and detector had stabilized.

The standards obtained from the extraction of the fortified serum were injected in duplicate, beginning with the least concentrated. Injections of sample extracts were interspersed. (See Fig. 2 for typical chromatograms.) The data from the fortified serum standards were used in a linear least-squares program to calculate the concentration of the unknown serum samples.

Dog studies

Two dogs, one male and one female, were given pentamidine, 4 mg/kg, by a 15-min intravenous infusion. Pre-dosage blood samples (10 ml) were taken, as well as samples at 1, 4, 24, and 48 h from each dog. The whole blood was allowed to coagulate, and the serum was separated by centrifugation. Each specimen was divided into three 1-ml aliquots, which were coded and analyzed on three different days in a blind study. Each time samples were analyzed, a set of at least four standards was included to provide a standard curve.

RESULTS AND DISCUSSION

Development and characterization of the method

Development of the best chromatographic conditions for HPLC analysis of pentamidine evolved over time. Since pentamidine is a polar compound with low solubility in organic solvents, a reversed-phase column was chosen. A microbore column (2.1 mm I.D.) was chosen to provide increased sensitivity after the discovery that human serum levels of pentamidine were usually less than 100 ng/ml when the recommended 4 mg/kg per day regimen was followed. The pH of the mobile phase (about 3) ensured that the pentamidine was completely protonated. An ion-pairing agent was necessary to elute pentamidine with reasonable peak shape; use of sodium heptanesulfonate resulted in a reasonable retention time at a lower pressure than did sodium pentanesulfonate. A concentration of 0.05 *M* sodium heptanesulfonate was needed to produce sharp peaks, although significant tailing still occurred. Results in a recently published paper [3] suggested that the addition of TEA might reduce the tailing; this proved to be the case. Tailing was almost completely eliminated and peak heights were doubled by the addition of the TEA. Phosphoric acid was added to adjust the pH to 3 after the TEA was added.

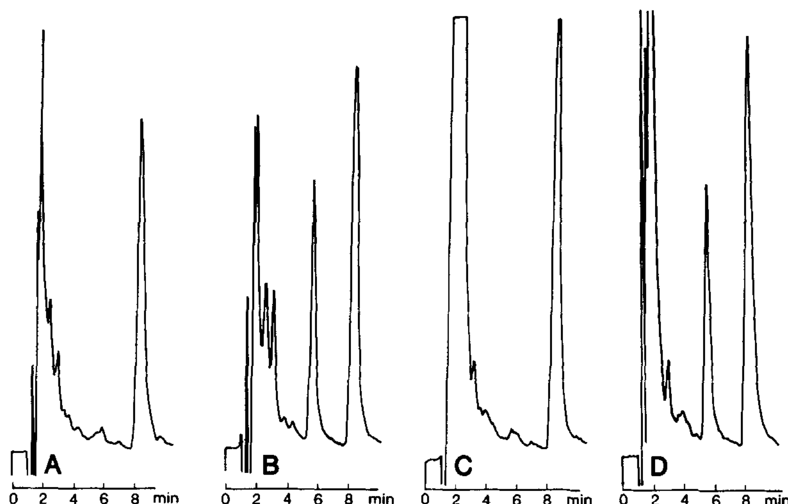

Fig. 2. Representative chromatographic traces. (A and B) Serum from a healthy human volunteer fortified at 0 and 150 ng/ml. (C and D) Pre- and post-dosing serum of patient treated with pentamidine. The retention time of pentamidine is 5.7 min and that of hexamidine, the internal standard, is 8.2 min.

The composition of the mobile phase finally chosen gave good separation between pentamidine and hexamidine. These conditions separate an interfering compound in certain serum extracts, which under other conditions yielded a peak that partially overlapped either the analyte peak or the internal standard peak, or both.

Very early in the development of this method the need became apparent for an internal standard to compensate for the variable recoveries often encountered with serum extractions. A sample of propamidine provided by its manufacturer proved not to be a good internal standard because the retention time was too short. Hexamidine (as the isethionate salt) proved to be an excellent internal standard, eluting shortly after pentamidine and separating well from other peaks in the serum extracts.

The extraction of pentamidine from serum proved to be difficult. Ethyl acetate, hexane—ether mixtures, butanol, chloroform, and methylene chloride extractions of fortified serum, made alkaline by addition of sodium hydroxide, yielded little or no pentamidine. An ion-pair extraction with DEHPA in chloroform has been used successfully to extract basic compounds from aqueous solutions [4]; this approach was taken for pentamidine. Chloroform containing 0.02 *M* DEHPA extracted both pentamidine and hexamidine from serum with acceptable recoveries. Higher concentrations of DEHPA cause difficulty in separating the organic and aqueous layers. The percentage recovery cannot be determined for this single step, because the pentamidine and hexamidine must be freed from the DEHPA by a back-extraction into 0.2 *M* hydrochloric acid before quantification is possible. The concentration of the aqueous hydrochloric acid is critical, since it was found that a lower concentration (0.02 *M*) works well for most, but not all, serum samples. The sera of a few patients were sufficiently different, possibly in lipid content, that neither pen-

tamidine nor hexamidine could be recovered from the chloroform solution with 0.02 *M* hydrochloric acid, but good recoveries resulted when 0.2 *M* hydrochloric acid was used. After this discovery, 0.2 *M* hydrochloric acid was used routinely in the back-extraction steps.

Extraction from the aqueous base is required to further purify and concentrate the analyte. The aqueous solution is made strongly basic (pH ca. 12) and extracted with methylene chloride. Chloroform is not used for this extraction, because a chemical reaction takes place when chloroform is in contact with strong base. Each time chloroform was used to extract the basic aqueous solution two new peaks appeared in the chromatogram, the ratio of the new peaks being the same as the ratio of pentamidine to hexamidine. The identities of the new compounds were not established, but they are not formed when methylene chloride is used for this final extraction. No difficulty exists when chloroform is used in the first extraction, because the aqueous layer is only weakly basic.

After the methylene chloride is evaporated, the residue is dissolved in 100 μl of an injection solvent that is chromatographically slightly weaker for reversed-phase operation (contains more water) than the mobile phase. The residue does not dissolve completely in 100 μl of mobile phase. The 100-μl volume of extract solution is sufficient for three injections (30 μl are used to overfill a loop with actual volume of 23.3 μl), although only two injections were routinely made.

The overall recovery of pentamidine, after three extractions, ranged from 17 to 32%, with a mean of 25% for eight samples. The samples, run on two different days, had been fortified with 127 ng of pentamidine and 148 ng of hexamidine. The percentage recovery of hexamidine was slightly higher, ranging from 34 to 68%, with a mean of 48% for these eight fortified samples. The ratio of pentamidine to hexamidine range from 0.456 to 0.535, with a mean value of 0.488 and a standard deviation of 5.9%. The percentage recovery did not differ significantly for samples fortified with pentamidine levels of 15.8, 31.6, 63.3, and 127 ng/ml.

Standard curves were linear over the range from 0 to 254 ng/ml, with correlation coefficients of 0.995 or greater (Table I). A new standard curve was determined for each new set of samples because of slight changes in the slope from day to day. The detection limit is 5 ng/ml with a signal-to-noise ratio of 5:1.

TABLE I

STANDARD-CURVE DATA FOR PENTAMIDINE

Pentamidine added (x) (ng/ml)	Area ratio pentamidine/hexamidine (y)	Pentamidine calculated* (x') (ng/ml)
0	0	0.289
63.3	0.200	63.0
127	0.394	124
190	0.624	196
254	0.800	251

*Calculated from the least-squares straight line, $y = mx + b$ (m = 0.00319, b = 0.000916); r^2 = 0.9986; n = 5.

TABLE II

SERUM LEVELS OF PENTAMIDINE IN DOGS AFTER A 15-min INTRAVENOUS INFUSION OF 4 mg/kg

Sample	Time after dose (h)	Pentamidine level (mean ± S.D., n = 3) (ng/ml)	Coefficient of variation (%)
Male dog*	1	51.0 ± 7.0	13.6
	4	11.1 ± 0.35	3.2
Female dog**	1	150 ± 6.24	4.2
	4	25.7 ± 1.39	5.4

*All pre-dose, 24-h, and 48-h samples were below the limit of detection, 5 ng/ml.
**The pre-dose and 24-h samples were below the limit of detection. The 48-h samples had a mean value of 7.27 ± 1.32 ng/ml, the significance of which is not yet clear.

Application of the method to experimental and clinical samples

An animal experiment was designed to test the new method and provide useful information on peak serum concentrations and the rate of drug elimination from serum (Table II). Dogs were chosen as the experimental animal so that sufficient serum would be available to run triplicate analyses for each data point.

The serum pentamidine concentrations of the two dogs were significantly different 1 h after injection, but the serum concentrations of both dogs fell to about 20% of the initial values within the next 3 h. After 24 h both dogs had serum concentrations below the detection limit. One dog showed an apparent slight increase in serum pentamidine concentration between the sample taken at 24 h and the one at 48 h. Further experiments are planned involving a larger number of animals and shorter time intervals between serum samples to see if this apparent increase is real. These additional experiments will also give more information about the time of the peak pentamidine concentration and the rapidity of the decline.

Serum samples from human patients receiving pentamidine isethionate were analyzed at various stages in the development of the method. Eleven serum samples, each taken from different patients 24 h after the tenth consecutive daily dose of pentamidine (4 mg/kg, intramuscularly), were found to have an average pentamidine level of 60 ± 34 ng/ml.

These data from human and dog studies support the findings of Launoy et al. [5], which showed that in white mice 50% of the injected dose is eliminated (total urinary and fecal) within 5 h.

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