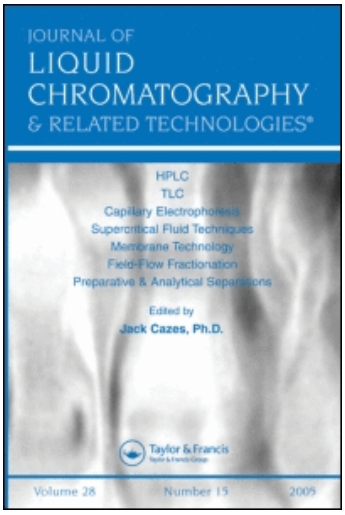

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AN IMPROVED HPLC METHOD FOR IDENTIFICATION AND QUANTITATION OF POLYAMINES AND RELATED COMPOUNDS AS BENZOYLATED DERIVATIVES

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ABSTRACT

A simple reversed phase HPLC method was developed for the determination of polyamines (putrescine, cadaverine, spermidine, spermine) and other polycations (n-acetyl putrescine, histamine, n-acetilspermine, agmatine), as their benzoylated derivatives. The advantages of this method, examined in various matrixes (hypothalamus, hypophyses, ovary, and seminal plasma), are its versatility, simplicity, and short time of determination of polyamines and related substances of importance in prokaryotes and eukaryotes, even in different matrixes, using a standard HPLC equipment (UV detection at 254 nm), with good resolution, sensitivity, and reproducibility.

INTRODUCTION

Polyamines are aliphatic polycations with widespread occurrence in eukaryotic and prokaryotic cells as well as in biological fluids.[1,2] It is accepted that they are implicated in a large variety of cell functions involving DNA repli-

cation, protein synthesis, and gene expression.[3,4] Thus, the determination of polyamines is necessary to clarify their role in normal as well as in pathological conditions. The increasing importance explains the numerous methods described during the last years for their quantitation.[4-16] Furthermore, the functions of each polyamine seem to be different, raising the necessity of having a method able to separate, identify, and quantify the polyamines and related compounds in a short analysis time. Some compounds have been related to polyamines because of their polycationic nature and degradation process (histamine) or their biosynthetic pathway (agmatine).

Previous studies have been conducted on TLC, but they use lengthy analyses and interferences were present. The HPLC analysis has solved this issue. Fluorescent detection have been chosen for many investigators because of its sensitivity, but benzoylation gave some advantages over dansylation (fluorescent detection): less secondary products of the reaction, detection by UV, a widely used detector, and that this method also works for agmatine.[12]

Previous works using benzoylated polyamines, used a short analysis time,[10-12] but they were often limited to a small number of polyamines. On the other hand, when more compounds were separated, the time required was longer and overlapping occurred frequently.[6-8]

In this work we describe an improved versatile and simple HPLC method for identification and quantitation of several compounds in different matrixes, with very good resolution, sensitivity, and reproducibility, in only thirty minutes.

EXPERIMENTAL

Chemicals

1,4-Diaminobutane dihydrochloride (Putrescine, Put), Spermidine trihydro-chloride (Spd), spermine tetra-hydrochloride (Spm) and agmatine sulphate (Agm) were supplied by RBI (Natick, MA, USA). 1,5 Diaminopentane (Cadaverine, Cad), Histamine dihydrochloride (Hist), N-acetil-putrescine (NaPut), N-acetyl-putrescine, N1-acetylspermine (NaSpm) and 1-6 diaminohexane were provided by Sigma (St. Louis, MO, USA). Chloroform and Benzoyl chloride were obtained from Carlos Erba (Milano, Italy). HPLC grade methanol, (JT Baker; Phillipsburg, NJ, USA) and water (eight time distilled) were filtered by 0.22 μm.

Equipment

Separation of benzoyl polyamines and related compounds was carried out with an HPLC Beckman (Berkeley, CA, USA), equipped with a Beckman pump

module 125, 20 μL Rheodyne loop injector (Cotati, CA, USA) and an Ultrasphere C18 column (250 mm X 4.6 mm I.D., 5 μm particle size) from Beckman. Detection of benzoyl polyamines was done using an UV Beckman detector, module 166, at 254 nm. The detector output was integrated and quantified on the System Gold (Beckman). All procedures were performed at room temperature.

Sample Preparation

Adult female Sprague Dawley rats were housed in an air-conditioned room with lights on at 07.00 hr. and off at 19.00 hr. and were given free access to laboratory chow and tap water. They were decapitated during morning hours and the brains quickly removed on ice. Trunk blood was collected, serum was separated and kept at –18°C. Hypothalami, including mediobasal hypothalamus and the suprachiasmatic-preoptic area were dissected as previously described;[17] *Pars distalis* of the adenohypophyses (neurohypophyses were discarded) and ovaries from the same rats were used. Tissues were kept at –70°C. They were homogenized in 4 % (v/v) perchloric acid (PCA, 10% v/w) in plastic Eppendorf tubes since polyamines bind to glass.[5]

After sonication, a way to release polyamines from protein aggregates,[9] samples were left overnight at 4°C, then centrifuged at 10.000 g, 20 min and supernatants separated for polyamine determination. The pellets were hydrolyzed in 3% PCA for DNA quantification as described by Burton et al.[18] Sperm were obtained from normal healthy adult volunteers.

Polyamine Derivatization Procedure

Benzoylation was done following the reaction of Schotten Baumann previously described for polyamines adapted in our laboratory.[5] Three hundred μL of 2N NaOH and 3 μL of benzoyl chloride were added to 100 μL of the supernatant mixed and incubated for 20 minutes at room temperature. The reaction was stopped by addition of 500 μL of a saturated solution of NaCl.

Polyamines were extracted in 500 μL of chloroform, since acetylated derivatives were poorly extracted by diethyl ether.[13] After centrifugation at 10000 g for 10 min., the chloroform phase was collected, evaporated to dryness, and redissolved in 100 μL of methanol 55%- H_20 45% (first mobile phase).

HPLC Analysis

The mobile phase was a mixture of methanol-water (55:45, v/v) with a resulting gradient 84%-16% at 23 min, then maintained isocratically until the

end of the 30 min elution. The flow rate was of 1.0 mL/min. Columns were allowed to stabilize for 10 min between samples.

RESULTS

Table 1 gives the different retention characteristics of eight polyamines found in biological tissues and fluids. A very good separation of all the polyamines was achieved (Figure 1), giving resolution values above acceptable limits (Rs >1.5). Aging of the column results in an increase in the width of the HPLC profile peaks; when the column was obstructed, with increase in pressure; values were delayed but resolution was not altered. This can be minimized by filtration or centrifugation of the sample.

Standard Curves

The calibration curves, absolute amount versus area under the curves, exhibited excellent linearity for all the polyamines (Table 2). The correlation coefficients were greater than 0.9988 for the concentration range investigated: 2, 10, 20, 200, 2000 pmol for Put, Cad and Spd, and 20, 100, 2000, 10000 pmol for the N acetylated compounds, Hist and Agm.

Table 1

Retention Time of the Different Polyamines*

Compound	Retention Time (min.)	Coefficients of Variation of the Retention Time (%)
N-acetyl putrescine	4.24	0.23
Putrescine	8.21	0.48
Cadaverine	9.06	0.38
N-acetyl spermine	9.93	0.21
Spermidine	12.26	0.15
Spermine	15.59	0.44
Histamine	17.04	0.43
Agmatine	26.52	0.14

* To determine these parameters at least 10 sets of individual standards were run.

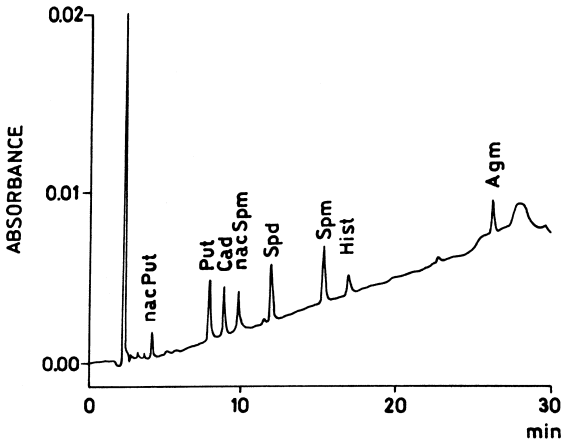

Figure 1. Chromatogram of 8 standards (for details see text).

Recovery and Precision

Recovery percentages of mixtures containing 800 pmol of each compound were quantified and recuperation was calculated with the formula:

Table 2

Linear Regression Parameters of a Set of Individual Standards*

Compound	Slope	Intercept	Correlation Coefficient
N-acetyl putrescine	585.89	19.47	0.99985
Putrescine	238.90	8.44	0.99932
Cadaverine	219.75	12.90	0.99931
N-acetyl spermine	252.81	13.23	0.99965
Spermidine	146.86	3.99	0.99991
Spermine	162.92	5.50	0.99973
Histamine	243.18	10.69	0.99948
Agmatine	306.29	8.00	0.99925

* Ranging from 10-2000 pmol for Put, Cad, Spd. Hist, andAgm, and 20-10000 pmol for the N-acetyl compounds.

$R = X_2 / X_1 \times 100$

X_1= weigh value. X_2= calculated value.

The procedure was repeated 3 times for 3 days and, in all cases, values were between 81% and 124 %.

Intra day coefficient of variation was calculated from 3 determinations of the mixture containing 800 pmoles of each compound. Inter day coefficient of variation were calculated from these results of 3 consecutive days (Table 3).

Matrix Effects

Quantification of standards, samples (hypothalami, adenohypophyses, ovary, and seminal fluid) or a mix of both (standards and samples), was done after derivatization (Table 4). When the sample values were subtracted of the standards plus sample value no differences in the standard determinations were observed due to the presence of the matrix (Figure 2).

Table 3

Relative Recovery from Nine Weighed Standards and Their Coefficients of Variation*

Compound	Recovery (%)	Intra-Day Mean CV %	Inter-Day Mean CV %
N-acetyl putrescine	85	2.3	3.7
Putrescine	111	2.5	2.7
Cadaverine	85	1.9	3.2
N-acetyl spermine	109	1.3	4.0
Spermidine	80	2.8	3.4
Spermine	103	1.4	3.3
Histamine	81	2.0	6.3
Agmatine	108	2.6	6.3

* Intra-day coefficients of variation were calculated from 3 determinations of a mixture containing 800 pmoles of each compound. Inter-day coefficients of variation were calculated from results of 3 consecutive days.

Table 4

Relative Recovery of Eight Polyamines from Four Different Matrixes*

Compound	Ovary	Adeno-Hypophisis	Hypothalamus	Seminal Fluid
N-acetyl putrescine	103	101	91	101
Putrescine	103	103	99	91
Cadaverine	111	97	104	101
N-acetyl spermine	107	104	87	100
Sperimidine	106	120	97	98
Spermine	114	115	89	130
Histamine	89	99	95	106
Agmatine	75	108	95	95

* Matrix value was subtracted from the value of matrix plus the added standard; divided by standard alone and multiplied by 100. Values were the average of three determinations.[27]

Selectivity

Addition of standards to matrixes does not change peak identification. Blank chromatogram (derivatization procedure over octadistilled water) shows no side reaction peaks in the region where a specific polyamine must appear.

Detection Limits

The detection limits, considered as the absolute amount that generated a signal 5 times the baseline were observed, in all cases, under the lowest curve points. They are 5 pmol for putrescine, cadaverine, spermine, and spermidine and 10 pmol for N acetylated compounds, histamine, and agmatine.

Internal Standard

The use of an internal standard is controversial when the procedure includes derivatization and extraction. Some authors favor this practice while others consider that it introduces imprecision.[19] In our system we used calibration curves (external standards), but we assayed an internal standard too. 1-6 Diaminohexane was adequate as a potential internal standard with a good differentiated retention time (10.9 min.) and regression parameters (X=306.04 x X-24.66. r=0.999).

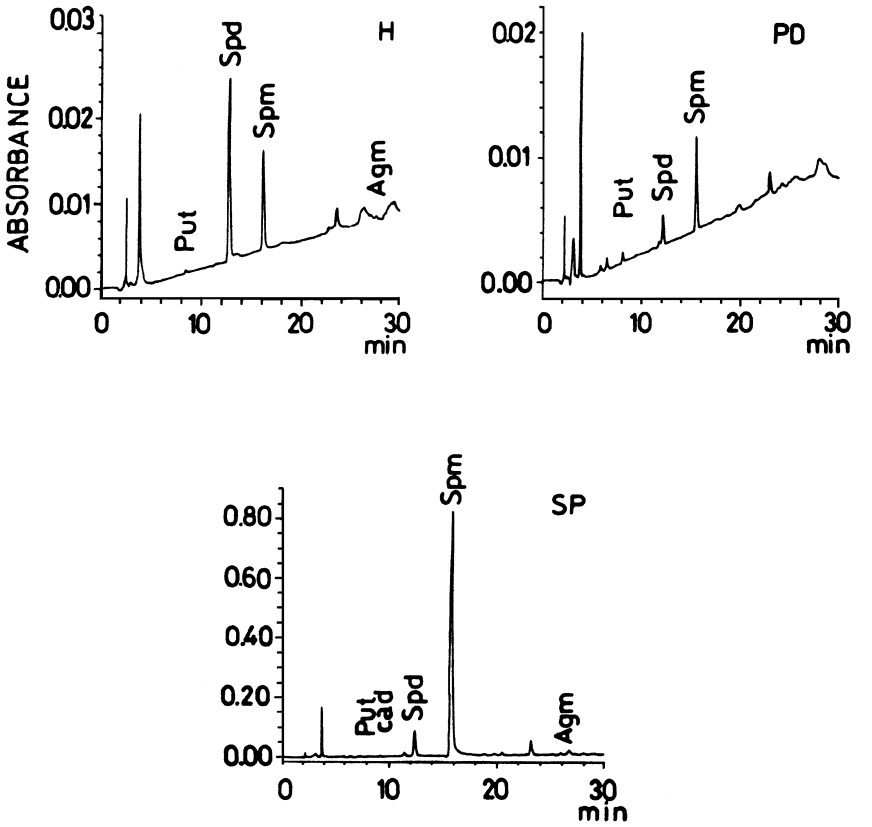

Figure 2. Chromatograms of H (Hypothalamus); pmol/whole tissue: Put: 93, Spd: 6615, Spm: 4145, Agm: 244), PD (*Pars distalis* of the adenohypophyses); pmol/whole tissue: Put: 109, Spd: 330, Spm: 1185 and SP (Seminal Plasm) nmol/mL: Put: 0.8, Cad: 0.9, Spd:1.4, Spm: 1616, Agm: 0.8.

DISCUSSION

Different HPLC methods for the determination of polyamines have been described, introducing improvements over previous methodologies. However, some of them required long analysis times, or only a small number of compounds were detected, mainly putrescine, spermidine, and spermine. In this work, a sensitive and reproducible HPLC method able to determine many polyamines and related substances in a short time is described. This is partic-

ularly important in view of the high number of compounds described, each of them with its own role in prokaryotes and eukaryotes. For example, agmatine first described in vegetables was found in mammals where it may act as a neuromodulator or neurotransmitter.[20] Agmatine could not be detected with methods using dansylation. Other techniques required a long time or detected only agmatine.[21] Histamine, although using different biosynthetic pathways, is also a polycation degraded by the diamine oxidase that can be determined simultaneously with our method. Simultaneous detection of polyamines and histamine is of importance in forensic medicine.[22] Furthermore, histamine has been related to inhibition of processes characteristic of polyamines, such as tumor growth and cell division.[23]

Determination of n-acetylderivatives could also be of importance in some medical studies and to determine precise metabolic pathways of polyamine.[24-26] All these compounds can be studied simultaneously with our technique and assayed in 3 different matrixes with good results.

In summary, the advantage of this method over others previously described, are versatility, simplicity, and short time determination of polyamines and related substances of importance, in different matrixes, using standard HPLC equipment, with good resolution, sensitivity, and reproducibility.

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