AN AUTOMATED MICROMETHOD FOR THE QUANTITATIVE ANALYSIS OF DI- AND POLYAMINES UTILIZING A SENSITIVE HIGH PRESSURE LIQUID CHROMATOGRAPHIC PROCEDURE

Laurence J. MARTON, Olle HEBY, and Charles B. WILSON
Naffziger Laboratories for Neurosurgical Research, Department of Neurological Surgery
University of California Medical Center, San Francisco, California 94143, U.S.A.

and

Patrick L. Y. LEE
Durrum Instrument Corporation, Palo Alto, California 94303, U.S.A.

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1. Introduction

With the increasing interest in the polyamines as they relate to the cellular events occurring in normal and neoplastic tissues, and to their potential use as marker substances for the presence of neoplasia, a sensitive, reproducible, and simple method for the determination of these compounds would represent an important technical advance.

The methods presently available have one or more of the following drawbacks: 1) lack of sensitivity; 2) interfering compounds; 3) tedious and time consuming sample preparation; and, 4) require lengthy analysis. The method to be described obviates these drawbacks.

2. Materials

2.1. *Instrumentation*

A Durrum D-500 (Durrum Instrument Corp.) amino acid analyzer was used with the standard 5 mm path length flow cell. All machine functions, including sample injection and calculations of peak areas, were accomplished by means of the built in PDP8/M computer (Digital Equipment Corp.). As many as 80 sample cartridges can be loaded for completely automated analysis.

2.2. *Resin*

Durrum DC-4A cation exchange resin (sulfonated polystyrene polymer with 8% cross linkage; bead diameter = 8.0 ± 0.5 μm) was packed to a height of 15 cm in a stainless steel 1.75 mm internal diameter column.

2.3. *Buffer*

A sodium citrate (0.90 M Na^+)–sodium chloride (1.75 M Na^+) mixture was prepared by dissolving 352.94 g of sodium citrate ·$2H_2O$, 409.08 g of NaCl, 20 ml of thiodiglycol, and 0.4 ml of liquefied phenol in distilled water to a final volume of 4 liters; concentrated HCl was then added to a final pH of 4.68 ± 0.02. The final Na^+ concentration was 2.65 M. The buffer was filtered through a Millipore filter (47 mm diameter, 0.45 μm pore size).

2.4. *Chemicals*

Ninhydrin reagent solution [1] (1.5 l dimethylsulfoxide, 0.5 l 4 M lithium acetate buffer, 40.0 g ninhydrin, and 1.250 g hydrindantin) (Pierce Chemical Co.) was obtained from Durrum Instrument Corp. Thiodiglycol was purchased from Pierce Chemical Co. Liquefied Phenol was purchased from Matheson, Coleman, and Bell. The hydrochlorides of putrescine, cadaverine, spermidine and spermine werd obtained from Calbiochem. ^{14}C-labeled di- and polyamines were obtained from New England Nuclear.

3. Methods

3.1. *Animals*

Four normal Fisher 344 male rats weighing 160–170 g were sacrificed and their brains, hearts, lungs, livers, spleens and kidneys were rapidly excised and frozen at −20°C. Four rats of the same sex, weight, and strain, that were bearing brain tumors transplanted from tissue culture [2] were sacrificed and their tumors excised and frozen.

3.2. *Extraction procedures*

Three spleens were each divided into three pieces which were homogenized with a Duall tissue grinder in 20 volumes of 4% (0.16 M) 5-sulfosalicylic acid, 0.20 M perchloric acid, and 10% (0.61 M) trichloroacetic acid, respectively. Following a 1 hr extraction at 0°C, the samples were centrifuged at 8000 *g* in an Eppendorf Micro Centrifuge for 10 min. The supernatants were used for analysis. The supernatant of the trichloroacetic acid extraction was divided into two aliquots, one of which was extracted 3 times with 3 volumes of diethyl ether to remove the excess trichloroacetic acid, the other was analyzed without diethyl ether extraction. The trichloroacetic acid-ether extraction procedure gave inaccurately higher values for the di- and polyamines than the other three procedures. For the remainder of the extractions we arbitrarily elected to use 5-sulfosalicylic acid.

3.3. *Amino acid analyzer parameters*

Elution of the di- and polyamines was accomplished in 35 min with the single 2.65 M Na^+ buffer, pH 4.68, at a flow rate of 18.5 ml/hr. The ninhydrin flow rate was 9.5 ml/hr. Column temperature was held isothermally at 66°C. Column pressure generated during analysis was approximately 2000 pounds per $inch^2$ gauge. Full scale deflection on the recorder was set at 0.1 O.D. units at 590 nm. Sample size was 50 μl.

Following each analysis the column was regenerated with a solution of 0.2 M NaOH, 0.67 mM Na_2EDTA, and 1.2 M NaCl, and then equilibrated with the 2.65 M Na^+ buffer.

* For the analysis of urinary polyamine levels, where less sensitivity is required and where a single buffer does not suffice for the separation of the polyamines from interfering substances, a 5 cm column was used with a two buffer system (0.20 M Na^+, pH 4.25, and 3.05 M Na^+, pH 4.68). The photometer was set at 1 O.D. full scale deflection.

4. Results and discussion

In order to analyze cellular polyamine levels in the small amounts of tissue that are obtainable from cell and tissue cultures, from tumors at early stages of growth, and from tumors of small size, such as experimental intracranial neoplasms, we have developed a method that allows for minimal sample handling, rapid analysis and high sensitivity.

The high sensitivity was achieved with a 0.1 O.D. full scale setting on the photometer. At this setting, fluctuations in the baseline resulted in malfunction of the integration parameters of the computer. Thus, it was not possible to utilize a multi-buffer system, in which baseline shifts occurred as a result of buffer changes, to achieve satisfactory separations and decreased analysis time*. Nevertheless, good separation was achieved using a 15 cm column and a single buffer.

Fig. 1 shows the separation of a mixture of 1 nmole each of putrescine, cadaverine, spermidine, and spermine. Their retention times were 6 min 36 sec,

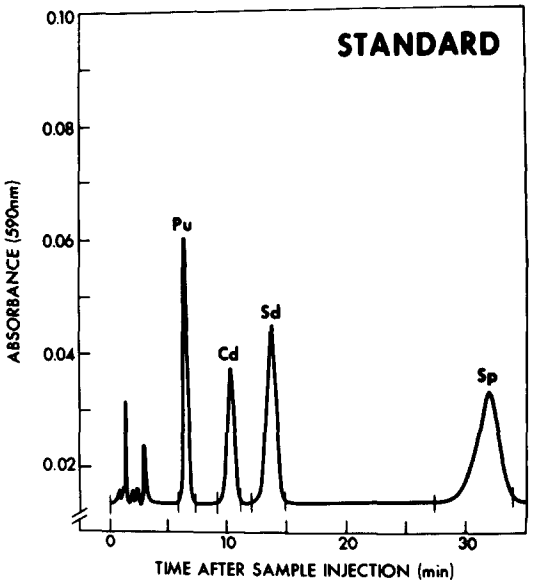

Fig. 1. Chromatogram of a standard solution containing 1 nmole each of putrescine (Pu), cadaverine (Cd), spermidine (Sd), and spermine (Sp).

Table 1
Coefficients of variation for standard analyses

Amount of each amine (pmoles)	Coefficient of variation (%)			
	Putrescine	Cadaverine	Spermidine	Spermine
1000	1.0	0.7	2.7	1.0
500	3.5	3.1	2.1	3.3
250	3.1	7.7	3.8	4.9
100	3.9	5.1	5.1	8.2
50	6.9	9.3	9.8	–
25	8.6	–	–	–

The coefficient of variation (S.D./mean, $n = 4$) was calculated from analyses of standard solutions containing all 4 amines at 25–1000 pmoles.

10 min 28 sec, 13 min 55 sec and 31 min 56 sec, respectively, with a coefficient of variation for each of only 0.3% ($n = 8$). Table 1 shows the coefficients of variation for the analyses of 25–1000 pmoles of mixtures of the amines. Better than 10% coefficient of variation was achieved down to 25 pmoles for putrescine, 50 pmoles for cadaverine and spermidine, and 100 pmoles for spermine. Analyses in the range studied (25–1000 pmoles) showed proportionality.

The use of trichloroacetic acid extraction followed by the removal of excess trichloroacetic acid by diethyl ether is one of the most frequently used methods for di- and polyamine extraction. Recently it has been reported that this method more efficiently extracts polyamines from tissues than the perchloric acid method [3]. We compared several of the known extraction procedures: 1) 0.20 M perchloric acid; 2) 4% (0.16 M) 5-sulfosalicylic acid; 3) 10% (0.61 M) trichloroacetic acid; and, 4) 10% (0.61 M) trichloroacetic acid followed by diethyl ether. Recovery data obtained by the addition of both cold and ^{14}C-labeled polyamines showed recovery rates of 96–102% for procedures 1–3, and of 116–119% for procedure 4. Table 2 shows that there are minimal differences among the first 3 procedures (these variations may be due to differences among the 3 portions of spleen). Procedure 4, however, results in higher putrescine, spermidine, and spermine values than those found with procedure 3, even though both procedures were carried out on the same tissue extracts. Probably ether extraction causes a concentration of the sample. We suggest the use of procedures that avoid ether extraction.

Quantitative recovery of di- and polyamines which were added at the time of homogenization, indicates

Table 2
Comparison of procedures for di- and polyamine extraction

Extraction procedure	Putrescine	Spermidine (nmoles/g, wet weight)	Spermine
Perchloric acid	34.6 ± 5.6	1175 ± 200	746 ± 97
Sulfosalicylic acid	28.8 ± 7.1	1221 ± 146	753 ± 53
Trichloroacetic acid	29.6 ± 4.9	1298 ± 173	803 ± 53
Trichloroacetic acid–ether	32.2 ± 7.0	1509 ± 221	959 ± 94

Rat spleens were homogenized in 20 vol of 0.20 M perchloric acid, 4% (0.16 M) 5-sulfosalicylic acid, and 10% (0.61 M) trichloroacetic acid, respectively, supernatants being used for analysis. Half of the trichloroacetic acid supernatant was extracted with diethyl ether to remove excess acid. Means ± S.D. of 3 spleens.

that there are no tissue factors that adversely effect the ninhydrin-color reaction. The identity of the putrescine, spermidine and spermine peaks were corroborated by gas-liquid chromatography and by thin-layer chromatography of the dansyl derivatives.

Table 3
Di- and polyamine concentrations in various rat organs

Organ	Putrescine	Spermidine	Spermine
	(nmoles/g, wet weight)		
Brain	9.6 ± 2.1	453 ± 24	255 ± 9
Heart	9.6 ± 1.8	302 ± 24	347 ± 16
Kidney	18.3 ± 3.7	562 ± 13	842 ± 8
Liver	9.0 ± 3.6	1041 ± 134	873 ± 45
Lung	36.4 ± 13.3	634 ± 106	357 ± 71
Spleen	29.6 ± 6.0	1262 ± 144	767 ± 52
Brain tumor	354 ± 102	771 ± 186	402 ± 68

Each organ was homogenized in 20 vol of 4% (0.16 M) 5-sulfosalicylic acid and the supernatants were used for the analyses. Means ± S.D. (n = 4).

Table 3 shows the concentrations of putrescine, spermidine, and spermine in various rat organs and in an experimental rat brain tumor. The spermidine and spermine values for the normal organs are within the range of those reported by other investigators [4, 5], however, the putrescine concentrations are significanlty lower [6–8]. A similar observation has been reported by others using methods applicable only to the assay of putrescine [9, 10]. In contrast to normal organs, the concentration of putrescine in the brain tumors was markedly elevated. This elevation was also seen when the same tumor was grown in the flank [11]. Fig. 2 shows comparative chromatograms of normal brain and of the experimental brain tumor.

Methods for di- and polyamine analysis have been the subject of several reviews [4, 12, 13]. Many of the methods listed in these reviews are specific only for a single di- or polyamine, and/or lack sensitivity and/or are extremely laborious. Table 4 lists a number of analytical procedures that are presently used for polyamine analysis and summarizes their relative merits.

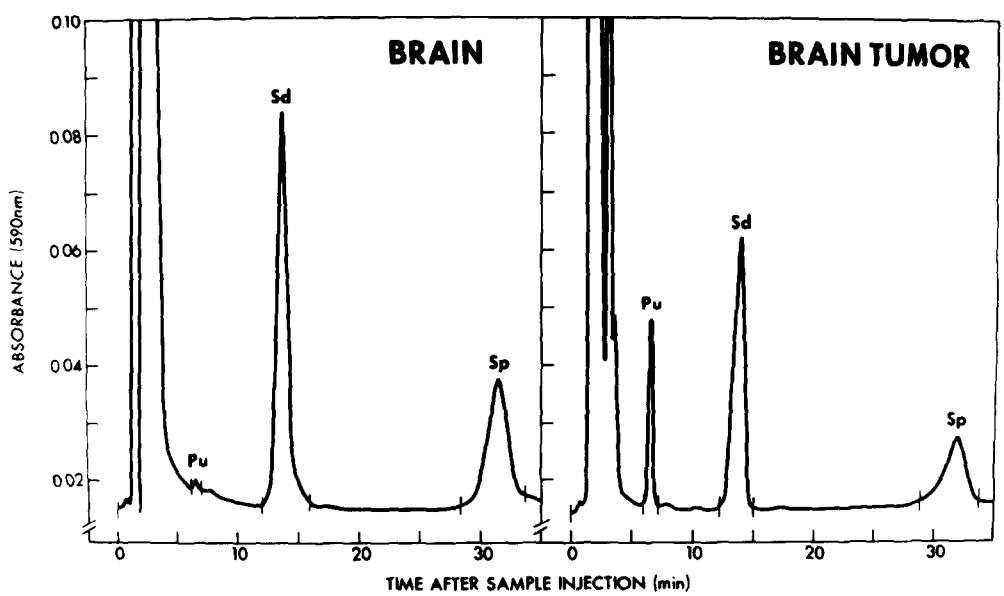

Fig. 2. Comparative chromatograms of 5-sulfosalicylic acid extracts of normal brain and brain tumor. The chromatogram of the normal brain represents 5 mg of tissue, and the chromatogram of the brain tumor represents 1.5 mg of tissue.

Table 4
Comparison of analytical procedures for the determination of putrescine, spermidine and spermine in biological materials

Analytical method	Derivative	Reagent	Length of procedure*	Sensitivity (pmoles)	Ref.
High pressure amino acid analyzer	–	Ninhydrin	Short	25– 100	(this study)
TLC-fluorometry	Dansyl	–	Long	25– 50	[12]
GLC-(flame ionization)	*N*-Trifluoroacetyl	–	Medium-Long	200– 600	[14, 15]
GLC-mass spectrometry	*N*-Trifluoroacetyl	–	Medium	200– 600	[16]
Conventional amino acid analyzer	–	Ninhydrin	Short	1000–2000	[17–20]
Paper electrophoresis spectrophotometry	–	Ninhydrin or Amido Black	Long	3000–5000	[6]

* Includes sample preparation, separation, and quantitation of putrescine, spermidine and spermine.

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