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POLYAMINES—AN IMPROVED AUTOMATED ION-EXCHANGE METHOD*

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SUMMARY

An accurate, precise, and improved automated cation-exchange chromatographic method with ninhydrin detection for the analysis of di- and polyamines (putrescine, cadaverine, spermidine, and spermine) has been developed. We have shown that different types of biological fluids such as urine, blood plasma, blood sera, tissue extracts, and cancer cell culture media can be analyzed under identical chromatographic conditions. The simplicity and precision of the method was achieved by eliminating the sample pre-separation and using an internal standard technique. Thus, not only has the sample preparation been simplified, but the accuracy and precision and sensitivity of the method have been greatly improved. Twenty-four unattended analyses were performed each day. With minor modifications of the instrument a two-fold analytical output can be achieved with analysis time cut to 30 min. The ruggedness and applicability of the method has been demonstrated in our laboratory during the past six months. More than two thousand urine and hundreds of other physiological samples have been analyzed by this method with a relative standard deviation from 3.3 to 7.8%, and recoveries of 94 to 97%.

This automated ion-exchange chromatographic method for the polyamines will be useful to researchers in biological markers programs for monitoring the course of cancer and effectiveness of chemotherapy.

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INTRODUCTION

The role of di- and polyamines (putrescine, cadaverine, spermine, and spermidine) as biological markers, whether for diagnosis of neoplasia [1—3] or as a means of monitoring efficacy of cancer chemotherapy [4], continues to attract the attention of cancer investigators. Their research has generated an ever increasing number and type of biological samples which have necessitated the continual development of more sensitive and expeditious polyamine analytical methods.

Most research emphasis has been placed upon the urinary excretion level of polyamines. Investigative work has now expanded to the point where the analysis of additional biological samples — blood serum and plasma, tissue extracts, and the media of cultured neoplastic cells are being requested by various researchers.

Numerous techniques have been developed for the determination of di- and polyamines; among them high-voltage paper electrophoresis, radioimmunoassay, enzymatic assay, and thin-layer chromatography. In most cases, however, not all of the polyamines are measured by each procedure, nor is adequate sensitivity and specificity obtained [5]. Precise and accurate determinations have not been adequately demonstrated with any of the methodologies.

Gas—liquid chromatography (GLC) [6] has proven sensitivity and resolution in polyamine analyses. The limitations of this method are encountered in the extensive sample cleanup procedures which make routine application of GLC impractical without the development of some automation.

In terms of accuracy, precision, simplicity, and rapidity of analyses, cation-exchange chromatography (CIE) utilizing an automated amino acid analyzer with ninhydrin detection apparently lends itself most readily to the analyses of a large number of samples. In 1971, Bremer et al. [7] first used such an instrument in the determination of polyamines. This method was significantly improved in sensitivity, separation, and speed by Marton et al. [8], Gehrke et al. [9], and Veening et al. [10].

The sample run time, for all three of these investigators, still remained at 90 to 120 min. Marton's method encountered interference problems and baseline shifts at high sensitivities, while Gehrke's procedure lacked the necessary sensitivity to analyze low levels of polyamines. Elution inconsistencies and relatively high minimum detection quantity for spermine were reported by Veening, nor had the method been substantiated by extensive application.

To answer the demand for the analysis of an ever increasing number and kind of biological samples for polyamines, this research has been directed toward the development of an improved method. Versatility and simplicity of analysis along with the required precision and accuracy were the goals of the investigation.

EXPERIMENTAL

Apparatus

A Beckman Model 121M amino acid analyzer (Beckman, Palo Alto, Calif., U.S.A.) provided with a 4.0 × 0.28 cm column packed with Beckman AA-20

resin, was used for the analyses. Column temperature was maintained at 65° throughout the analysis. The detector was set at a wavelength of 570 nm with a 1.2 cm flow cell with an output of 0.1 O.D./100 mv.

The data handling system was a Hewlett-Packard 3352B laboratory data system consisting of: a Hewlett-Packard 2100 computer with 16 K of memory; 18652A analog-to-digital converters (A/D); ASR33 teletype; and a 2748B high-speed photo reader (Hewlett-Packard, Maryland Heights, Mo., U.S.A.).

The urine sample aliquots and the deproteinized cell culture media were placed in 16 × 75 mm PTFE-lined screw cap Pyrex culture tubes obtained from Corning Glass Works (Corning, N.Y., U.S.A.). The blood plasma and sera aliquots were put into Corex (Corning) 30-ml round-bottom centrifuge tubes. Samples and buffered hydrolysates were shaken on a Vortex Genie mixer purchased from Scientific Products (Evanston, Ill., U.S.A.). Centrifugation was done in an automatic Servall superspeed centrifuge (Ivan Servall, Norwalk, Conn., U.S.A.) or in an Eppendorf Model 3200/30 microcentrifuge (Brinkman, Westbury, N.Y., U.S.A.).

Hydrolysis of samples was done in either a constant temperature oven from Aloe Scientific (Division of Brunswick, St. Louis, Mo., U.S.A.) or in a custom built proportional temperature controlled heating block (Science Instrument Shop, University of Missouri, Columbia, Mo., U.S.A.). An aluminum block (37 long × 15 wide × 8 high cm) with 44 holes (1.8 cm diameter × 3.5 cm deep) was heated by four CE 200-X 400 W heaters from Watlow (St. Louis, Mo., U.S.A.). The temperature was maintained at 145° ± 1° by a RFL temperature controller from RFL Industries (Boonton, N.J., U.S.A.).

Samples and buffered hydrolysates were dried either on a Temp-Blok module heater from Lab-Line Instruments (Melrose Park, Ill., U.S.A.) or on a Vaspilator rotary evaporator purchased from Rinco (Greenville, Ill., U.S.A.). Water was distilled from a Corning Model AG-11 all glass still equipped with a 45 l automatic collection system (9-035-65).

Sterilization of the water used in buffer preparation was accomplished by passage through a twin-90 (0.22 μm) filter from Millipore (Bedford, Mass., U.S.A.). The buffers were filtered through a Millipore GSWP04700 (0.22 μm) filter supported by a Millipore disc filter holder (XX1004730). The buffered hydrolysates were filtered through a DAWP01300 filter (0.65 μm). The deproteinized cell culture media, blood plasma, and blood sera were filtered through Millipore GSWP01300 filters (0.22 μm).

Reagents

Putrescine, cadaverine, spermidine, and spermine were obtained as hydrochloride salts from Calbiochem (Los Angeles, Calif., U.S.A.). 3,3'-Diaminodipropylamine was purchased from Aldrich (Milwaukee, Wis., U.S.A.). Ninhydrin and hydrindantin were obtained from EM Labs (Elmsford, N.Y., U.S.A.). Lithium hydroxide · H_2O, glacial acetic acid, dimethylsulfoxide, and sodium chloride, all reagent grade, were purchased from J.T. Baker (Phillipsburg, N.J., U.S.A.). Reagent-grade sodium citrate · H_2O, disodium ethylenediamine tetraacetate, and 5-sulfosalicylic acid were obtained from Fisher Scientific (St. Louis, Mo., U.S.A.).

Buffers. Two liters each of the buffers were prepared using deionized,

TABLE I

COMPOSITION OF EACH BUFFER

Reagent	Buffer per l					
	Sample	A	B	C	D	NaOH
pH, ±0.03	5.90	8.20	7.00	6.00	6.00	—
Na^+ Concentration (*N*)	0.20	0.20	1.60	2.50	3.50	0.2
Sodium citrate · $2H_2O$ (g) (0.2 *N*)	19.6	19.6	19.6	19.6	19.6	0
Sodium chloride (g)	0	0	81.83	134.4	192.9	0
Phenol (ml)	1.0	1.0	1.0	0	0	0
Na_2EDTA (g)	20.0	0	0	0	0	10.0

doubly distilled water that had been sterilized by passage through a Millipore twin-90 filter (0.22 μm). Glassware and buffer containers were treated with Chlorox (Chlorox, Oakland, Calif., U.S.A.), washed with 1 *N* HCl, and finally rinsed with the sterilized deionized doubly distilled water, prior to use.

All of the buffer solutions were boiled for 30 min, then filtered through a 0.22-μm filter before final pH and volume adjustments were made. Adjustments of pH were made with 6 *N* HCl. See Table I.

Ninhydrin. The ninhydrin solution was prepared according to the method of Moore [11]. Lithium acetate buffer (4 *M*) was first made by adding 336 g of $LiOH \cdot H_2O$ to 800 ml of doubly distilled water in a beaker. The solution was stirred until half of the $LiOH \cdot H_2O$ was dissolved, then 568 ml of glacial acetic acid were added. The lithium acetate solution was cooled, transferred to and brought almost to volume in a 2-l volumetric flask. A 1.0-ml aliquot of the buffer was withdrawn, diluted with 3.0 ml of doubly distilled water and its pH determined. If the pH was not 5.20 ± 0.05, it was adjusted with 2 g $LiOH \cdot H_2O$ or 2 ml acetic acid per 2.0 liters for each 0.01 pH unit. The lithium acetate buffer was made to volume, filtered through a 0.22-μm filter and 225 ml of the buffer were combined with 675 ml of dimethylsulfoxide (DMSO). Nitrogen containing <10 ppm of oxygen was then bubbled through the solution for at least 15 min.

Ninhydrin (18.0 g) was added to the above DMSO—lithium acetate solution while stirring. The bubbling of nitrogen was continued until all of the ninhydrin was in solution. Just prior to use the ninhydrin was reduced by the addition of 0.56 g of hydrindantin while stirring and bubbling the nitrogen through the solution for ca. 3 h.

Instrument and chromatography conditions

Beckman AA-20 resin with a bed size of 40.0 × 2.8 mm was used to separate the di- and polyamines. Flow-rates of the buffers and the ninhydrin were 8.8 and 4.4 ml/h, respectively. A column temperature of 65° and a reaction bath temperature of 100° ± 1° was maintained. The polyamines reacted with the ninhydrin for 2.8 min and were then detected at 570 nm in a 12.0 mm flow cell. Sensitivity was set at 0.1 a.u.f.s. A 50-μl volume of the sample filtrate or supernatant was injected on the column for analysis.

TABLE II

PREPARATION OF SINGLE STOCK SOLUTIONS

Polyamine	MW	g/100 ml	μmoles/ml
Pu·2HCl	161.1	1.611	100.0
Cd·2HCl	175.1	1.751	100.0
I.S.*	131.2	1.312	100.0
Sd·3HCl	254.6	1.273	50.0
Sp·4HCl	348.2	1.161	33.3

*3,3'diaminodipropylamine free base.

Calibration standard solutions

The hydrochloride salts of the polyamines used for the preparation of the standard solutions were stored under refrigeration at 4° and dried for one day in a desiccator with reduced pressure of less than 70 μ at room temperature prior to weighing.

Single compound stock solutions were prepared to yield concentrations of 100 μmoles/ml each of putrescine (Pu), cadaverine (Cd), internal standard (I.S.) (3,3'-diaminodipropylamine), 50 μmoles/ml of spermidine (Sd), and 33 μmoles/ml spermine (Sp). Exactly 10.0 ml of each of the single compound stock solutions were made to a volume of 2000 ml with 0.1 *N* HCl. Exactly 5.0 ml of this solution was brought to a volume of 100 ml with 0.05 *N* HCl. Final polyamine concentrations as the free bases are given in Table II. Working standard solutions were prepared of 25.0 nmoles/ml for Pu, Cd, and I.S.; 12.5 nmoles/ml for Sd; and 8.35 nmoles/ml for Sp.

It was observed that the stock solutions were not stable when stored for a few days if pH >7. Storage at 4° and at pH 2 was required. At these conditions the polyamine solutions were stable for three months.

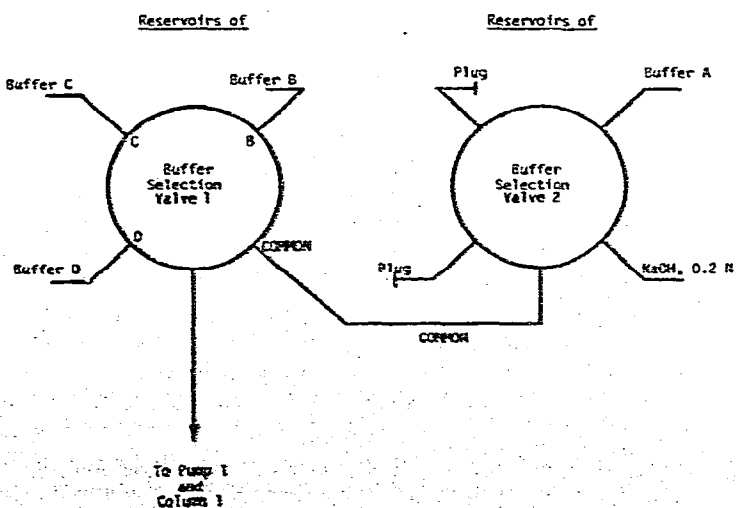


Fig. 1. CIE buffer selection valve sequence. For buffer composition see Table I.

CIE analysis sequence

Table III describes the sequence of events for the automated CIE analysis and Fig. 1 shows the modifications of the buffer selection valves employed in the instrumentation for polyamine analysis.

Procedure

Urine. Homogeneous 2.00-ml aliquots of 24-h urine collections were placed in 16 × 75 mm Pyrex screw-cap tubes and evaporated to dryness under purified nitrogen gas at 65 to 70° in a heating block. Exactly 2.00 ml of 6 *N* HCl containing 50 nmoles of 3,3′-diaminodipropylamine (I.S.) were added to each dried sample. The tubes were tightly capped with PTFE-lined screw caps, and then placed in a 110° oven for 16 h. After hydrolysis the HCl was evaporated from the samples to dryness at 70° under a gentle stream of nitrogen gas followed by the addition of 1.00 ml of the sample buffer. The buffered hydrolysates were thoroughly mixed and then placed under refrigeration at 4° for a minimum of 4 h.

TABLE III

CIE INSTRUMENTAL ANALYSIS SEQUENCE

Step 1 for starting instrument only. Steps 2—12 are the actual analytical time. Steps 13 and 14 are for instrument shut down only.

Step	Step time (min)	Event
1	15.0	Buffer pump No. 1 on. Ninhydrin pump on. Buffer selection valve 1 to buffer D position. Ninhydrin diverter valve to reaction coil position. Column No. 1 effluent to reaction coil.
2	0.1	Stop program recycling. Sample transfer pump on. Buffer selection valve 2 to NaOH position. Buffer selection valve 1 to common.
3	4.9	This completes sample transfer to the injection loop.
4	3.0	Buffer selection valve 2 to buffer A position to equilibrate column.
5	7.0	Ninhydrin diverter valve to drain position. Buffer pump No. 2 on.
6	0.1	Start sample transfer to column. Start computer. Start data reduction system.
7	0.9	Complete transfer of sample from injection loop to column. Column No. 1 effluent to drain. Column No. 2 effluent to reaction coil.
8	13.0	Buffer selection valve 1 to buffer B position.
9	2.0	Column No. 1 effluent to reaction coil. Buffer pump No. 2 off.
10	18.0	Ninhydrin diverter valve to reaction coil position. Buffer selection valve 1 to buffer C position.
11	10.9	Buffer selection valve 1 to buffer B position.
12	0.1	Start program recycling.
13	8.0	Buffer selection valve 1 to common. Buffer selection valve 2 to buffer A position.
14	15.0	Ninhydrin pump off. Ninhydrin diverter valve to drain. Program shut down.

Total Run time: 60.0

The samples were filtered through a Millipore 0.45—0.65-μm filter or centrifuged on the Eppendorf Microcentrifuge for 5 min at 12,000 *g*, and then 50 μl of the filtrate or supernatant were placed on the CIE column for analysis.

Cell culture media. Homogeneous 5.00-ml aliquots of cell culture media were placed in 30 ml centrifuge tubes to which were added 1.00 ml of 20% (w/v) sulfosalicylic acid. The tubes were then thoroughly shaken, heated at 70° for 30 min, and than centrifuged at 12,100 *g* for 10 min at 4°. The deproteinized supernatants were transferred to 16 × 75 mm Pyrex screw-cap tubes and then evaporated to dryness in a 70° heating block under a nitrogen gas sweep. Exactly 2.00 ml of 6 *N* HCl containing 5.00 nmoles of I.S. were added to the dried samples. They were then tightly capped with PTFE-lined screw caps and hydrolyzed at 120° for 40 h. The samples were periodically shaken during hydrolysis.

After hydrolysis, the tubes were placed under refrigeration for 30 min. While maintaining a temperature of 0° with an ice bath, the hydrolysates were filtered through glass wool plugs into 16 × 75 mm screw-cap Pyrex tubes. The hydrolysis tubes and the glass-wool plugs were both washed with ca. 1 ml of ice cold 6 *N* HCl, and the washes were combined with the filtrates and then taken to dryness at 70° under a purified nitrogen gas flow. The dried samples were redissolved with sonication in 1.00 ml of doubly distilled water, prior to CIE analysis, then 50 μl placed on the analyzer.

Blood plasma and serum. Same sample preparation method as for cell culture media except 16 h of hydrolysis at 110° was used.

Pork tissue extracts. Ground tissue portions (100 g) were homogenized in a blender with 150 ml of 0.6 *N* $HClO_4$ and then centrifuged at 2000 *g* for 20 min. The tissue residues were homogenized once again with an additional 150 ml of 0.6 *N* $HClO_4$, centrifuged, and the two supernatants were combined and refrigerated at 4°. Filtration through glass-wool to remove suspended fat followed, and the pH of the filtrates was then adjusted to 6 with 6 *N* KOH. To facilitate the precipitation of the perchlorate salts, the sample solutions were cooled (4°) and the salts removed with filtration through Whatman No. 5 filter paper. The filtrates were then frozen, lyophilized, and redissolved in 50 ml of doubly distilled water. The salts were once again removed by filtration and the filtrates made to volumes of 100 ml with doubly distilled water (extract A).

Free polyamines. Determinations of the free polyamines in the tissue extracts were made by taking exactly 1.00 ml aliquot of extract A and adding an internal standard solution of exactly 1.00 ml of 0.1 *N* HCl containing 50.0 nmoles of 3,3'-diaminodipropylamine. Then 50 μl were placed on the CIE column for analysis.

Total polyamines. The total polyamine contents of the extracts were determined by placing exactly 1.00 ml aliquots of extract A in 16 × 75 mm Pyrex screw-cap tubes and then evaporating to dryness at 70° under a nitrogen gas sweep in a heating block. Exactly 1.00 ml of 6 *N* HCl containing 50.0 nmoles of internal standard was then added to the dried samples. After being tightly capped with PTFE-lined screw caps, the tubes were hydrolyzed at 110° for 16 h and then dried at 70° in a heating block under a nitrogen gas flow. The dried hydrolysates were redissolved in 2.00 ml of doubly distilled water for CIE analysis, and 50 μl were placed on the CIE column for analysis.

Precision, recovery and accuracy

Initially the precision of the CIE chromatography and the instrumentation was established by the multi-analysis of a working standard solution. The precision of the method was then demonstrated by the repeated analysis of two normal pooled urine collections. Additionally, during the routine application of the method in analyses of urine from cancer patients, 10% of the samples were chosen at random for duplicate independent analyses.

Recovery experiments were routinely made by determining the initial level of polyamines in a random 10% of the samples and then spiking these samples with known amounts of all the polyamines and re-analysis by CIE.

Drying time and temperature

The effects of time and temperature of drying on the polyamines in hydrolysates were investigated. Twelve aliquots of a urine hydrolysate were prepared and divided into four sets of three each. The first set was taken just to dryness at 70°, the second set was taken to dryness at 70° and then allowed to remain in the heating block for an additional hour. The third set was taken just to dryness at 100° and then held in the heating block for an additional hour. At all times during the drying all of the hydrolysate aliquots were kept under a gentle stream of nitrogen. The dried hydrolysates were then taken through the remainder of the sample preparation procedure and analyzed.

Hydrolysis

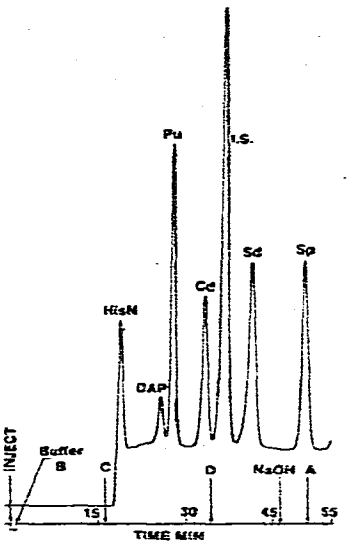
Ten replicate aliquots of a pooled normal urine collection were drawn and divided into two equal groups. One group was hydrolyzed at 110° for 16 h while the five other aliquots were hydrolyzed at 150° for 4 h. Sample preparation for both groups was then completed followed by CIE analysis of the polyamines.

In addition, a group of twelve 24-h urine collections from cancer patients were also used to evaluate the modified hydrolysis. Duplicate aliquots were drawn from each of the samples and one taken through the routine hydrolysis method (110° for 16 h) while the other was hydrolyzed at 150° for 4 h. Determination of the polyamines followed completion of the sample preparation.

RESULTS AND DISCUSSION

Fig. 2 shows the separation, resolution and order of elution of the various di- and polyamines at the nmole level. Marton et al. [12], in their analyses of cerebrospinal fluids, reported the virtual co-elution of histamine with cadaverine between putrescine and spermidine. In our method, histamine elutes well ahead of not only cadaverine, but putrescine as well, leaving cadaverine clearly resolved.

The sensitivity of analysis with the Beckman 121M instrumentation was demonstrated. Quantification at the ngram level for the calibration mixture of di- and polyamines was done at a recorder setting of 0.01 a.u.f.s. with negligible noise interference. Linearity of the ninhydrin color response was observed from 17 pmoles to 25 nmoles placed on the column.



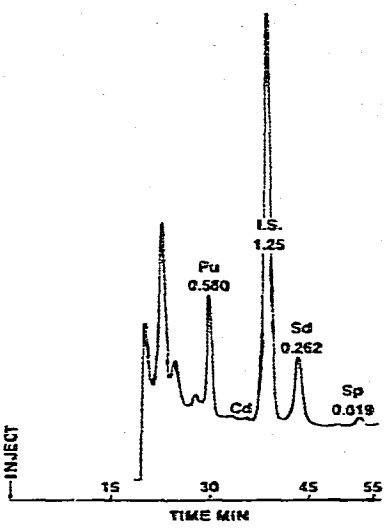


Fig. 2. CIE chromatogram of a calibration mixture of di- and polyamines. Conditions: injection, 50 μl; detection, 570 nm, 0.10 a.u.f.s.; column, 40 × 2.8 mm, packed with Beckman AA-20 resin, temperature, 65°; flow-rate, 8.8 ml/h; reaction, 10-in. coil, 2.8 min, 100°. Sample units: Pu, 1.25; Cd, 1.25; I.S., 1.25; Sd, 0.625; Sp, 0.418 nmoles.

Fig. 3. CIE chromatogram of polyamines in urine of breast cancer patient. Sample, 0.10 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

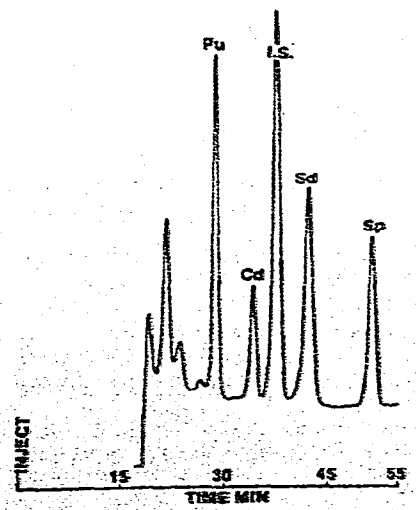


Fig. 4. CIE chromatogram of breast cancer patient urine spiked with polyamines. Sample 0.10 ml; further conditions as in Fig. 2.

COMPUTER REPORT FOR POLYAMINE ANALYSIS IN URINE

REPORT NO. 286 — 121M PA's ANAL.
CHAN# 6 METHOD: PA'S — SAMPLE: URINE
IST3 = 25.000,R NM/ML

RT	AREA	NM/ML	NAME
26.53	16508	3.142	DAP
27.89	161815	22.664	PU
32.62	15908	3.084	SD
36.24	426304		&IS
40.06	107073	6.289	SD
49.47	21850	1.098	SP

TOTAL AREA = 749.458

COMPUTER PROGRAM FOR POLYAMINE ANALYSIS

```
1.  CHAN, PROC, RPRT, RDVC
    6, ISTD, ME, TI
2.  SAMP, UNTS, TITLE
    URINE, NM/ML, 121M PA'S ANAL.
3.  #PKS, RTM, PRG
    25, 54.00, YES,
4.  MIN AR, MV/M, DLY, DVT, DIL-FTR%
    2000. .020. 22.0. 0.00. 100.00
5.  REF-RTW, %RTW, ID-LVL, RF-UNK
    .50, 1, 2000, 0.000
6.  # KAN PKS
    7
```

#	TIME	AMT		FACTOR		NAME
1	26.54	1.0000E	1	= 3.2457E	0	DAP
2	27.90	2.5000E	1	= 2.3883E	0	PU
3	32.61	2.5000E	1	= 3.3055E	0	SD
4	36.25	2.5000E	1	= 1.0000E	0	&IS
5	40.01	1.2500E	1	= 1.0015E	0	SD
6	49.42	8.3500E	0	= 8.5654E-	0	SP
7	60.00	1.0000E	0	= 0.0000E	0	#DUMMY

```
7.  # EVENTS
    5
```

	TIME,	EVENT	
1	24.00	B	Reset baseline at end of peak
2	26.20	B	
3	27.90	B	
4	44.00	I	Reset baseline
5	49.40	B	

Fig. 5. Computer report and program for polyamine analysis in urine. The parameters of the computer method for polyamine analysis are divided into seven categories.

(1) Calculation procedure: CHAN: channel assigned to this method (No. 6); PROC: calculation procedures (internal standard method); RPRT: report types (Medium type); RDVC: report device (Teleprinter No. 1).

(2) Sample identification: SAMP: sample description (urine); UNTS: units of concentration to be reported (nmoles/ml); TITLE: title of the method (Polyamine analysis Beckman 121M).

(3) Chromatography conditions: NO. OF PKS: the maximum number of peaks presented in the analysis (25 peaks); RTM: computer run time (54.0 min); PRG: chromatography program mode (stepwise program).

(4) Integration parameters: MIN AR: minimum area of a peak (2000 μV · sec); MV/M: slope threshold (0.02 mV/min); DLY: delay of integration (22.0 min); DVT: dead volume holding time (0.00 min); DIL-FTR%: %dilution (factor in 100%).

(5) Identification procedures: REF-RTW: reference peak (I.S. peak) retention time window (0.50 min), %RTW: retention time window for known peaks (1.0%); ID-LVL: identification-level of the known peaks (2000 μV · sec); RF-UNK: response factor for unknown peaks (0.00).

(6) Information for the identification and calculation of known peaks: TIME: absolute retention time of known peaks (min); AMT: concentration of known peaks (nmoles/ml); FACTOR: response factor of known peaks as 1/RWR,

$$\frac{1}{RWR} = \frac{\text{(Area of I.S.)}}{\text{(Area of compound)}} \times \frac{\text{Conc. of Compound}}{\text{Conc. of I.S.}}$$

NAME: name of known compounds.

(7) The integrator time events: B: force baseline next valley; I: force baseline immediately.

Fig. 3 is a chromatogram of a typical urine sample; in this case that of a diagnosed breast cancer patient. It is noted that potentially interfering diaminopropane has been adequately separated from putrescine to allow accurate analysis. Fig. 4 shows the same urine sample spiked with di- and polyamines prior to hydrolysis. A representative computer report and method for a urine sample analysis is presented in Figure 5. The computer method parameters are also defined and presented.

The CIE chromatograms of polyamines in blood plasma and sera are presented in Figures 6 and 7, respectively, and show quantitation at much lower levels of the di- and polyamines. 1,6-Diaminohexane commonly encountered in plasma and sera must be separated from spermine to allow for an accurate analysis. A chromatogram of spiked blood serum is presented in Fig. 8.

The in vitro culturing of bladder cancer cells in Minimum Essential Medium (MEM) yielded the chromatogram shown in Fig. 9. The supernatant of the cultured tumor cells was analyzed to determine the excreted polyamines.

The versatility of the method is once again demonstrated in the analyses of meat tissue extracts, typical chromatograms of which are presented in Figures 10 and 11. Both free and total di- and polyamine determinations were made on extracts of pork tissue. Relatively high levels of spermine are seen in the unhydrolyzed extract. The conjugated components of spermidine and spermine add significantly to their total in the hydrolyzed extract.

Excellent precision and accuracy for the chromatography and instrumentation were established by the ten replicate analyses of the working standard solution. The statistical parameters of the analyses are presented in Table IV.

The precision of the method was determined in two aspects. The sample matrix independent precision is presented in Table V in which five independent

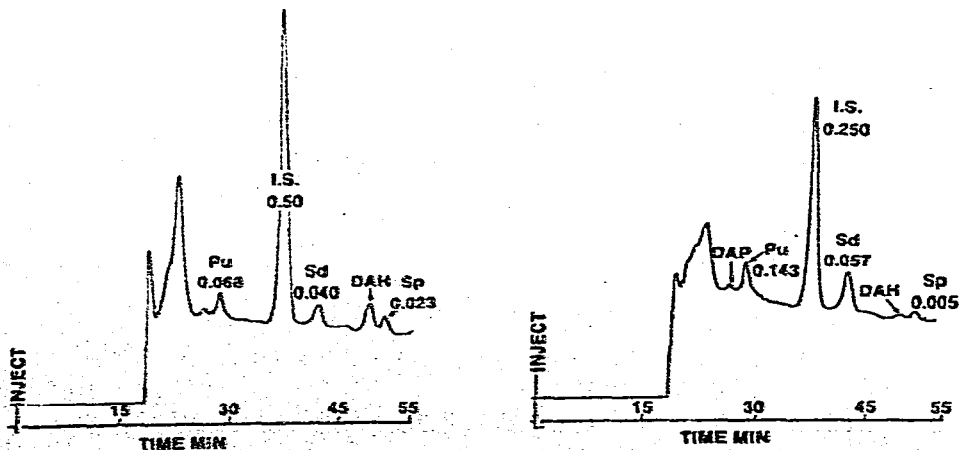


Fig. 6. CIE chromatogram of polyamines in pooled human plasma. Sample, 0.25 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

Fig. 7. CIE chromatogram of polyamines in pooled human serum. Sample, 0.25 ml; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

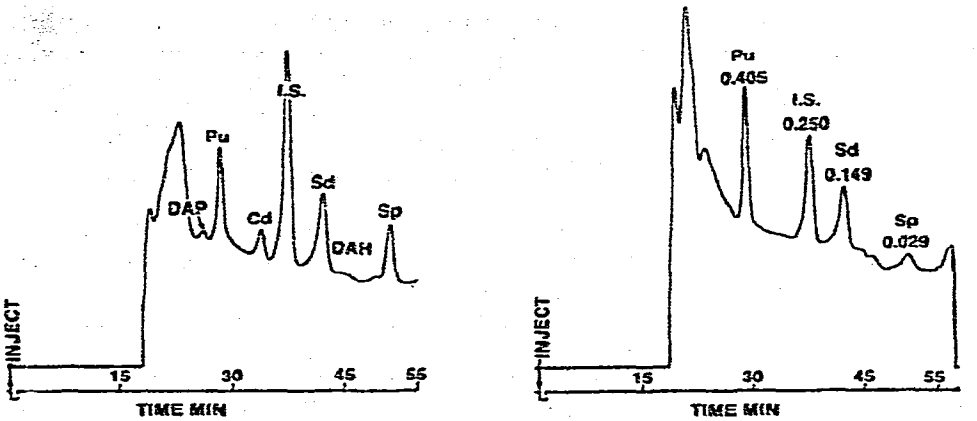


Fig. 8. CIE chromatogram of pooled human serum spiked with polyamines. Sample, 0.25 ml; further conditions as in Fig. 2.

Fig. 9. CIE chromatogram of polyamines in control media cultured with bladder cancer cells. Sample, 0.25 ml of MEM media; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

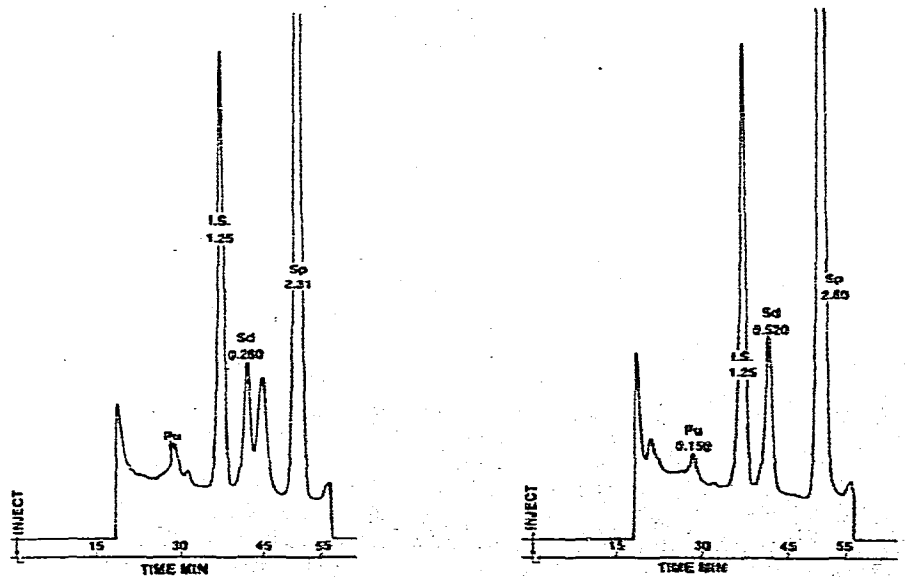


Fig. 10. CIE chromatogram of free polyamines in meat tissue extract. Sample, 25 mg; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

Fig. 11. CIE chromatogram of polyamines in hydrolyzed meat tissue extract. Sample, 25.0 mg; further conditions as in Fig. 2. The sample units at the peaks are given in nmoles.

TABLE IV

PRECISION OF RETENTION TIME AND RESPONSE (n = 10)

	Retention time (min)				Response (nmoles/ml)			
	Pu	Cd	Sd	Sp	Pu	Cd	Sd	Sp
$\bar{x}$	27.90	32.61	40.01	49.43	24.93	25.12	12.53	8.45
S.D.	0.008	0.010	0.004	0.010	0.144	0.244	0.035	0.070
R.S.D. (%)	0.03	0.03	0.02	0.02	0.58	0.90	0.28	0.80

TABLE V

PRECISION STUDY ON THE SAME AND DIFFERENT SAMPLES FOR POLYAMINES IN URINE

$\sigma = \sqrt{[\Sigma(x_1 - x_2)^2]/2P}$; R.S.D. (%) = $\sigma/\bar{x} \times 100$, P = number of pairs, $\bar{x}$ = mean for a population.

Sample 1 (n = 5)	nmoles/ml			
	Pu	Cd	Sd	Sp
$\bar{x}$	10.40	2.36	4.99	0.522
σ	0.092	0.057	0.068	0.010
R.S.D. (%)	0.88	2.4	1.4	1.9
Sample 2 (n= 5)				
$\bar{x}$	35.48	30.10	16.93	10.60
σ	0.430	0.202	0.169	0.114
R.S.D. (%)	1.2	0.67	1.0	1.1
Different samples (n = 21)				
$\bar{x}$	15.5	3.44	7.35	0.994
σ	0.806	0.113	0.407	0.078
R.S.D. (%)	5.2	3.3	5.5	7.8

TABLE VI

RECOVERY OF POLYAMINES FROM CANCER PATIENT URINE

Sample (n = 17)	Recovery (%)			
	Pu	Cd	Sd	Sp
$\bar{x}$	93.8	96.8	96.0	97.2
σ	6.46	4.29	5.11	5.55
R.S.D. (%)	6.89	4.43	5.32	5.71

analyses were made on the same sample on different days, and that for the sample matrix dependent precision for analyses on different samples in Table V. As we expected the sample matrix affected the precision of the method. The relative standard deviations for the sample matrix independent analyses ranged from 0.67 to 2.4%; whereas, the relative standard deviations for the sample matrix dependent analyses were from 3.3 to 7.8%.

Consistent recoveries of 94 to 97% were achieved in day to day routine operation (Table VI) with a relative standard deviation of 4 to 7%.

During the analyses of over 1000 urine samples less than 1% of the samples have shown interfering unknown peaks requiring manual correction. In these instances chemotherapeutic drugs or their metabolites are possibly the cause. A systematic study of the various cancer chemotherapeutic drugs and their effects on polyamine analysis might well be of value in identification of the interferences occasionally encountered in polyamine determinations.

Quantitation of putrescine and spermine in the 16 h, 110° cell culture media hydrolysate was difficult due to the elution of interfering peaks at nearly the same retention time. The interferences were successfully removed on hydrolysis for 40 h at 120 ± 1°. The stability of the polyamines under these rigorous conditions was verified by the recovery (89—107%) of all polyamines added to urine and processed as for samples.

Only a limited number of blood plasma and sera samples have been analyzed,

TABLE VII

THE EFFECT OF TIME AND TEMPERATURE ON DRYING URINE HYDROLYSATES

Three aliquots of a pooled urine control were evaporated at each of the conditions given.

Polyamine	nmoles/ml			
	Drying at 70° and 100°			
	70 *	70 + 1 h **	100° *	100° + 1 h **
Pu				
$\bar{x}$	10.50	10.80	10.73	11.14
σ	0.06	0.05	0.08	0.02
R.S.D. (%)	0.5	0.5	0.8	0.1
Cd				
$\bar{x}$	2.54	2.54	2.56	2.67
σ	0.11	0.06	0.06	0.13
R.S.D. (%)	4.2	2.6	2.3	4.9
Sd				
$\bar{x}$	5.15	4.98	5.12	5.10
σ	0.06	0.06	0.04	0.06
R.S.D. (%)	1.2	1.2	0.8	1.2
Sp				
$\bar{x}$	0.51	0.51	0.52	0.47
σ	0.04	0.04	0.03	0.04
R.S.D. (%)	8.8	8.8	5.8	8.8

*The samples were taken just to dryness at 70° and 100°.

**The samples were held at 70° and 100° for 1 h after dryness.

however, sensitivity and recoveries (96—105%) associated with the serum polyamine analyses appear superior to those obtained with other methodologies. Previous investigations [13, 14] employed a tedious *n*-butanol extraction of the serum hydrolysates with 50% recoveries reported. By elimination of the alcohol extraction step the method was greatly simplified and made more rapid, accurate, and reliable as well.

An extremely high spermine to putrescine ratio was observed in all the meat tissue extracts. This is a reversal of the spermine to putrescine ratio consistently found in urine. This observation may indicate different metabolic pathways of these two molecules.

The ruggedness of the analytical part of the method was demonstrated by drying of the hydrolysates at different time and temperatures (Table VII). No noticeable difference in the polyamine results was observed on drying at 70° or 100° and holding at either temperature for 1 h.

Roach and Gehrke [15] reported that the maximum yield for all of the protein amino acids was obtained on hydrolysis at 145 ± 2° for the minimal time of 4 h. An attempt was made to apply this rapid method to the hydrolysis of conjugated polyamines in urine [16].

Somewhat higher results were obtained on hydrolysis at 150°—4 h as compared to the commonly used 110°,—16 h (Table VIII). A systematic hydrolysis study is underway to determine the significance of hydrolysis time and temperature.

A program is underway on the use of a fluorescent detection system with the collaboration of Beckman Instrument Company to determine sensitivity and reliability by measurement at low levels.

Comments on the method

All glassware and containers used in the preparation of buffers and their storage must be maintained scrupulously clean and sterilized to avoid mold contamination.

To retard mold formation and maintain stability of standards and buffers, they should always be kept under refrigeration at 4°.

An alternative procedure for the preparation of the sodium citrate buffer solution is to prepare a 2.00 *N* stock solution which is then boiled for ca. 30 min for sterilization. This will prolong the mold-free lifetime of the buffers. Ten fold dilutions can then be made to prepare the other working buffer solutions.

Adjustments of the pH of the buffers should be made to within 0.03. This is especially important for buffer B which affects the separation of diaminopropane and putrescine.

The complete dissolution of the hydrindantin generally requires ca. 3 h of constant stirring under nitrogen at room temperature. Complete solubility can be verified by looking at the solution with a strong light during mixing.

Working standard solutions of the di- and polyamines should be freshly prepared every two weeks.

To assure accuracy and precision of this method, thoroughly mixed samples for homogeneity, accurate aliquots, and exact amounts of internal standard are critical.

TABLE VIII

THE EFFECT OF HYDROLYSIS TIME AND TEMPERATURE ON THE ANALYSIS OF POLYAMINES IN URINE

Urine	Concentration (nmoles/ml), hydrolysis temperature and time							
	Pu		Cd		Sd		Sp	
	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h	110°, 16 h	150°, 4 h
Normal, 1								
$\bar{x}$ ($n = 4$)	10.90	11.78	2.36	2.45	4.93	5.22	0.471	0.513
σ	0.13	0.20	0.06	0.15	0.11	0.06	0.02	0.04
R.S.D. (%)	1.2	1.7	2.6	6.2	2.2	1.1	4.1	7.1

If only limited volumes of cell culture media, blood plasma, or sera are available 2.00 ml can be analyzed, if the sample is reconstituted in 0.40 ml of buffer prior to analysis.

The 100 g of pork tissue cited in the method were necessary to obtain a representative sample. Routine polyamine analyses were made on 1.0 g of tissue.

To ensure accurate and precise analyses from day to day, a pooled, normal control sample with pre-determined polyamine levels was prepared and analyzed with each group of samples (ca. 50).

Internal standard solutions of 3,3'-diamino-dipropylamine containing 5.00 and 50.0 nmoles/ml were prepared on dilution of the stock solution. This solution is very stable under acidic conditions.

Time and temperature conditions for the drying of hydrolysates were found not to be critical.

Each day check all instrument controls for proper settings of flow-rate, temperature of reaction coil and chromatographic column, and column pressure.

In our laboratory the ion-exchange column has been used for more than one thousand analyses without increased pressure or deterioration.

CONCLUSIONS

An improved chromatographic method for the automated CIE analysis of the di- and polyamines, putrescine, cadaverine, spermidine, and spermine has been developed utilizing the highly sensitive and versatile Beckman Model 121M amino acid analyzer and ninhydrin detection. The unique chromatographic conditions and sample preparation enable the analysis of polyamines in a wide array of biological samples in less than 1 h per analysis. The precision, accuracy, and routine applicability of the method has been demonstrated. We believe this method provides the precise, accurate, and simple means of determining polyamines in biological fluids in support of research for bio-markers in cancer.

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