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DETERMINATION OF POLYAMINES IN HUMAN URINE BY AN AUTOMATED ION-EXCHANGE METHOD*

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SUMMARY

A sensitive and reliable automated cation-exchange method for the determination of polyamines in human urine has been developed and applied to samples from both normal subjects and patients bearing various types of cancer. A relatively large number of cancer patient urine samples are currently being analyzed, and these data will be the subject of a later paper.

INTRODUCTION

Biomedical interest in the di- and polyamines, putrescine, spermidine, and spermine has been stimulated by the reports of Russell *et al.*[1,2], which indicated that cancer patients excrete elevated urinary levels of these polyamines. These investigators also reported that the polyamine elevations were observed in patients with a number of different types of cancer, and that removal of a portion of the tumor mass by surgery resulted in a definite decrease in the polyamine excretion level. These results were obtained after 6 *N* HCl hydrolysis of urine followed by extraction of the polyamines with basic 1-butanol, separation by high-voltage paper electrophoresis, and estimation of the amounts present by a ninhydrin-spectrophotometric method.

As the electrophoretic analytical method has been found subject to problems of specificity, accuracy, and sensitivity, cation-exchange chromatography (CIE) appeared to offer both the improved resolving power and sensitivity for ascertaining the amount of each polyamine in urine samples. Bremer and Kohne and others[3–5] have reported buffer systems for the analysis of the polyamines by CIE; Marton *et al.*[6,7] more recently reported analysis of the polyamines in the urine from normal and cancer patients by CIE.

This paper describes our development of an automated CIE method for the

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quantitative analysis of polyamines in human urine from normal and cancer patients. This described CIE method was developed in parallel with our previously reported gas–liquid chromatographic method for the analysis of urine[8] to serve as a complementary and confirmatory technique for studying the urinary excretion levels of polyamines. A large number of cancer patient urine samples are currently being analyzed, and the results obtained from this study will be the subject of a later report.

EXPERIMENTAL

Apparatus

A Bio-Cal Model BC-200 amino acid analyzer (Bio-Cal, Richmond, Calif., U.S.A.) was used, with a 7.5 × 0.9 cm column packed with Aminex A-5 (12–15 μ) spherical resin (Bio-Rad Labs., Richmond, Calif., U.S.A.). The resin column was maintained at 65° throughout the analysis. The analyzer was also equipped with a Chromatronix ROSV-1.0 twenty-loop, refrigerated, automatic injection valve (Chromatronix, Berkeley, Calif., U.S.A.).

A Model MV-3 HP 3-port motor valve (MER Chromatographic, Mountain View, Calif., U.S.A.) was inserted between the end of the resin column and the ninhydrin reaction coil. This valve was controlled by the electronic programmer of the analyzer through a relay (24 V d.c.), and allowed the effluent from the analytical column to be directed either to the detector system or to waste. The chromatographic peak areas were determined with an Infotronics Model 110-A digital integrator (Infotronics, Houston, Texas, U.S.A.) with baseline display which was connected between the 570-nm colorimetric detector and the Honeywell (Ft. Washington, Pa., U.S.A.) Elektronik 15 multipoint recorder with a 50 mV, 0–2 O.D. span.

PTFE filter discs of 10 μ and 5-μ stainless-steel screens, both 0.9 cm in diameter, were obtained from MER Chromatographic. A stainless-steel screen and a PTFE filter (5–10 μ) were placed at the bottom of the resin column, and a PTFE filter was placed on top of the resin bed. For filtering the hydrolysates of the urine samples, 0.5-μ Solvinert filters were purchased from Millipore (Bedford, Mass., U.S.A.), as were Millipore Twin-90 filter units (0.22 μ) for filtration of the deionized water. Whatman Gamma-12 in-line filters of 3.0 μ were used for filtering the prepared buffers, and were obtained from A. H. Thomas, Philadelphia, Pa., U.S.A.

A Temp-Blok module heater for evaporation of samples was purchased from Lab-Line Instruments (Melrose Park, Ill., U.S.A.) and a Cary recording spectrophotometer was used from Applied Physics (Pasadena, Calif., U.S.A.).

Reagents

Putrescine, cadaverine, spermidine, and spermine were obtained as their hydrochloride salts from Calbiochem (Los Angeles, Calif., U.S.A.). 3,3'-Diaminodipropylamine was purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Ninhydrin was obtained from EM Labs. (Elmsford, N.Y., U.S.A.), and titanous chloride, pentachlorophenol, and Brij 35 solution were obtained from Pierce (Rockford, Ill., U.S.A.). A.R.-grade sodium citrate·2 H_2O, and sodium acetate·3 H_2O were obtained from Fisher Scientific (St. Louis, Mo., U.S.A.), as were ethylene glycol monomethyl ether and disodium ethylenediamine tetraacetate. Sodium chloride, Baker Analytical Reagent, was obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.).

Buffers

The buffers were prepared in 20-liter lots (Table I) to ensure reproducibility during the analyses.

The deionized water used for preparation of the buffers was sterilized by passage through a Millipore Twin-90 filter (0.22 μ), and all glassware and buffer containers were treated with chlorox prior to use, then thoroughly rinsed with sterile deionized water. The pH of the buffer solutions was adjusted with either A.R.-grade concentrated HCl or 50% NaOH, and all buffers were filtered through 3.0-μ Whatman Gamma-12 filters and the dissolved air removed under vacuum prior to loading into the instrument.

TABLE I

COMPOSITION OF EACH BUFFER PER LITER

Reagent	*Buffers*				
	Sample	*A*	*B*	*C*	*D*
pH	5.80	8.80	5.80	5.80	5.80
Na^+ concentration, *N*	0.35	0.35	1.35	2.35	3.50
Sodium citrate·$2H_2O$, g	34.31	34.31	34.31	34.31	34.31
25% Brij solution, ml	4.0	4.0	4.0	4.0	4.0
Pentachlorophenol, ml	0.1	0.1	0.1	0.1	0.1
Sodium chloride, g	0	0	58.44	116.9	184.1
Na_2EDTA, g	20.0	0	0	0	0

The ninhydrin solution was prepared according to the modified method of Spackman *et al.*[9], using titanous chloride in place of stannous chloride[10].

Calibration standard solutions of the polyamines containing 1.00 mg of each polyamine (as the free base) per ml of 0.1 *N* HCl were prepared and maintained under refrigeration at 4°. Calibration analyses were conducted after dilution of this stock solution to 4 μg/ml with the diluting buffer.

The flow-rate of the buffers during the analyses was maintained at 120 ml/h, with a ninhydrin flow-rate of 60 ml/h. The reaction coil was held at 100° ± 1° and the detector flow cell length was 23 mm.

CIE analysis sequence

Table II describes the sequence of events of the CIE analysis. The sequence of events is controlled automatically, and requires a minimum of operator effort. This time sequence will vary depending on the time required for each instrument to perform the buffer change.

Preparation of urine samples for analysis

Aliquots of 4.00 ml of 24-h urine collections were placed in 16 × 75 mm Pyrex screw-cap tubes and evaporated to dryness under streams of purified nitrogen gas at 60–70° in a heating block. 3.00 ml of 6 *N* HCl containing 15.0 μg of 3,3'-diaminodipropylamine (as internal standard, I.S.) were added, the tubes tightly capped with PTFE-lined screw caps, and then placed in a 110° oven for 16-h hydrolysis. After hydrolysis, the HCl was evaporated from the samples at 60° with a nitrogen gas sweep,

TABLE II

CIE ANALYSIS SEQUENCE

Time (min)	*Event*
0	The buffer pump is started, the three-way valve to waste activated, and the sample injected
0 to 10	Buffer A is pumped through the resin column to waste.
10 to 30	Buffer B is pumped through the column to waste.
at 30	The three-way valve is activated, switching the column effluent from waste to the ninhydrin reaction coil. The ninhydrin pump, integrator, and recorder are started.
30.5 to 52	Buffer C is pumped through the column.
52 to 75	Buffer D is pumped through the column.
75 to 85	0.4 *N* NaOH containing 0.25% EDTA is pumped through the column.
85 to 90	Buffer A is again pumped through the column.
at 90 to 120	The ninhydrin pump, integrator, and recorder are turned off. Buffer A continues.
at 120	The buffer pump is turned off, and the programmer automatically recycled.

and 3.0 ml of the sample buffer were added. EDTA was added to the sample-diluting buffer to complex multivalent metal ions in the sample, thereby increasing the efficiency and life of the analytical resin column. The sample tubes were then shaken manually and placed in an ultrasonic bath to ensure thorough mixing. The pH of each sample was then determined, and adjusted to between 5 and 6 with a few drops of 40% NaOH using an external indicator. The sample solutions were then refrigerated at 4° for a minimum of 4 h, then filtered through 0.5-μ Millipore Solvinert filters. *Ca.* 1.0 ml of the filtrate was then injected into the instrument for analysis.

RESULTS AND DISCUSSION

The aim of this study was to modify the method developed by Bremer and Kohne[5] and to improve the speed, sensitivity, and reliability of the CIE technique for the analysis of di- and polyamines in human urine.

The effects of pH and ionic strength of the buffer solutions on the affinities of the polyamines for the CIE resin were studied in the early part of this research. At pH less than 11, the polyamines were all strongly retained by the resin. The use of eluents containing increased salt concentrations greatly reduced the retention time of the di- and polyamines, with the relative affinity of each polyamine remaining unchanged with salt concentrations up to 3 *N* Na^+.

A rapid elution scheme was then developed using a high pH (8.80), low ionic strength (0.35 *N* Na^+) buffer to elute other ninhydrin-positive components of urine, such as amino acids, which have a weaker affinity for the resin than the di- and polyamines. After injection of a urine sample, these extraneous ninhydrin-positive components proceed through the column much more rapidly than the polyamines, and are diverted to waste during the first 30 min of the analysis. Failure to vent these components resulted in interferences which made the determination of the polyamines most difficult.

The di- and polyamines were eluted with buffers of increasing ionic strength (Table I), with the effluent containing these components directed through the ninhydrin mixing coil and colorimetric detection system.

The di- and polyamines were reacted separately with ninhydrin, and the absorption spectra obtained over the range of 400 to 620 nm. A single absorption band with a maximum at 565 nm was observed for each polyamine. The 570-nm channel of the analyzer was thus well suited for the detection of the polyamines.

The linear range of the analytical system was evaluated from 2 to 50 μg of each di- and polyamine, and the relative weight response (RWR) of each remained essentially constant, indicating good linearity. The $RWR_{PA/I.S.}$ values were: putrescine, 1.10; spermidine, 1.26; and spermine, 1.32. On an equimolar, $RMR_{PA/I.S.}$ basis, the response of spermidine was twofold greater than that of putrescine, and the response of spermine was threefold the response of putrescine.

Fig. 1 presents a typical chromatogram of a standard calibration mixture of the four di- and polyamines, plus the internal standard, 3,3'-diaminodipropylamine.

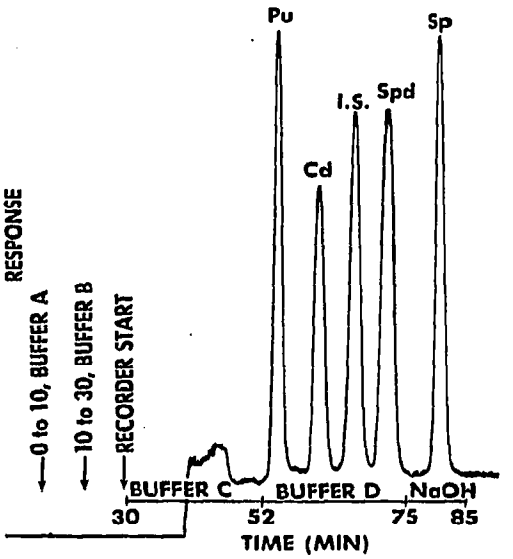

Fig. 1. CIE analysis of a standard calibration mixture of the four di- and polyamines. Internal standard: 3,3'-diaminodipropylamine. Sample injected: 6 μg of each polyamine. Abbreviations: see Table III.

The detector output is displayed on a Corning Model 845 recorder with a 50-mV range (Corning, Corning, N.Y., U.S.A.). Fig. 2 shows the chromatogram obtained from the analysis of a urine sample from a patient with diagnosed breast cancer. The values ranged from 1.93 mg per 24 h for putrescine to only a trace amount of cadaverine in this sample. 1,3-Diaminopropane and 1,6-diaminohexane, which may be present at low levels in human urine, are separated from the polyamines with this method. 1,3-Diaminopropane is eluted before putrescine, and 1,6-diaminohexane is eluted between spermidine and spermine.

In order to evaluate the precision and accuracy of the analytical method, known amounts of each polyamine were added to urine samples prior to hydrolysis. Aliquots of the same 24-h urine collections were also analyzed to determine the amount of each polyamine initially in the sample, thus allowing a calculation of the per cent recovery

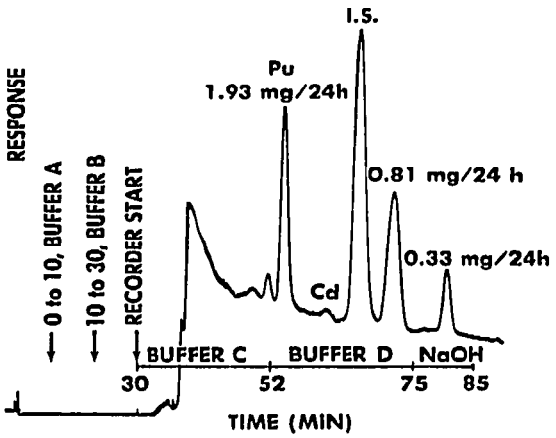

Fig. 2. CIE analysis of a urine sample from a patient with diagnosed breast cancer. Internal standard: 3,3'-diaminodipropylamine. Sample injected: 1.25 ml of hydrolyzed urine. Abbreviations: see Table III.

of the added polyamines. Fig. 3 shows the analysis of a breast cancer patient urine sample spiked with 2.5 μg/ml of each polyamine.

Table III lists the results of a recovery study with urine from patients with various types of cancer, and the recoveries of the di- and polyamines are seen to be essentially quantitative.

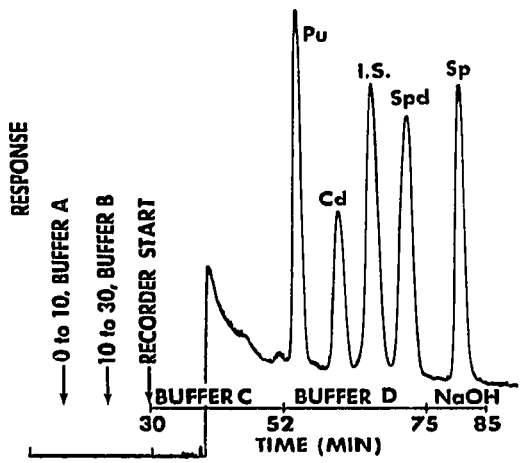

Fig. 3. CIE analysis of a breast cancer patient urine sample spiked with 2.5 μg of each polyamine. Internal standard: 3,3'-diaminodipropylamine. Sample injected: 1.25 ml of hydrolyzed urine. Abbreviations: see Table III.

Further, a study was made to determine the precision of the total analytical procedure by preparing and analyzing two independent hydrolysates of each of twelve 24-h urine collections. Six collections were obtained from normal individuals and six from patients with diagnosed lung cancer. The results are given in Table IV, with the

TABLE III

RECOVERY OF POLYAMINES FROM CANCER PATIENT URINE*

Abbreviations: Pu = patrescine; Cd = cadaverine; Spd = spermidine; Sp = spermine.

NCI Sample No.	*Diagnosis*	*μg/ml of each polyamine added***	*Recovery (%)*			
			Pu	*Cd*	*Spd*	*Sp*
190	Breast cancer	2.5	101	98	100	103
202	Colon cancer	2.5	102	97	94	96
208	Breast cancer	2.5	97	96	96	99
213	Lung cancer	2.5	90	100	89	81
223	Breast cancer	2.5	90	92	90	90
228	Stomach cancer	2.5	99	90	94	93
233	Breast cancer	2.5	93	96	97	101
241	Stomach cancer	2.5	93	101	99	104
246	Colon cancer	2.5	99	105	96	106
Average			96	97	95	97

* Analyzed by CIE.

** 10.0 μg of each polyamine added to 4.0 ml of urine prior to hydrolysis with 6 *N* HCl, 110°, 16–18 h. The equivalent of *ca.* 1 ml of urine was used per CIE analysis.

TABLE IV

PRECISION OF ANALYSIS FOR POLYAMINES IN HUMAN URINE BY CIE*

For abbreviations, see Table III.

Sample No.	*Diagnosis*	*Pu*		*Spd*		*Sp*	
		μg/ml	*mg***	*μg/ml*	*mg***	*μg/ml*	*mg***
89	Normal	3.37	2.2	3.07	2.0	0.65	0.5
		3.42		3.06		0.71	
90	Normal	2.21	3.1	1.48	2.0	0.20	0.3
		2.26		1.49		0.30	
91	Normal	2.12	3.1	1.22	1.8	0.22	0.3
		2.04		1.23		0.19	
92	Normal	2.46	2.1	1.38	1.2	0.50	0.4
		2.38		1.35		0.49	
93	Normal	0.68	1.1	0.74	1.2	0.14	0.2
		0.65		0.76		0.10	
94	Normal	1.47	1.7	1.23	1.4	0.48	0.5
		1.35		1.15		0.29	
95	Lung cancer	1.64	1.5	1.03	1.0	0.31	0.3
		1.57		1.08		0.38	
96	Lung cancer	1.98	1.8	3.18	2.8	0.83	0.7
		1.81		2.89		0.64	
97	Lung cancer	3.27	4.5	2.32	3.2	1.50	2.1
		3.59		2.54		1.80	
98	Lung cancer	1.47	1.2	1.31	1.1	0.16	0.1
		1.38		1.22		0.15	
99	Lung cancer	0.89	1.8	0.90	1.9	0.08	0.2
		0.74		0.86		0.08	
100	Lung cancer	1.27	3.8	0.63	2.0	0.15	0.5
		1.09		0.60		0.18	
R.S.D. Av.***, %		5.1		4.4		18.1	

* Hydrolyzed with 6 *N* HCl at 110°, 16–18 h.

** mg/24 h calculated as average of two independent hydrolysates.

*** R.S.D. $= \left(\frac{\Sigma (X_1 - X_2)^2}{2P}\right)^{\frac{1}{2}} / \text{av.} \times 100.$

μg/ml of each polyamine found in each hydrolysate listed. The relative standard deviation for putrescine was 5.1%; spermidine, 4.4%; and spermine, 18.1%. The low levels of spermine present in urine are reflected in the increased R.S.D. Also listed are the mg/24 h values of the polyamines, with putrescine ranging from 4.5 to 1.1, spermidine from 3.2 to 1.0, and spermine from 2.1 to 0.1 mg/24 h.

CONCLUSIONS

An automated CIE method has been developed for the analysis of the di- and polyamines putrescine, cadaverine, spermidine, and spermine in human urine. Sample preparation prior to analysis is not lengthy, as other ninhydrin-positive urine components are separated from the polyamines by the analytical system. The effects of pH and ionic strength of the buffer systems were studied and optimized, resulting in a total analysis and column regeneration time of 120 min.

Studies were conducted to determine the recovery of polyamines added to urine, with the average recovery of each polyamine being: putrescine, 96%; cadaverine, 97%; spermidine, 95%; and spermine, 97%.

The developed method was found to be precise, with relative standard deviations from analyses of independent hydrolysates being 5.1% for putrescine, 4.4% for spermidine, and 18.1% for spermine. Spermine is generally found at much lower levels than putrescine or spermidine, thus a higher R.S.D.

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REFERENCES

1 D. H. Russell, *Nature (London)*, 223 (1971) 144.
2 D. H. Russell, C. C. Levy, S. C. Schimpff and I. A. Hawk, *Cancer Res.*, 31 (1971) 1555.
3 K. H. Schull, J. McConomy, M. Vogt, A. Castillo and E. Farber, *J. Biol. Chem.*, 241 (1966) 5060.
4 D. R. Morris, K. L. Koffron and C. J. Okstein, *Anal. Biochem.*, 30 (1969) 449.
5 H. J. Bremer and E. Kohne, *Clin. Chim. Acta*, 32 (1971) 407.
6 L. J. Marton, J. G. Vaughn, I. A. Hawk, C. C. Levy and D. H. Russell, in D. H. Russell (Editor), *Polyamines in Normal and Neoplastic Growth*, Raven Press, New York, 1973, p. 367.
7 L. J. Marton, D. H. Russell and C. C. Levy, *Clin. Chem.*, 19 (1973) 923.
8 C. W. Gehrke, K. C. Kuo, R. W. Zumwalt and T. P. Waalkes, in D. H. Russell (Editor), *Polyamines in Normal and Neoplastic Growth*, Raven Press, New York, 1973, p. 343.
9 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
10 L. B. James, *J. Chromatogr.*, 59 (1971) 178.