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SOLID-PHASE EXTRACTION AND DETERMINATION OF DANSYL DERIVATIVES OF UNCONJUGATED AND ACETYLATED POLYAMINES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY: IMPROVED SEPARATION SYSTEMS FOR POLYAMINES IN CEREBROSPINAL FLUID, URINE AND TISSUE

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SUMMARY

A sensitive and simple liquid chromatographic assay with fluorometric detection for unconjugated and acetylated polyamines in biological fluids is described. After precolumn derivatization with dansyl chloride, unconjugated polyamines and acetylated polyamines were extracted by elution from a Bond-Elut C_{18} column and then separated on a reversed-phase column with gradient elution. The complete analysis of unconjugated putrescine, spermidine, and spermine in either hydrolyzed urine, cerebrospinal fluid or tissue could be accomplished within 20—26 min, while the simultaneous analysis of unconjugated poly-

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amines and monoacetylpolyamines could be completed within 40 min. Unhydrolyzed urine and cerebrospinal fluid required a Bond-Elut cation-exchange clean-up before dansylation. Standard curves for the assay were linear up to 20 nmol/ml, and the within-day and day-to-day coefficients of variation were between 1.1 and 4.6% and between 1.6 and 11.8%, respectively. Results obtained with the method were compared with results obtained with a well established modified amino acid analyzer method for urine, tissue and cerebrospinal fluid samples. The correlation coefficients between these two methods were in the range 0.933—0.996. Detection limits between 50 and 150 fmol were achieved for unconjugated and acetylated polyamines. Of more than twenty drugs and amines tested for possible interference with the assay, only normetanephrine was found to have the same retention time as the internal standard 1,6-diaminohexane.

INTRODUCTION

Polyamines are found in all cells and are involved in growth and differentiation [1, 2]. Increased intracellular concentrations of polyamines are associated with increased rates of cell proliferation, and elevated polyamine levels are frequently found in the urine, serum and cerebrospinal fluid (CSF) of patients with cancer [3, 4]; however, the usefulness of polyamines as tumor markers has been limited. In general, it appears that polyamine determinations have at best a minor role in the early diagnosis of cancer, but may be of some use for monitoring the progression of disease or response to therapy of patients harboring tumors [3]. CSF polyamine levels have been used clinically to monitor the progression or response to therapy of patients harboring medulloblastoma [5]. Because of the continued study of the clinical utility of the polyamine assay, a growing interest in the basic intracellular functions of these compounds, and the use of polyamine biosynthesis inhibitors in cancer and parasite chemotheraphy, improved assay methods for the determination of polyamine levels are useful.

Most liquid chromatographic (LC) procedures that have been reported for the measurement of polyamines require either pre-column [6—10] or post-column [11—13] derivatization for fluorescence detection. Although many methods have been reported for the separation and quantitation of free and conjugated polyamines in tissues and biological fluids, CSF polyamines are not easily assayed with the LC methods currently available. Modified amino acid analyzers have been used successfully to determine polyamines levels in CSF and other biological fluids and tissue homogenates. However, the cost of instrumentation limits the use of amino acid analyzer techniques.

In this paper we describe a simple, precise, accurate and specific gradient reversed-phase LC procedure for the simultaneous determination of dansyl derivatives of free and monoacetylated polyamines in CSF, urine and tissue homogenates. Results obtained with the LC method correlate excellently with results obtained with an established, well tested amino acid analyzer technique [14].

EXPERIMENTAL

Chromatography

Analysis was performed on a Series 3 liquid chromatograph equipped with a

Model 650-10 fluorescence detector and a Model LC 100 column oven (all from Perkin-Elmer, Norwalk, CT, U.S.A.). A reversed-phase Ultrasphere ODS 5-μm, 15 cm × 4.6 mm column (for unconjugated polyamines) or an Ultrasphere ODS 5-μm, 25 cm × 4.6 mm column (for both unconjugated and acetylated polyamines) obtained from Beckman (Berkeley, CA, U.S.A.) and an RP-18 spheri-guard column from Rainin Instruments (Woburn, MA, U.S.A.) were mounted in the oven. Chromatograms were recorded on a chart recorder or on a Sigma 10 data system (Perkin-Elmer). Samples were injected into a Rheodyne 7125 valve (Rheodyne, Cotati, CA, U.S.A.) mounted on the chromatograph. A block heater (Lab. Line Instruments, Meltrose Park, IL, U.S.A.) was used for temperature control during derivatization. A Vac-Elut apparatus and Bond-Elut C_{18} and Bond-Elut SCX (strong cation exchange) extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). A flexi-dry freeze dryer (FTS Systems, Hicksville, NY, U.S.A.), Eppendorf micro test tubes (1.5 ml polypropylene) and an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY, U.S.A.) were used.

Reagents and standards

All inorganic reagents were analytical grade. Methanol and acetonitrile (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Stock potassium citrate buffer contained 178.75 g potassium chloride, 28.75 g potassium citrate and 3 ml of 12 *M* hydrochloric acid per l, pH 5.56 (2.57 *M*). The buffer was diluted to 10 and 20 m*M* for use.

A working solution of dansyl chloride (100 mg/ml in acetone, Pierce, Rockford, IL, U.S.A.) is prepared by diluting 0.2 ml of the commercial stock solution to 2 ml with acetone to yield a concentration of 10 mg/ml. This solution is stable for 24 h at 4°C in a foil-wrapped glass tube. The polyamine stock solution is prepared by dissolving 10 mg each of putrescine, cadaverine, spermidine, spermine, monoacetylputrescine, N^1-acetylspermidine, N^8-acetylspermidine and N^1-acetylspermine (Sigma, St. Louis, MO, U.S.A.) in 200 ml of 0.1 *M* hydrochloric acid. This solution is stable indefinitely when frozen at −20°C in 20-ml aliquots. The stock solution is diluted with 0.1 *M* hydrochloric acid to final concentrations of 100, 200, 300, 400 and 1000 pmol per 50 μl, the volume derivatized for analysis. Refrigerated diluted standards are stable for at least two weeks. The internal standard 1,6-diaminohexane dihydrochloride (Sigma) was prepared by dissolving 2 μmol in 100 ml of 0.1 *M* hydrochloric acid and then diluting this five-fold to a concentration of 200 pmol per 50 μl. Refrigerated diluted internal standard is stable for at least two weeks. For CSF and unhydrolyzed urine, 1,7-diaminoheptane dihydrochloride (Sigma) dissolved in 0.1 *M* hydrochloric acid at a concentration of 200 pmol per 50 μl was used as the internal standard. This diluted internal standard is stable for at least two weeks at 4°C.

Solvents

Solvents for gradient I were acetonitrile and 10 m*M* phosphate buffer, pH 4.4 Various mixtures of these mobile phase solvents were obtained using the gradient mixer on the liquid chromatograph. Solvents for gradient II were acetonitrile and methanol (185:15) and 10 m*M* phosphate buffer, pH 4.4. The

TABLE I

COMPOSITION OF GRADIENTS I AND II

Gradient I*			Gradient II**		
Elution time (min)	Percentage of solvent		Elution time (min)	Percentage of solvent	
	A	B		A	B
0	45	55	0	35	65
14	80	20	25	60	40
15	90	10	30	64	36
20	90	10	34	90	10
			40	90	10

*Gradient I: solvent A, acetonitrile; solvent B, 10 m*M* phosphate buffer, pH 4.4. Equilibration time, 6 min; total time, 26 min.
**Gradient II: solvent A, acetonitrile—methanol (185:15); solvent B, 10 m*M* phosphate buffer, pH 4.4. Equilibration time, 6 min; total time, 46 min.

gradients listed in Table I were found to be appropriate for the separation of polyamines and their monoacetyl derivatives. Gradient I was selected for the separation of hydrolyzed urine, hydrolyzed CSF and tissue polyamines. The flow-rate is 2 ml/min and the column is heated to 50° C. The column effluent is monitored at an excitation wavelength of 340 nm and an emission wavelength of 515 nm at a sensitivity of 0.3. Gradient II was selected for the separation of acetylated polyamines in unhydrolyzed urine, CSF and tissues.

Preparation of samples

Urine (hydrolyzed). To 200 μl of urine in a 1.5-ml Mini-Aktor tube (Applied Science Labs., State College, PA, U.S.A.) add 200 μl of 12 *M* hydrochloric acid and cap using PTFE tape on the threads. Hydrolyze at 110° C for 14--16 h and lyophilize the hydrolysate. Dissolve residue in 200 μl of 4% 5-sulfosalicylic acid and centrifuge for 5 min. Transfer 50 μl of supernatant to a polypropylene tube and derivatize the sample using the reaction conditions described below.

Urine or CSF (unhydrolyzed). To 300 μl of unhydrolyzed urine or CSF in a 1.5-ml polypropylene tube add 100 μl of 1,7-diaminoheptane, the internal standard (200 pmol per 50 μl) and 500 μl of 20 m*M* K^+ buffer (pH 6.2) to adjust the pH of the solution to between 3 and 4. This solution is transferred to a strong cation-exchange Bond-Elut SCX column, which is activated by washing with two column volumes each of methanol, water and 10 m*M* K^+ buffer (pH 6.2). After the SCX column has drained, wash it with two column volumes each of water and 10 m*M* K^+ buffer. The acetylated and free polyamines are then eluted with two 300-μl volumes of 20% acetone in 2.57 *M* K^+ buffer (pH 5.56).

Tissue culture. Wash cells free of tissue culture medium or trypsin solution with phosphate-buffered saline. Spin cells into a pellet, pour off saline and absorb excess saline with a Kim-wipe or cotton swab, being careful not to touch the pellet. Add 200—250 μl of 8% 5-sulfosalicylic acid for every 10^6 cells in the pellet; trichloroacetic or perchloric acid may also be used. Sonicate

for 20—30 s and allow to stand in ice for 60 min, mixing once at 30 min. Transfer material to 400-μl microfuge tubes and spin for 5 min at 12 000 *g*. Transfer 50 μl of each supernatant into another polypropylene tube for derivatization. Solid tissue samples can be used after homogenization.

CSF (hydrolyzed). To 1.0 ml of CSF in a 1.5-ml Mini-Aktor tube, add 1 drop of 3.0 *M* hydrochloric acid and lyophilize. Add 0.5 ml of 6.0 *M* hydrochloric acid to the residue, cap tightly used PTFE tape and hydrolyze at 110°C for 14—16 h. Lyophilize the hydrolysate, dissolve the residue in 150 μl of 4% 5-sulfosalicylic acid and centrifuge for 5 min. Transfer 50 μl of the supernatant to a polypropylene tube for extraction. To the supernatant add 50 μl of 1,7-diaminoheptane (internal standard, 200 pmol per 50 μl), 100 μl of 0.2 *M* potassium hydroxide and 500 μl of 20 m*M* K^+ buffer (pH 6.2). The final pH of this solution is adjusted to between 3 and 4 with 0.2 *M* potassium hydroxide or 0.1 *M* hydrochloric acid. Transfer this mixture to a Bond-Elut cation-exchange SCX column (SCX, a strong cation-exchange column that is activated by washing with two column volumes each of methanol, water and 20 m*M* K^+ buffer, pH 6.2). After the SCX column has drained, wash it with two column volumes each of water and 10 m*M* K^+ buffer. The polyamines are then eluted with two 300-μl volumes of 20% acetone in 2.57 *M* K^+ buffer (pH 5.56).

Derivatization and extraction of dansyl derivatives

In a polypropylene tube, 50 μl of 1,6-diaminohexane, internal standard (200 pmol per 50 μl), 200 μl of saturated sodium carbonate and 200 μl of dansyl chloride (10 mg/ml) are added to 50 μl of the supernatant for urine or tissue and to the entire supernatant for unhydrolyzed urine and CSF. (1,7-Diaminoheptane, internal standard, is added instead of 1,6-diaminohexane before the SCX column extraction, when it is used.) Cap the tube and vortex for 15 s, then incubate at 70°C for 10 min. Cool the tube to room temperature and transfer contents to a Bond-Elut C_{18} column on a Vac-Elut apparatus. (The Bond-Elut C_{18} column is activated by washing with two column volumes each of methanol and water.) After the column has drained, wash with two column volumes of water. The dansylated polyamines are then eluted with 500 μl of methanol. A 10-μl aliquot of the methanol eluate is injected onto the liquid chromatograph for urine and tissue samples and 50—100 μl are injected for CSF. The dansyl derivatives are stable, and they can be stored in darkness at 4°C for up to a month without appreciable loss (< 10%).

RESULTS AND DISCUSSION

Optimum conditions for derivatization

The optimum conditions for the assay were determined by varying reagent concentration, reaction temperature, reaction time, pH and the composition of buffers. In initial experiments we found that a dansyl chloride concentration of 1.25 mg/ml was decolored within 2 min of being added to urine samples, which indicated that the amount of reagent present in solution was not high enough to fully derivatize the polyamines and other reactive compounds present in urine. A concentration of 10 mg/ml, however, resulted in excessively large reagent peaks in chromatograms; 100 μl of a 250 mg/ml proline solution

added at room temperature for 10 min reacted with excess dansyl chloride and decreased the size of the reagent peaks. The time of derivatization at 70°C was varied between 5 min and 1 h and results were compared to derivatization overnight at room temperature. Derivatization was complete after 10 min at 70°C. Increased reaction times were often accompanied by a decrease in the peak height of the dansylated polyamines and an increase in background peaks. If the temperature was raised above 80°C, there was substantial decomposition of the dansylated polyamines. We selected 70°C for 10 min as the optimum conditions for derivatization.

The pH of the reaction mixture was critical for complete derivatization. Below pH 9, derivatization was not complete and slow. The optimum pH for this reaction was found to be between 9.5 and 11. To assure that all samples, hydrolyzed or not, were at the appropriate pH for derivatization (10.5), the pH of samples was adjusted by addition of 200 μl of saturated sodium carbonate.

Extraction of dansyl derivatives

When a 10-μl aliquot of the crude derivatized mixture was injected onto a reversed-phase column, a large number of peaks were observed near the solvent front; peaks corresponded to polar reaction products that are the result of the excess dansyl chloride in the crude mixture. The presence of early eluting peaks was reduced considerably by selectively isolating non-polar dansyl derivatives of polyamines on a Bond-Elut C_{18} extraction column. The isolation procedure was simplified sufficiently by solid-phase extraction such that a batch of ten samples could be processed in approximately 5 min. This extraction method also prolonged the useful life of the analytical column; by replacing the guard column after every 150 analyses, the same analytical column was used to analyze more than 1500 samples.

For CSF and unhydrolyzed urine samples, it was necessary to isolate free and acetylated polyamines with a Bond-Elut SCX column before derivatization. Without cation-exchange clean-up, there were too many interfering peaks near the putrescine and internal standard peaks.

Chromatography

The effects on resolution of the composition of the mobile phase, the pH of the mobile phase, gradient time, column length and column temperature were evaluated by injecting approximately 1 ng each of the polyamine dansyl derivatives. Variation of the mobile phase included different ratios of acetonitrile–phosphate buffer and acetonitrile—methanol—phosphate buffer, and different times for gradient elution. While the elution order of polyamines and monoacetylpolyamines was unaffected, the retention time and resolution depended on the time of gradient elution or the concentration of acetonitrile. The resolution between N^1-acetylspermidine and N^8-acetylspermidine was not optimum with an acetonitrile–phosphate buffer gradient and a 150 × 4.6 mm ODS column, but baseline separation of these two isomers could be achieved by adding 7.5% methanol to acetonitrile and using a 250 × 4.6 mm ODS column. Gradient II was selected for the simultaneous analysis of free and mono-

acetylated polyamines and had to be used for analysis of unhydrolyzed urine or CSF samples. Gradient I was used for the analysis of free polyamines from tissue and hydrolyzed urine, and slightly modified for hydrolyzed CSF.

Separations with gradient I

The chromatogram for the separation of a standard mixture of polyamines is shown in Fig. 1. Chromatograms for the separation of polyamines from a tissue homogenate and from a urine hydrolysate processed as described above are shown in Fig. 2A and B. Under these conditions, monoacetylputrescine elutes close to the solvent front, while peaks for N^1- and N^8-monoacetyl-

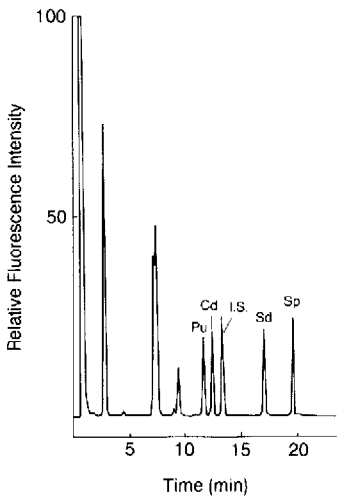

Fig. 1. Chromatogram of the dansyl derivatives of putrescine (Pu), cadaverine (Cd), spermidine (Sd) and spermine (Sp). Each peak, including 1,6-diaminohexane (internal standard, I.S.), represents about 1 ng of polyamine injected (gradient I).

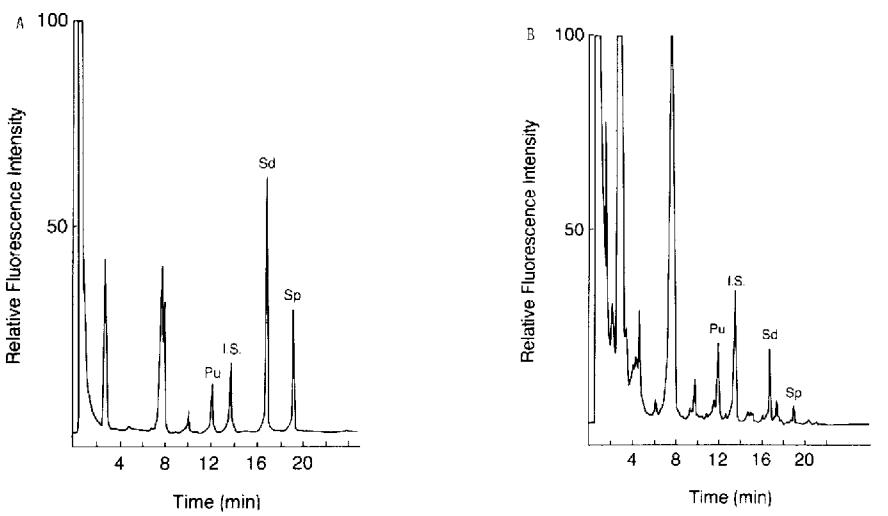

Fig. 2. (A) Chromatogram of dansylated polyamines in tissue; (B) separation of polyamines from human urine hydrolysate using gradient I. Peaks as given in Fig. 1.

spermidine are not resolved. Thus, gradient I (Table I) is suitable only for the analysis of unconjugated polyamines in tissue homogenates and urine hydrolysates. For hydrolyzed CSF, the composition of gradient I was slightly modified by increasing the acetonitrile concentration from 45 to 80% in 20 min instead of in 14 min. The chromatogram for the separation of polyamines from a CSF hydrolysate is shown in Fig. 3.

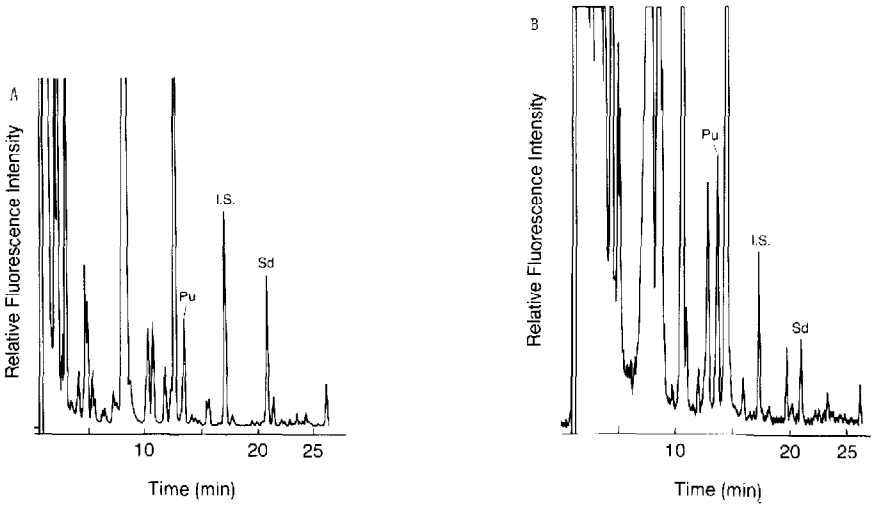

Fig. 3. (A) Chromatogram of a hydrolyzed human CSF sample containing 367 pmol/ml putrescine (Pu) and 447 pmol/ml spermidine (Sd). (B) Chromatogram of a hydrolyzed human CSF sample with 1140 pmol/ml putrescine (Pu) and 372 pmol/ml spermidine (Sd). Chromatograms obtained using a modified gradient I with 1,7-diaminoheptane as internal standard (I.S.).

Separations with gradient II

The chromatogram for the separation of a standard mixture of polyamines and monoacetylpolyamines with gradient II is shown in Fig. 4. The chromatogram in Fig. 5 shows the separation of polyamines and acetylpolyamines from an unhydrolyzed urine sample using gradient II. Elution with gradient II improved the separation for unhydrolyzed urine sufficiently that peaks for putrescine and monoacetylputrescine, and N^1- and N^8-acetylspermidine could be resolved. Gradient II is also useful for the analysis of unhydrolyzed CSF and of tissue when acetylated polyamines are to be quantitated.

Detection limits

Minimum detection limits for dansyl derivatives are determined by injecting 20 pg of each polyamine and determining the signal-to-noise ratio. Less than 20 pg (equal to 0.12, 0.08 and 0.06 pmol for putrescine, spermidine and spermine, respectively) could be detected at a signal-to-noise ratio of 4.

Precision

The within-run and day-to-day precision for ten determinations of two different concentrations of polyamines in urine and CSF pools are listed in Tables II and III. The within-day coefficient of variation (C.V.) ranged from

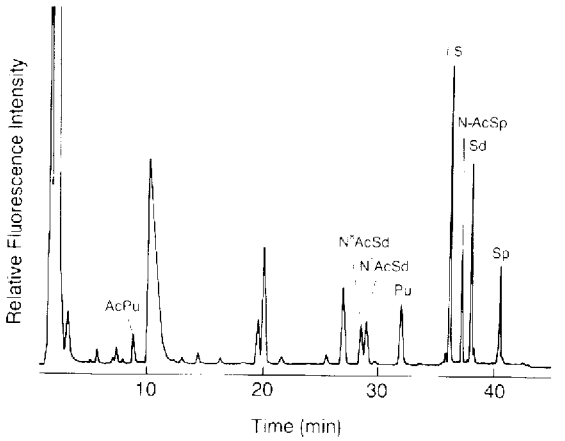

Fig. 4. Separation of polyamines and monoacetylpolyamines using gradient II. Each peak, including 1,7-diaminoheptane (internal standard, I.S.), represents about 500 pg of derivative injected. Peaks: AcPu = acetylputrescine; N^8AcSd = N^8-acetylspermidine; N^1AcSd = N^1-acetylspermidine; Pu = putrescine; N-AcSp = N-acetylspermine; Sd = spermidine; Sp = spermine.

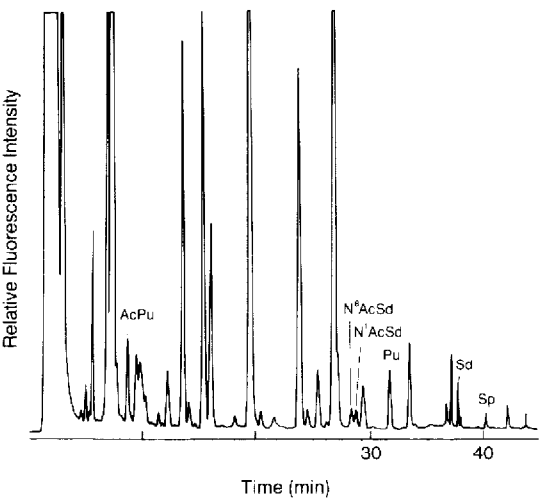

Fig. 5. Separation of polyamines and acetylated polyamines from unhydrolyzed human urine (gradient II). No internal standard was used in this separation. Peaks as given in Fig. 4.

1 to 4% for urinary polyamines, and from 3 to 5% for putrescine and from 1 to 5% for spermidine in CSF. The day-to-day C.V. ranged from 2 to 5% for urinary polyamines and from 8 to 12% for putrescine and spermidine in CSF. Spermine is rarely detected in CSF.

Background

Five hydrolyzed urine samples without 1,6-diaminohexane, the internal standard, added were assayed using gradient I to determine if samples had peaks at the elution time of the internal standard that would interfere with quantitation. A peak eluted at the same time as the internal standard, but it was too small to interfere with accurate quantitation; the background

TABLE II

WITHIN-RUN PRECISION (n = 10)

Polyamine	Concentration (mean ± S.D.)	C.V. (%)
Urine (nmol/ml)		
Putrescine	20.5 ± 0.54	2.6
	114.0 ± 3.0	2.6
	205.6 ± 4.1	2.0
Cadaverine	8.3 ± 0.21	2.5
	82.8 ± 0.93	1.1
	167.9 ± 2.75	1.6
Spermidine	9.45 ± 0.30	3.1
	64.8 ± 1.04	1.6
	127.4 ± 3.80	3.0
Spermine	6.2 ± 0.25	4.1
	39.9 ± 0.89	2.2
	89.2 ± 3.56	4.0
CSF (pmol/ml)		
Putrescine	217.6 ± 5.9	2.7
	309.8 ± 4.6	1.5
Spermidine	169.5 ± 7.9	4.6
	280.8 ± 3.5	1.3

TABLE III

DAY-TO-DAY PRECISION (n = 10)

Polyamine	Concentration (mean ± S.D.)	C.V. (%)
Urine (nmol/ml)		
Putrescine	20.5 ± 0.53	2.6
	113.9 ± 2.95	2.5
	205.5 ± 4.1	2.0
Cadaverine	3.3 ± 0.15	4.5
	83.8 ± 2.45	2.9
	161.7 ± 3.04	1.9
Spermidine	9.1 ± 0.21	2.3
	66.9 ± 2.76	4.1
	122.2 ± 1.94	1.6
Spermine	2.08 ± 0.12	6.0
	43.1 ± 2.0	4.6
	85.5 ± 2.6	3.0
CSF (pmol/ml)		
Putrescine	250 ± 29.5	11.8
	358 ± 29.6	8.3
Spermidine	202 ± 20.9	10.3
	317 ± 35.8	11.3

calculated for this peak from these samples was < 0.05 pmol. Tissue samples analyzed by this method with either gradient had cleaner backgrounds than urine or CSF. No peaks were present in the chromatograms of tissue extracts that would interfere with the use of either internal standard. Chromatograms

for hydrolyzed CSF samples with no internal standard assayed with gradient I had an unidentified peak that eluted at the same time as 1,6-diaminohexane that interfered with accurate quantitation of CSF polyamines. 1,7-Diaminoheptane could be used as an internal standard, however, without any observed interference.

Recovery and linearity

Unconjugated polyamines and monoacetylpolyamines were added to pools of urine and CSF in the amounts listed in Tables IV and V. A constant amount of 1,6-diaminohexane or 1,7-diaminoheptane was added to each sample, which was then processed as described above. Concentration and peak-area ratios were linearly related over this range. Analytical recoveries for polyamines ranged from 90 to 108% for hydrolyzed urine and from 81 to 117% for hydrolyzed CSF. Analytical recoveries for monoacetylated polyamines ranged from 88 to 125% for unhydrolyzed CSF.

TABLE IV

RECOVERY OF POLYAMINES (n = 5)

Polyamine	Added	Recovered	Recovery (%)
Urine (nmol/ml)			
Putrescine	11.36	10.56	93
	22.72	21.93	96
	56.8	53.62	94
	113.6	113.33	98
	227.2	216.75	95
Cadaverine	9.8	9.02	92
	19.6	18.8	96
	49.0	46.6	95
	98.0	93.7	96
	196.0	185.0	94
Spermidine	6.9	6.7	97
	13.8	13.4	97
	34.5	34.5	100
	69.0	66.1	96
	138.0	135.8	98
Spermine	4.95	4.65	94
	9.9	9.5	96
	24.75	24.2	98
	49.5	47.2	95
	99.0	96.3	97
CSF (pmol/ml)			
Putrescine	100	117	117
	200	202	101
	500	484	96.8
	1000	814	81.4
Spermidine	100	91	91
	200	225	112.5
	500	488	97.6
	1000	918	91.8

TABLE V

RECOVERIES OF ACETYLPOLYAMINES

CSF acetylpolyamines	Added (pmol/ml)	Recovered (pmol/ml)	Recovery (%)
N-Acetylputrescine	250	305	122
	500	625	125
	1000	957	96
N^1-Acetylspermidine	250	266.3	106.5
	500	522.8	104.2
	1000	995.2	99.5
N^8-Acetylspermidine	250	284.5	113.8
	500	532.3	106.5
	1000	1016.8	101.7
N^1-Acetylspermine	250	238.5	95.4
	500	461.4	92.3
	1000	936.5	93.7

Accuracy

The accuracy of this assay was assessed by comparing the results obtained with this method to those obtained with a modified amino acid analyzer method [14]. Results of regression analysis for the two methods for urine, tissue and CSF are listed in Table VI. Obviously, the results obtained with these methods compare most favorably.

TABLE VI

CORRELATION OF PROPOSED LIQUID CHROMATOGRAPHY METHOD (y-AXIS) WITH REFERENCE AMINO ACID ANALYZER METHOD (x-AXIS)

Polyamine	Sample	Parameters
Putrescine	Tissue	n = 36, r = 0.996, slope = 0.899, y-intercept = 0.084 nmol/ml
Spermidine	Tissue	n = 36, r = 0.987, slope = 0.864, y-intercept = 0.118 nmol/ml
Spermine	Tissue	n = 43, r = 0.985, slope = 0.767, y-intercept = 0.991 nmol/ml
Putrescine	Urine	n = 51, r = 0.986, slope = 0.822, y-intercept = —1.24 nmol/ml
Spermidine	Urine	n = 51, r = 0.971, slope = 0.884, y-intercept = 0.31 nmol/ml
Putrescine	CSF	n = 73, r = 0.978, slope = 0.982, y-intercept = 5.17 pmol/ml
Spermidine	CSF	n = 73, r = 0.933, slope = 0.869, y-intercept = 34.0 pmol/ml

Interference

The compounds listed in Table VII were tested for possible interference with the assay. Normetanephrine was found to have the same elution time as

TABLE VII

RETENTION TIMES OF POLYAMINES AND SOME DRUGS BY GRADIENT I

Compound	Retention time (min)	Compound	Retention time (min)
Agmatine	8.8	5-Hydroxytryptamine	14.6
Amikacin	N.D.*	Norepinephrine	18.2
Cadaverine	12.5	Methamphetamine	14.0
Deoxyepinephrine	N.D.	Normetanephrine	13.5
Amphetamine	11.4	Putrescine	11.7
1,3-Diaminopropane	11.2	1,6-Diaminohexane (I.S.)**	13.5
Dopamine	N.D.	Spermidine	17.2
Gentamicin	19.5, 20.5, 20.9	Spermine	19.8
Histamine	13.0	Tobramycin	18.4

*N.D. = Not detectable.
**I.S. = Internal standard.

1,6-diaminohexane, the internal standard. The concentration of this amine is normally low and should not interfere with the assay. The peak present at the elution time for 1,6-diaminohexane in hydrolyzed urine samples assayed without internal standard corresponds to the elution time for normetanephrine. This peak corresponded to a concentration of less than 0.05 pmol, an amount that would not interfere with quantitation. Additionally, 1,3-diaminopropane is baseline-separated from putrescine with gradients I and II (Table VII).

CONCLUSION

There is increasing interest in determining both intracellular and physiological fluid polyamine concentrations. A variety of LC pre-column or post-column derivatization methods for the assay of polyamines have been reported, but many of them are complicated or tedious, and few, if any, give reproducible results for CSF polyamine determinations. Modified amino acid analyzers are currently used to analyze polyamines levels in CSF, but the cost of instrumentation frequently limits the utility of this technique. In this paper we report a simple and specific gradient reversed-phase LC procedure for free and monoacetylated polyamines that uses pre-column derivatization with dansyl chloride. Sample clean-up employs either a Bond-Elut C_{18} column alone or in conjunction with a Bond-Elut SCX column, a procedure that has not previously been reported for LC analysis of polyamines. The method is relatively rapid, requiring only 26 min for tissue and hydrolyzed urinary polyamine determinations and 46 min for CSF or unhydrolyzed urine polyamine determinations. Results obtained with this procedure compare well with results obtained with the established amino acid analyzer technique for urine, tissue and CSF polyamines.

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