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MEASUREMENTS OF POLYAMINES AND THEIR ACETYLATED DERIVATIVES IN CELL EXTRACTS AND PHYSIOLOGICAL FLUIDS BY USE OF AN AMINO ACID ANALYZER

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SUMMARY

A fast and sensitive method for the determination of free polyamines and their acetylated derivatives is presented. The separation is carried out on a Durrum DC-6A cation-exchange resin with an automated amino acid analyzer. The determination is based on a stepwise elution with a sodium chloride—sodium citrate buffer system. Detection is done by fluorescence of the *o*-phthaldialdehyde—polyamine conjugates. The sensitivity is in the picomole range. No prior purification step is needed. The method has been applied to cell extracts and urine samples.

INTRODUCTION

The significance of polyamines in biochemical and physiological processes has stimulated the development of a large number of methods for their rapid and sensitive determination [1]. Ion-exchange column chromatography with automated instruments is one of the most suitable methods for routine analysis of the polyamines in cell extracts and body fluids. The methods described to date have been exclusively concerned with the separation of free polyamines [2, 3]. However, recently, evidence has been accumulating that points to the importance of acetyl derivatives of polyamines in cell metabolism. The monoacetyl derivative of putrescine has been found in the human brain [4] and in various other tissues [5]; spermidine is excreted in human urine, almost exclusively as acetylspermidine both in normals and cancer patients [6, 7].

A method of separating both the conjugates and the free polyamines has been described recently [8]. This method is based on pre-separation on Dowex resin, derivatisation by dansylation and subsequent thin-layer chromatography.

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For a rapid quantitative determination of the conjugates and the free polyamines no routine method is as yet available. We describe here the separation of free and conjugated polyamines by ion-exchange chromatography in a single step using the automatic amino acid analyzer. The application of this method to cell extracts and human urine is shown.

EXPERIMENTAL

Chemicals

Chemicals for buffers and reagents were obtained from Merck (Darmstadt, G.F.R.) and were of analytical grade quality. The non conjugated polyamine hydrochlorides were from Sigma (Munich, G.F.R.). Acetylputrescine and N^1-acetylspermidine were kindly donated by Dr. N. Seiler, Centre de Recherche Merell International (Strasbourg, France).

Sample preparations

Cell extracts were obtained from the gram-positive *Bacillus subtilis* or from the slime mould *Dictyostelium discoideum*.

B. subtilis, strain W 23, was grown in Tris-buffered glucose salt medium [9] to a density of 0.5 E_{578}. An aliquot of 5 ml from a 20-ml shaking culture was centrifuged without chilling and the cells were washed twice using 5 ml of distilled water in each case. Polyamines were twice extracted from the cells with 500 μl 10% trichloroacetic acid (TCA) for 1 h at 4°C with shaking.

The slime mould *Dictyostelium discoideum* was grown in axenic medium [10] to a density of $3 \cdot 10^6$ cells per ml. Cells were harvested by centrifugation, washed twice with buffer (2 m*M* Na_2HPO_4—14.7 m*M* KH_2PO_4, pH 6.7) and twice extracted for 1 h at 4°C with 100 μl of 10% TCA per ml cell suspension. One-milliliter samples of human urine were filtered through a Millipore filter (0.22 μm) and diluted with 500 μl 0.1 *N* hydrochloric acid, 250 μl ethanol and 250 μl buffer 1 (see Table I) specially adjusted to pH 1.9 (urine dilution A). A 100-μl aliquot of urine sample A was diluted further with 100 μl buffer 1 (urine dilution B). Various amounts of these urine samples were used for analysis.

TABLE I

COMPOSITION OF BUFFER AND CONDITIONS OF ELUTION

Number of buffer	Sodium chloride*	Sodium citrate dihydrate*	Ethanol (%)	Final pH	Temperature (°C)	Time (min) of programmed buffer change
1	—	0.04	5	2.90	73	35
2	0.2	0.3	5	6.20	68	20
3	0.9	0.3	5	5.55	68	22
4	3.0	0.3	5	5.55	68	29

*Molarity of Na^+.

Conditions of polyamine analysis

All analyses were run on a Biotronik LC 6000 E instrument. The 10 × 0.5 cm column was filled with Durrum DC-6A resin (11 μm) up to a bed height of 7.5 cm. Conditions of the analysis: temperature, elution time and the composition of eluting buffers were as indicated in Table I. The back pressure during a complete analysis did not exceed 43 kP/cm^2 in the buffer pump and 13 kP/cm^2 in the reagent pump. The flow-rates were 42 ml/h for the buffers and 28 ml/h for the reagent. Buffers were made with water that had been deionised, double distilled and cleaned through a LOBAR B column (Merck) or a Milli Q apparatus. The buffers were adjusted to a pH of about 0.5 units above the final value with 6 *N* hydrochloric acid. After filtration of the buffer through a Millipore filter (0.22 μm) the final pH was adjusted and the ethanol added.

The reagent was prepared by dissolving 400 μg of *o*-phthalaldehyde in 20 ml ethanol, and adding this solution to a nitrogen-saturated solution of 74.2 g boric acid, 60.0 g potassium hydroxide, 5 ml Brij 35 (30%, w/v) and 5 ml β-mercaptoethanol in 2 l water.

The column was regenerated with 0.6 *N* sodium hydroxide for 15 min and subsequently equilibrated with buffer 1 for 20 min. After each run the resin was allowed to expand for 5 min.

RESULTS

Standardisation of the procedure

Marton and Lee [3] have described the basic procedure for the separation of the non-conjugated polyamines by use of an amino acid analyzer. They have shown that the reaction product of polyamines with *o*-phthalaldehyde gives a linear absorption curve over a wide concentration range. The relationship between sample concentration and relative peak area is also linear for acetylputrescine and acetylspermidine in the 100—2000 pmol range.

The coefficients of variation (C.V.) of the integrated areas were calculated from five runs of a standard mixture of the free and the acetylated polyamines (500 pmol each) (Table II).

TABLE II

COEFFICIENTS OF VARIATION

In each case $n = 5$. A standard mixture containing 500 pmol each of the free and the acetylated polyamines was used for each run.

Peak*	Integrated area ($\bar{X}$)	S.D.	C.V. (%)
Ac-Put	252 033	8 265	3.2
Ac-Spd	244 895	3 934	1.6
Put	182 209	1 910	1.0
Spd	480 048	10 156	2.1
Sp	307 236	7 279	2.3

*For abbreviations, see legend to Fig. 1.

An integrator is coupled to the fluorimeter and quantifies the peaks. The integrator was calibrated every day with a standard mixture of 500 pmol of each polyamine.

Recovery was measured by adding 500 pmol of each polyamine to a test sample, and was found to be 92—105%.

From a standard solution containing 500 pmol each of free and acetylated polyamines, 3 nmol of an amino acid standard (Hamilton) containing 19 amino acids and about 10 nmol ammonia, all the polyamines and their derivatives can be separated (Fig. 1). From 5—10 pmol of spermidine and 15—20 pmol of the other polyamines up to 2000 pmol can be measured accurately.

Buffer 1 elutes the acidic and neutral amino acids in two peaks and separates the basic amino acids. The most important prerequisite is the sufficient separation of ammonia and acetylputrescine, since in human urine ammonia is in large excess over acetylputrescine. Buffer 2 elutes some basic amines, which have not been characterised further. Buffer 3 separates acetylspermidine, 1,3-diaminopropane and putrescine, whereas buffer 4 elutes spermidine and spermine.

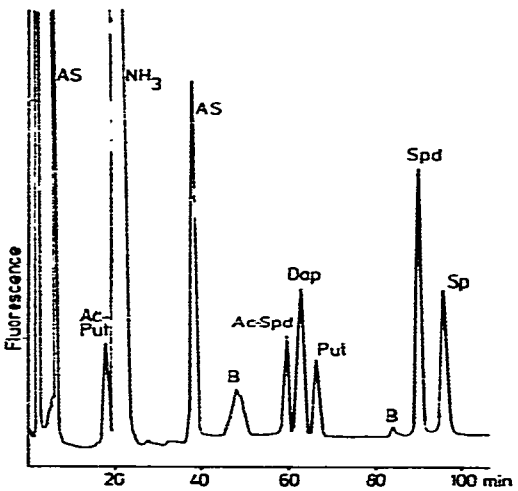


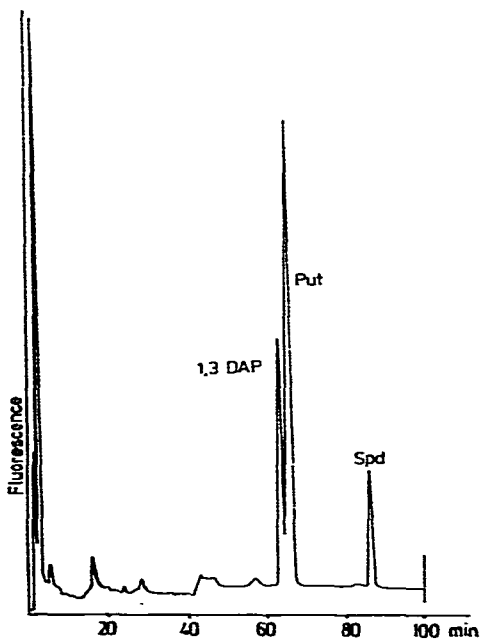
Fig. 1. Chromatogram of a standard mixture of polyamines (500 pmol each), amino-acid mixture (3 nmol) and ammonia (10 nmol). Peaks: AS = amino acid mixture; Ac-Put = monoacetylputrescine; NH_3 = ammonia; Ac-Spd = N^1-monoacetylspermidine; Dap = 1,3-diaminopropane; Put = putrescine; Spd = spermidine; Sp = spermine; B = peaks occurring occasionally after buffer change. (N^8-monoacetylspermidine has exactly the same retention time as N^1-monoacetylspermidine.)

Application of polyamine analysis

The polyamine content was estimated in cell extracts of *Bacillus subtilis*. The chromatographic profile of an extract of *B. subtilis*, grown in a minimal medium shows that spermidine synthesis predominates in these cells whereas putrescine is found only in trace amounts. Acetylated polyamines are not present in the extracts.

The slime mould *Dictyostelium discoideum* represents a good system for the study of cell development. The cells can be grown vegetatively in a maximal medium and development can be induced by starvation. The chromatogram of a sample of an extract of *Dictyostelium discoideum* is shown in Fig. 2. This lower eukaryote contains spermidine, putrescine and high amounts of 1,3-diaminopropane, but no spermine.

The analysis of free polyamines and their conjugates in human urine needs two separate runs. Because of the large excess of amino acids and ammonia, the simultaneous determination of acetylputrescine with all the other polyamines is not possible. In the first run we determined the concentration of acetylputrescine in a 30-min chromatogram with buffer 1 only. Normally a 10-μl sample of urine dilution B is sufficient to permit quantification and good separation from amino acids and ammonia (Fig. 3). The complete disappearance of the acetylputrescine peak after hydrolysis shows that no other compound interferes with this peak. The other polyamines were determined with 150 μl of urine dilution A; buffer 2 is used as the starting buffer (Fig. 4).



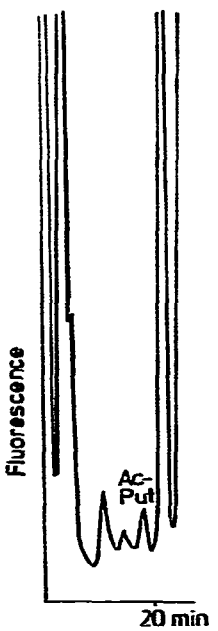


Fig. 2. Chromatogram of trichloroacetic acid extracted polyamines from *Dictyostelium discoideum* (20-μl sample). Peaks: 1,3 DAP = 1,3-diaminopropane; Put = putrescine; Spd = spermidine.

Fig. 3. Chromatogram of a sample of human urine (normal male individual); 10 μl urine dilution B to determine acetylputrescine (Ac-Put).

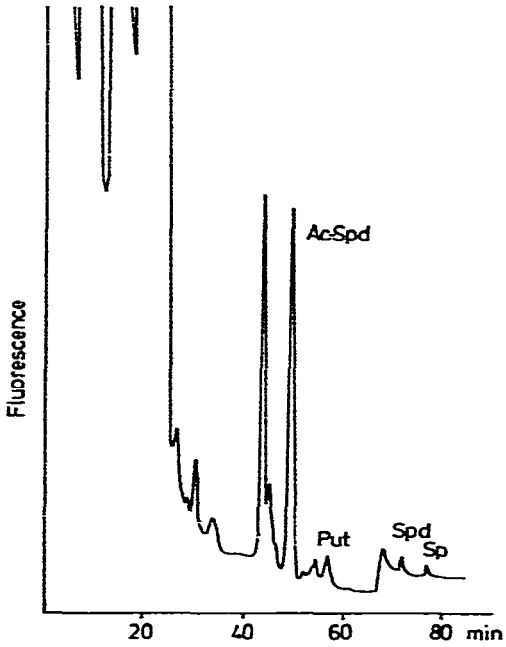


Fig. 4. Chromatogram of a sample of human urine, 150-μl sample of urine dilution A, buffer 2 is used as the starting buffer. Peaks: Ac-Spd = N^1-monoacetylspermidine; Put = putrescine; Spd = spermidine; Sp = spermine.

CONCLUSION

In the present work we describe a method which permits the separation and the quantitative determination of the free polyamines as well as their acetylated derivatives, on a Durrum DC-6A resin using an automatic amino acid analyzer. The advantages are that prior purification of the polyamines is not necessary. This prior purification step is often time-consuming and entails the risk of losing compounds present in trace amounts. Reproducibility and sensitivity of the method are excellent. The reason for the peaks occasionally occurring after buffer change has not yet be discovered — most probably they are due to contaminations in the buffer chemicals. The method is now being used in our laboratory to study the influence of polyamines on cell development. In a clinical study we are using it to investigate the excretion of polyamines in the urine of cancer patients undergoing chemotherapy.

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