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Short Communication

Simple high-performance liquid chromatographic assay for polyamines and their monoacetyl derivatives

Gennaro Taibi* and Maria Rita Schiavo

Institute of Biological Chemistry, University of Palermo, Via Del Vespro 129, 90127 Palermo (Italy)

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ABSTRACT

A rapid reversed-phase high-performance liquid chromatographic method, using pre-column derivatization with benzoyl chloride and ultraviolet detection at 254 nm, was developed for the simultaneous measurement of polyamines and their monoacetyl derivatives. Calibration curves were linear for concentrations from 1.25 to 25 nmol/ml. The method was employed to assay these compounds in chick embryo retina explants using organic solvent extraction and 1,7-diaminoheptane as an internal standard. This simple and sensitive method can be applied to routine determinations of these compounds in various biological samples.

INTRODUCTION

Putrescine, spermidine and spermine, which are essential for normal growth and differentiation [1–3], are commonly present in significant amounts in both prokaryotic and eukaryotic cells. Acetylpolyamines are also present in the cells, although their biological function is still unclear [4]. It has been ascertained that the intracellular concentration of these acetylated forms vary greatly, according to the proliferative status of the cell and also in response to a wide range of stimuli [5–7]. High concentrations of polyamine acetyl derivatives have been found in malignant breast tissues, whereas they are not detectable in normal tissues [8]. N^1-Acetylspermidine has also been identified as an excretion product of cultured cells [9], where acetylation seems to be a prerequisite for excretion [10,11].

Seiler and co-workers [12–14] have demonstrated that, in several tissues, putrescine can be synthesized not only from ornithine but also by an alternative route involving spermidine and spermine. The latter pathway depends on a two-step mechanism: the first step is the conversion of spermine or spermidine into their N^1-acetyl derivatives, by spermine/spermidine N^1-acetyltransferase (N^1SAT), and the second is the oxidation of the monoacetylpolyamine through the action of a polyamine oxidase (PAO), that leads to the formation of spermidine or putrescine.

A rapid and sensitive assay for the simultaneous detection of polyamines and their conjugated forms is an important prerequisite to study the role of these metabolites in the regulation of cell proliferation and differentiation. This paper reports a new reversed-phase high-performance

* Corresponding author.

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liquid chromatographic (HPLC) procedure for the direct determination of natural polyamines and their acetylated derivatives extracted from chick embryo retina explants.

EXPERIMENTAL

Chemicals

Polyamines and their monoacetyl derivatives (hydrochloride forms) were purchased from Sigma Chimica (Milan, Italy). All commercial reagents (A-grade or for chromatographic use) were from Farmitalia Carlo Erba (Milan, Italy).

Instruments

A Gilson gradient analytical liquid chromatograph (Series 4000) equipped with a Rheodyne 7125 syringe-load injector (20-μl loop) and a variable-wavelength UV detector (Gilson Model 116) and controlled by Gilson 715 HPLC system controller software running on an IBM PS/2 computer was used for this study. Chromatographic separations were achieved using a Spherisorb ODS2 column (15 cm × 0.46 cm I.D.; 3 μm particle size) (Phase Separations, Deeside, UK).

Sample preparation

Chick embryo retinas were accurately separated from the pigment epithelium, washed and placed in pre-warmed culture medium at 37°C. Retinas were incubated at 37°C in 4 ml of serum- and glutamine-free Eagle's minimum essential medium (MEM) (pH 7.4) supplemented with 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES), penicillin (100 U/ml) and streptomycin (100 μg/ml). After incubation for 4 h, retinas were collected by centrifugation at 500 *g* for 1 min, resuspended in one volume of 0.2 *M* perchloric acid (20 mg wet weight per ml), containing 20 μ*M* 1,7-diaminoheptane as internal standard, and homogenized. Aliquots (100 μl) of the homogenate were always drawn up for protein determination [15]. Samples were sonicated at 10-s bursts (10 μm amplitude), in a Soniprep 150 (MSE), and centrifuged at 20 000 *g* for 15 min. The resulting supernatants were collected and stored in a polypropylene tube at −20°C.

In order to obtain the polyamine derivatives, samples were neutralized with potassium hydroxide, centrifuged to remove potassium perchlorate, and then submitted to benzoyl chloride derivatization using a partially modified version of the method of Redmond and Tseng [16].

Derivatization procedure

Samples (1.0 ml) were treated by addition of 2 *M* sodium hydroxide (1.0 ml) and 5 μl of benzoyl chloride. The mixture was briefly vortex-mixed and, after 20 min, saturated sodium chloride solution (2.0 ml) was added. The samples were then extracted with diethyl ether (2.0 ml) and centrifuged. The upper organic phase was withdrawn and washed with 0.1 m*M* sodium hydroxide (2.0 ml), in order to completely eliminate benzoylated amino acids and other water-soluble compounds. At this stage, a few milligrams of anhydrous sodium sulphate were added to the diethyl ether extract in order to eliminate residual water. After centrifugation, the ethereal phase was separated from the precipitate and then evaporated under a nitrogen stream. The remaining residue was dissolved in 2 ml of methanol solution (62%).

Chromatographic separation

Eluent components were degassed by bubbling with helium prior to use. Samples were filtered through a Millex-FG_{13} filter (0.2 μm pore size), and 20-μl aliquots were injected onto a 3-μm Spherisorb ODS2 column (15 cm × 0.4 cm I.D.). The polyamines and their acetylated derivatives were eluted with 62% methanol (v/v) (flow-rate 1.0 ml/min) at room temperature (*ca.* 20°C) and detected at 254 nm (detector sensitivity 0.01 a.u.f.s.).

Individual compounds were quantitated using calibration curves previously generated from standard solutions. Recoveries of single compounds were determined by the percentage recovery of the internal standard (see *Sample preparation*). Each determination was performed in triplicate.

RESULTS AND DISCUSSION

Several HPLC methods have been reported for polyamine determinations [17–23]. Most of them require a programmed gradient elution system, elution times between 20 and 50 min, thermostatted columns and post-column derivatization; this implies additional costs as well as a lengthy procedure.

Porta *et al.* [24], using the classical Schotten–Bauman benzoylation method, as described by Redmond and Tseng [16], separated the natural polyamines putrescine, cadaverine, spermidine and spermine but were unable to separate and measure their monoacetyl derivatives.

The technique described in this paper offers higher sensitivity as consequence of a reduction in column size (15 cm × 0.4 cm I.D.) as well as in particle diameter (3 μm). Concomitant with the use of a smaller column length and reduced flow-rates was the reduction in the total volume in which each component was eluted. For this reason the separation and quantitation of benzoylated polyamines and their monoacetylated forms was possible in our experiments.

The procedure was first tested using a mixture of the following standards: N-acetylputrescine, N-acetylcadaverine, N^1-acetylspermidine, putrescine, cadaverine, N^1-acetylspermine, spermidine, 1,7-diaminoheptane and spermine. A linear relationship was established between polyamine concentration and peak height in the concentration range 1.25–25 μM. For each concentration three samples were analysed and linear regression parameters (estimate intercept = b_0; estimate slope = b_1) and coefficients of correlation (r) were calculated. They were as follows: N-acetylputrescine, $b_0 = -0.26$, $b_1 = 307.69$, $r = 0.99$; N^1-acetylspermidine, $b_0 = -0.330$, $b_1 = 511.21$, $r = 0.99$; N^1-acetylspermine, $b_0 = -0.699$, $b_1 = 587.89$, $r = 0.99$. The same relationship was also ascertained between polyamine concentration and peak area (data not shown).

In order to establish the reproducibility of the method, a standard mixture of derivatized polyamines containing 20 nmol/ml was diluted 1:1, 1:3 and 1:7. For each dilution three samples were analysed. The same run was repeated for a period of three weeks, and intra- and inter-day coefficients of variation (C.V.) were calculated (Table I).

TABLE I

INTRA- AND INTER-DAY REPRODUCIBILITY OF HPLC ASSAY OF POLYAMINES AND THEIR MONOACETYLDERIVATIVES

An aliquot (0.1 ml) of a standard polyamine mixture (200 μM), after derivatization, was dissolved in 1 ml of methanol solution (62%). After this, the standard solution was diluted 1:1; 1:3 and 1:7. For each dilution, three samples were analysed. The same run was repeated for three weeks.

Compound	Intra-day C.V. (%)	Inter-day C.V. (%)
N-Acetylputrescine	2.93	4.35
N-Acetylcadaverine	2.75	3.96
N^1-Acetylspermidine	3.13	5.17
Putrescine	1.64	2.35
Cadaverine	0.95	2.15
N^1-Acetylspermine	2.18	4.42
Spermidine	1.42	3.31
1,7-Diaminoheptane	0.87	1.59
Spermine	1.51	2.60

The elution pattern of polyamines and their monoacetyl derivatives is shown in Fig. 1. Spermine was eluted from the column at 8.20 min and elution was usually stopped after 10 min. An additional 2 min was required, before the following analysis, for column and detector stabilization. Using this procedure we were also able to detect histamine when it was present in the samples; it was eluted after spermine, with a retention time of 10.35 min (data not shown). The retention times of various polyamines were reproducible (± 0.02 min).

The method has been used for the determination of acetylated and non-conjugated polyamines in chick embryo retina explants (seven days old). The intracellular concentrations and elution pattern of polyamines and their monoacetyl derivatives are reported in Table II and Fig. 2, respectively.

This method may be considered not fully satisfactory because of the overlap between N^1-ace-

TABLE II

POLYAMINE CONCENTRATIONS IN CHICK EMBRYO RETINAS

Retinas (seven days old) were treated as in Experimental. Results are the mean + S.D. of three separate experiments.

Compounds	Concentration (nmol/mg of protein)
N-Acetylputrescine	8.187 ± 0.51
N^1-Acetylspermidine	6.175 ± 0.46
Putrescine	6.287 ± 0.26
Spermidine	3.187 ± 0.11
Spermine	3.550 ± 0.21

tylspermidine and N^8-acetylspermidine (data not shown). However, it has been previously demonstrated that N^8-acetylspermidine is not a product of the metabolic interconversion of polyamines [6].

The advantages of our HPLC procedure are as follows: (1) the neutralized perchloric acid supernatant is benzoylated directly without any prepurification; (2) the entire HPLC run requires *ca.* 10 min for each sample; (3) no gradient elution is required; (4) the separation is achieved at room temperature.

This method proved to be efficient, reproducible, time-saving, cost-effective and simple; therefore it appears to be suitable for many routine applications.

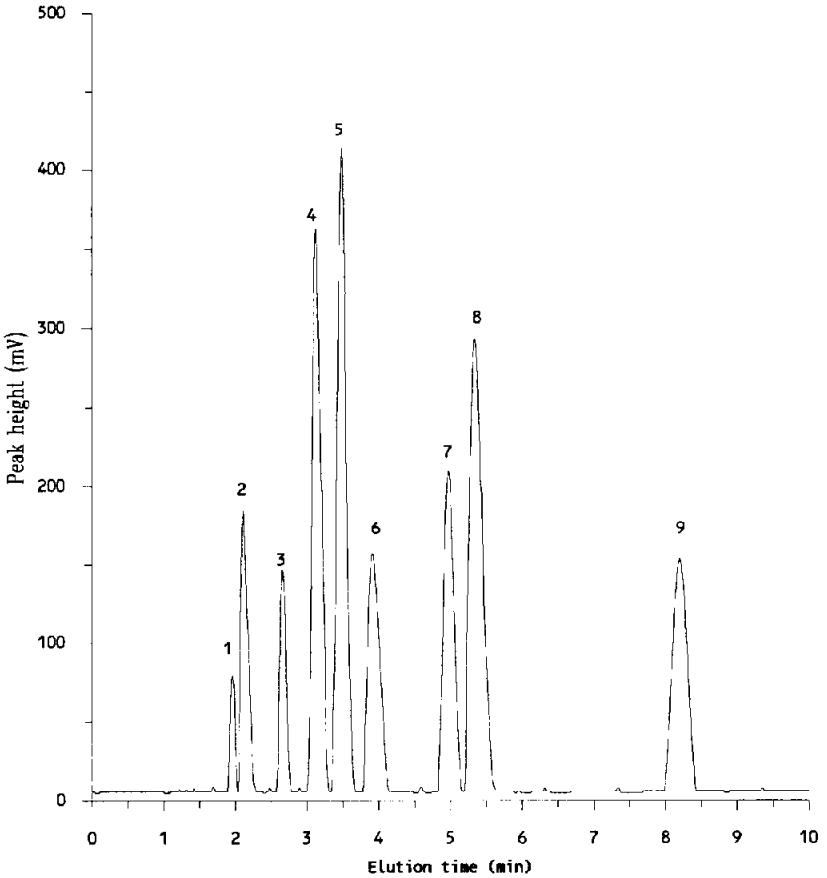

Fig.1. HPLC elution profile of benzoylated polyamines and their monoacetylated forms (standard mixture). Amount of each compound, 0.25 nmol per 0.020 ml. Peaks: 1 = N-acetylputrescine; 2 = N-acetylcadaverine; 3 = N^1-acetylspermidine; 4 = putrescine (1,4-diaminobutane); 5 = cadaverine (1,5-diaminopentane); 6 = N^1-acetylspermine; 7 = spermidine [N^1-(3-aminopropyl)putrescine]; 8 = 1,7-diaminoheptane (internal standard); 9 = spermine [N^1,N^4-bis(3-aminopropyl)-putrescine].

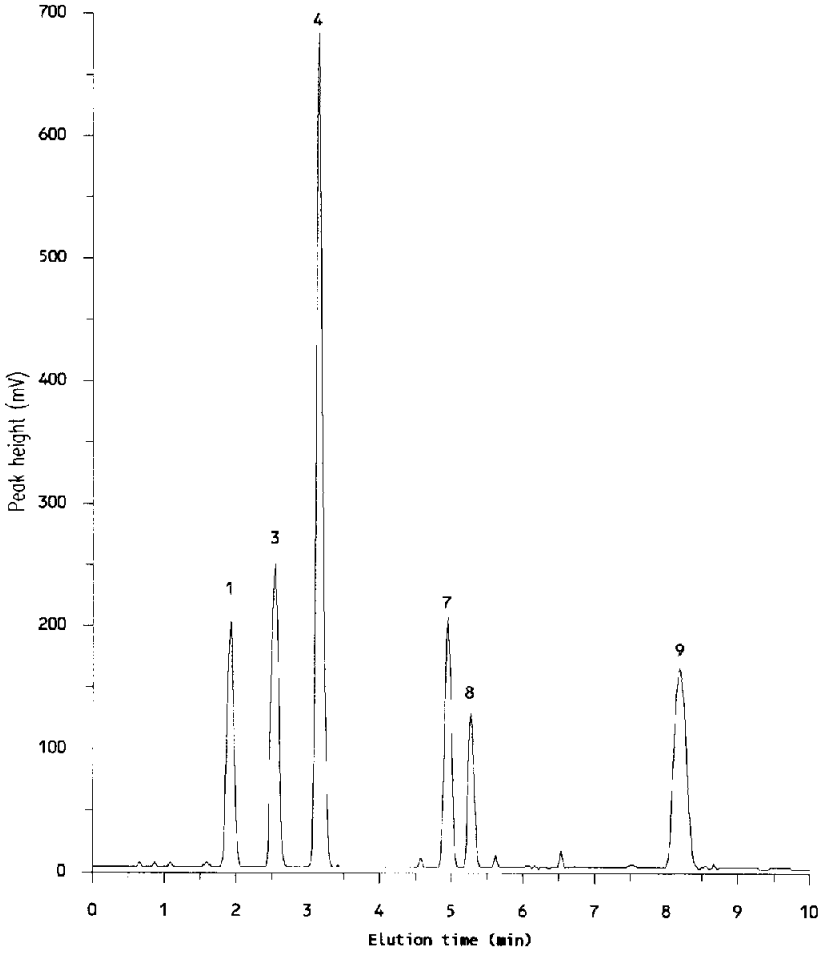

Fig. 2. HPLC elution profile of benzoylated polyamines in seven-day-old embryo retina explants. Polyamines were analysed as described in sample preparation. Peak numbering as in Fig. 1.

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