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DETERMINATION OF MONOACETYLDIAMINES AND -POLYAMINES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure is described for the determination of monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine in human urine. The procedure is based on the high-performance liquid chromatographic separation of the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) derivatives of these amines using two different chromatographic modes. Monoacetyl-1,6-diaminohexane was used as an internal standard. The amines were extracted from urine using a silica gel cartridge. The dansyl monoacetylpolyamines were separated from the mixture of dansyl derivatives of urinary amines on a bonded-phase CN column using a programmed solvent gradient elution. The dansyl acetylpolyamines were rechromatographed on a silica gel column.

This chromatographic procedure was used for the determination of the concentration of N^1-acetylspermidine, N^8-acetylspermidine and monoacetylputrescine in the urine of healthy volunteers and cancer patients.

INTRODUCTION

The polyamines are present in human urine predominantly as conjugates that produce the free amines after hydrolysis [1]. In most studies in which urinary polyamines were utilized as markers of cancer, the urine was hydrolyzed prior to analysis to liberate the polyamines from these conjugates [1, 2]. N^1-Acetylspermidine (N^1-Ac-Spd), N^8-acetylspermidine (N^8-Ac-Spd) and monoacetylputrescine (Ac-Put) were identified as the major excretory form of spermidine (Spd) and putrescine (Put) in the urine of cancer patients and healthy volunteers [3—6]. Furthermore, a majority of the cancer patients studied were found to have a higher ratio of N^1-Ac-Spd to N^8-Ac-Spd in the 24-h urine than did healthy volunteers [5]. These findings suggested that the urinary concentrations of the acetylpolyamines may provide a more precise marker of cancer than the urinary levels of total polyamines obtained after hydrolysis of urine [5].

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A variety of procedures are available for the determination of the polyamines in biological samples [7]. However, there are only two procedures which have been successfully used for the quantitation of acetylpolyamines in urine [5, 6]. Both procedures are based on the formation, separation and quantitation of the dansyl derivatives of the acetylpolyamines from urine. A combination of two-dimensional thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) was used for the separation and determination of the dansyl polyamines in previous studies in our laboratory [5]. Recently, Seiler and Knödgen [6] described an elegant procedure for the determination of the naturally occurring monoacetyl derivatives of di- and polyamines in human urine using TLC. These authors obtained results which are in general agreement with some of the previously published findings [5].

In the present paper a procedure for the HPLC determination of the dansyl derivatives of urinary acetylpolyamines is described. This procedure has many advantages over the procedure previously utilized in our laboratory [5]. These advantages include the use of an internal standard for the quantitation of the polyamine, the replacement of the isoamyl alcohol extraction with a purification procedure utilizing Sep-Pak^{R} silica gel cartridges and the use of a sequential HPLC separation using two different chromatographic modes.

EXPERIMENTAL

Materials

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) were purchased from Sigma (St. Louis, MO, U.S.A.). Monoacetylputrescine hydrochloride was obtained by using a published procedure [8]. N^{1}-Acetylspermidine, N^{8}-acetylspermidine and monoacetyl-1,6-diaminohexane (Ac-DAH) were prepared using procedures developed in our laboratory and which will be published elsewhere. Chloroform, isopropanol, hexane and methylene chloride were HPLC grade solvents and were obtained from Fisher (Itasca, IL, U.S.A.) or Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Sep-Pak® silica cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

Urine sample preparation

Twenty-four-hour urine samples were collected in polyethylene bottles and stored under toluene at 4°C. A 6-ml aliquot was pipetted into a plastic cup and a 100-μl portion of 1 m*M* solution of acetyl-1,6-diaminohexane dihydrochloride was added. The sample was adjusted to pH 9.0 with 1.0 *N* sodium hydroxide solution using an automatic titrator (Radiometer, Copenhagen, Denmark). A 2-ml aliquot was pipetted into a syringe and added to a silica gel Sep-Pak which had been previously washed with 5 ml of water. Water (5 ml) was passed through the Sep-Pak and the eluent was discarded. The polyamines were eluted with 10 ml of 0.1 *N* hydrochloric acid and the eluent was collected in a 50-ml centrifuge tube. The eluent was evaporated to dryness at 40°C in vacuo using an evapo-mix (Buchler Instruments, Fort Lee, NJ, U.S.A.). The residue was dissolved in 0.5 ml of 0.6 *N* hydrochloric

acid and mixed with 0.5 ml of a saturated solution of sodium carbonate and 2 ml of a solution of 7.5 mg/ml of Dns-Cl in acetone. The centrifuge tubes were stoppered and placed in an ultrasonic bath for 2.5 h. A 0.2-ml aliquot of a proline solution (5.0 g per 100 ml) was added to each tube and the tubes were placed in the ultrasonic bath for an additional 5 min. The solution was evaporated to dryness at 40°C in vacuo. The residue in each tube was mixed vigorously for 20 sec with a vortex mixer with 1.0 ml of water and 5.0 ml of toluene. The tubes were centrifuged at 900 *g* for 20 min. The toluene layer was transferred to a 12-ml conical centrifuge tube. The toluene extract was evaporated to dryness at 40°C in vacuo. The residue was mixed with 0.5 ml of 0.1 *N* sodium hydroxide and extracted with 2.5 ml of toluene. The toluene extract was evaporated to dryness and the residue was stored at —20°C until analysis.

HPLC analysis

HPLC was performed on a system composed of a U6K injector, two Model 6000 solvent delivery systems and a Model 660 solvent programmer (Waters Assoc.), a LDC Model 1209 FluoroMonitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.). The separation of the dansyl monoacetylpolyamines was carried out on a Micropak CN-10 column (25 × 2.5 mm I.D., particle size 10 μm; Varian, Palo Alto, CA, U.S.A.) with a solvent composed of *n*-hexane—isopropanol (100:3) as solvent A and *n*-hexane—methylene chloride—isopropanol (10:5:1) as solvent B. The residue of dansyl monoacetylpolyamines was dissolved in 125 μl of methylene chloride and a 50-μl aliquot of the resulting solution was injected into the system. The sample was eluted with a programmed solvent gradient using the linear gradient curve No. 6. The gradient changed from 100% of solvent A to 100% of solvent B in 15 min at a flow-rate of 3 ml/min. The elution was continued in the isocratic mode with solvent B for an additional 5 min. The dansyl monoacetylpolyamines eluted after 15—20 min and the column eluent corresponding to these compounds was collected in a conical centrifuge tube. The column was allowed to re-equilibrate with solvent A for 5 min before a second sample was injected.

The column fraction corresponding to the dansyl monoacetylpolyamines was evaporated to dryness. The residue was dissolved in 100 μl of chloroform and 25 μl of the resulting solution were injected into the system. The separation of the dansyl monoacetylpolyamines was carried out on a silica gel column Ultrasphere-Si, 150 × 4.4 mm I.D., packed with 5-μm silica gel (Altex, Berkeley, CA, U.S.A.) or a 250 × 4.6 mm I.D. column packed with 10-μm silica gel, Alltech Assoc. (Deerfield, IL, U.S.A.) with a solvent composed of chloroform—isopropanol (100:6). The sample was eluted in the isocratic mode with a programmed flow-rate using the concave gradient curve No. 9. The flow-rate changed from 1 ml/min to 2 ml/min in 10 min and was maintained at 2 ml/min for an additional 10 min.

Calibration curve

Aliquots (3 ml) from a 24-h urine sample obtained from a healthy male volunteer were pipetted into plastic cups and 100 μl of a 1 m*M* solution of

Ac-DAH were added to each sample. A 3-ml aliquot of either water or one of the three standard solutions of acetylpolyamines, which contained various concentrations of Ac-Put (5—40 nmole/ml), N^1-Ac-Spd (1—8 nmole/ml) and N^8-Ac-Spd (1—8 nmole/ml), was added to each aliquot of urine. The aliquots were treated as described under Urine sample preparation and HPLC analysis. The ratios of the peak heights of each of the dansyl monoacetylpolyamines to that of the internal standard (dansyl monoacetyl-1,6-diaminohexane) were calculated and plotted as shown in Fig. 1. Regression analysis was used to determine lines of best fit to the data points. The slopes of these lines, together with the ratios of the peak heights of the dansyl monoacetylpolyamines to that of the internal standard, were used for the calculation of the concentrations of the acetylpolyamines in the urine samples.

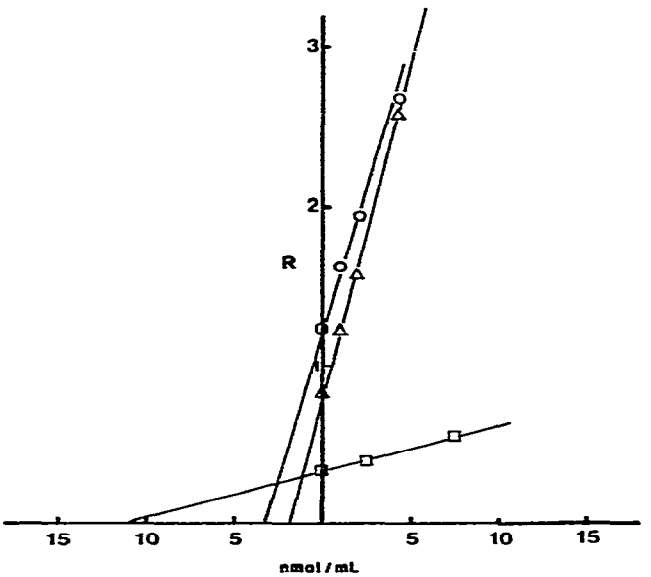

Fig. 1. Correlation graphs for the determination of the monoacetylpolyamines in urine using the method of standard additions. R = ratio of the height of the monoacetylpolyamine peak to the height of the internal standard peak. ○, N^1-Ac-Spd; △, N^8-Ac-Spd; □, Ac-Put. The data on the graph indicate that the urine sample used contained N^1-Ac-Spd, 3.44; N^8-Ac-Spd, 1.91; and Ac-Put, 11.01 nmole/ml.

RESULTS

A number of compounds were evaluated for use as an internal standard in the analysis of urinary acetylpolyamines. Ac-DAH was selected for several reasons. Ac-DAH is chemically similar to the naturally occurring monoacetylpolyamines which resulted in similar recoveries in the extraction and dansylation steps of the analysis. Secondly, the retention volume of this compound on the CN column was similar to those of the natural monoacetylpolyamines. This allowed the collection of only one fraction of the column eluent which contained the natural monoacetylpolyamines and the internal standard. Third-

ly, the retention volume of Ac-DAH on the silica gel column was intermediate between that for N^1-Ac-Spd, N^8-Ac-Spd and Ac-Put. Finally, the Ac-DAH peak was sufficiently separated from the peak due to acetylcadaverine, which was present in small quantities in the urine of some cancer patients.

The acetylpolyamines were extracted from urine, which had been adjusted to pH 9.0, using a silica gel cartridge (Sep-Pak). This procedure is similar in principle to that described by Grettie et al. [9] for the extraction of polyamines from plasma. The use of silica gel for the extraction of polyamines was much more convenient and gave higher recoveries than the extraction procedure used in our laboratory in previous studies [5]. The use of the silica gel cartridge was also more convenient and provided better reproducibility in our hands, than the use of columns prepared according to the procedure described by Grettie et al. [9].

The dansyl derivatives of the extracted polyamines were formed using a standard procedure. The residues of the dansyl polyamines were treated with base to remove interfering substances as suggested by Seiler and Knödgen [6].

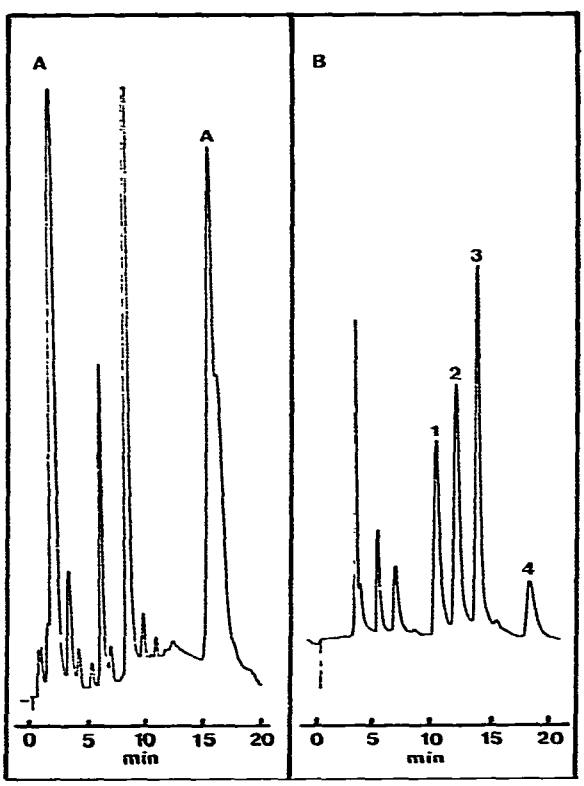

Fig. 2. (A) HPLC separation of the dansyl derivatives of a standard solution of monoacetylpolyamines (Peak A) on a Micropak CN-10 column (250 × 2.5 mm I.D., Varian Aerograph). (B) HPLC separation on a silica gel column (250 × 4.6 mm I.D., 10-μm particle size, Alltech) of the dansyl derivatives obtained after concentration of the Micropak CN-10 column eluent corresponding to peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-DAH (internal standard); 4 = Ac-Put.

This treatment resulted in a relatively clean preparation of the dansyl derivatives.

The crude mixture of the dansyl derivatives of the urinary polyamines was separated on a bonded-phase CN column using a programmed solvent gradient elution [10]. A typical separation of the dansyl derivatives of a synthetic mixture of N^1-Ac-Spd, N^8-Ac-Spd, Ac-Put and Ac-DAH (internal standard) is shown in Fig. 2. Although partial separation of the monoacetylpolyamines could be obtained using different solvent gradient conditions, we used separation conditions to obtain all the acetylpolyamines in a single peak (peak A in Fig. 2A). The eluent corresponding to peak A was collected and concentrated. The residue was separated on a silica gel column (Fig. 2B). This figure demonstrates the excellent separation between N^1-Ac-Spd and N^8-Ac-Spd.

Fig. 3A represents the chromatogram obtained from the urine of a healthy male volunteer. It is clear from this chromatogram that the acetylpolyamines are present in human urine. Also, a number of fluorescent substances are

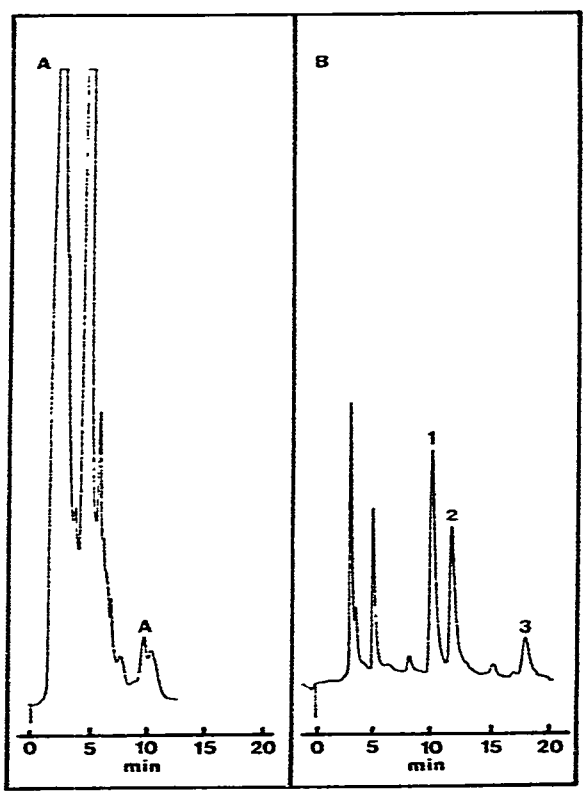

Fig. 3. (A) HPLC separation of the dansyl derivatives obtained from the urine of a healthy male volunteer on a Micropak CN-10 column (250 × 2.5 mm I.D., Varian Aerograph). Peak A corresponds to the naturally occurring monoacetylpolyamines. (B) HPLC separation on a silica gel column (250 × 3.6 mm I.D., 10-μm particle size, Alltech) of the dansyl derivatives obtained after concentration of the fraction of column eluent corresponding to peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-Put.

present in the sample which have similar retention volumes to the dansyl polyamines. These substances interfere with the dansyl polyamine peaks and make the quantitation of the dansyl polyamines impossible. The dansyl acetylpolyamines peak was rechromatographed on a silica gel column and is shown in Fig. 3B. Fig. 4 illustrates the chromatograms obtained from the urine of a cancer patient.

The working correlation graph for the determination of the acetylpolyamines in urine was prepared using the standard addition method (Fig. 1). This figure illustrates the linear relationship between the amount of acetylpolyamine added and the ratio of its peak height to that of the internal standard. The correlation coefficients (r) were calculated by regression analysis and found to be 0.99 for N^1-Ac-Spd, 0.99 for N^8-Ac-Spd and 0.92 for Ac-Put.

This chromatographic procedure was used to determine the concentration of N^1-Ac-Spd, N^8-Ac-Spd and Ac-Put in the urine of several healthy volunteers and cancer patients. The urinary concentrations of the acetylpolyamines of five representative patients are shown in Table I. The values in Table I are in agreement with those previously published for these compounds.

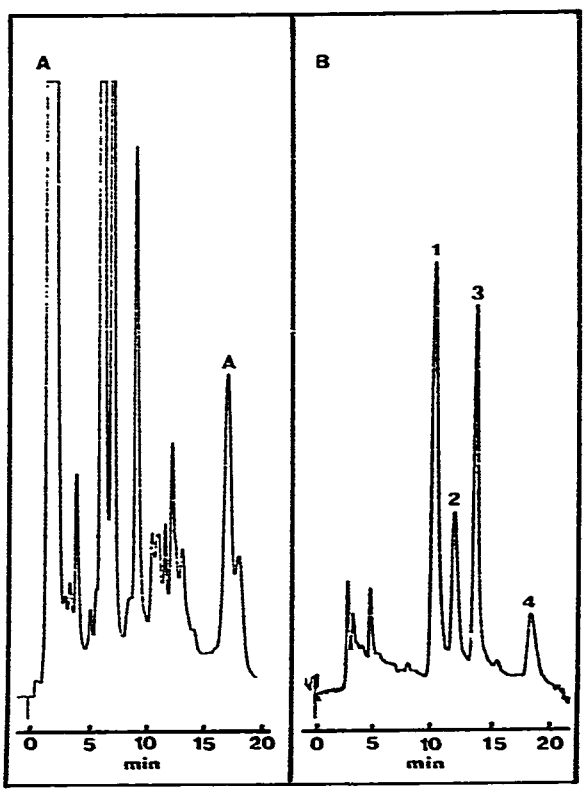

Fig. 4. (A) HPLC separation on a Micropak CN-10 column, of the dansyl derivatives obtained from the urine of a cancer patient. Peak A corresponds to the naturally occurring monoacetylpolyamines. (B) HPLC separation on a silica gel column of the dansyl derivatives obtained after concentration of the fraction of column eluent corresponding to Peak A. Peaks: 1 = N^1-Ac-Spd; 2 = N^8-Ac-Spd; 3 = Ac-DAH (internal standard); 4 = Ac-Put.

TABLE I

PRETREATMENT CONCENTRATIONS OF MONOACETYLPOLYAMINES IN THE 24-h URINE OF CANCER PATIENTS

Patient	Acetylpolyamine conc. (μmole per 24 h)				Ratio*
	N^1-Ac-Spd	N^8-Ac-Spd	Ac-Put	Total	
C.W.	5.2	2.5	22.0	29.7	2.1
R.B.	4.9	1.7	8.0	24.6	2.9
J.C.	9.1	1.7	17.4	28.2	5.4
E.L.	3.5	1.4	19.4	24.3	2.5
C.C.	18.3	7.0	24.7	50.0	2.6

*Ratio N^1-Ac-Spd to N^8-Ac-Spd.

It should be pointed out that the ratio of N^1-Ac-Spd to N^8-Ac-Spd in the urine of the majority of cancer patients examined so far was greater than 2.0. This ratio was found to be 1.0 in the urine of healthy volunteers [5, 6].

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