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Short communication

Rapid and simultaneous high-performance liquid chromatography assay of polyamines and monoacetylpolyamines in biological specimens

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Abstract

A rapid, resolutive and reproducible reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for polyamines and acetylpolyamines by adopting pre-column derivatization with benzoyl chloride. In a single run lasting less than 15 min ten polyamines were separated as well as traces of benzoic acid, methylbenzoate and benzoic anhydride. These contaminants, produced during the derivatization reaction, were almost all eliminated by washing steps envisaged in the same procedure. This simple and sensitive method can be applied to routine determination of polyamines in biological samples. A fine application of this procedure to the determination of endogenous content of polyamines in chick embryo retina was reported.

Keywords: Polyamines; Monoacetylpolyamines

1. Introduction

Polyamines are present in significant amounts in both prokaryotic and eukariotic cells. Their role in the regulation of main functions in cell growth and cell differentiation has drawn the attention of many researchers. Their involvement in DNA replication, gene expression, protein synthesis and cell surface receptor functions has been widely accepted [1–5]. Over-production of polyamines can be cytotoxic to cells [6] or, in the presence of negative growth signal, can induce apoptosis [7]. Acetylpolyamines are also present in cells and it has been ascertained that the intracellular concentration of these acetylated forms varies greatly, according to the proliferative status of the cell and also in response to a wide range of stimuli [8–10]. High concentrations of acetylpolyamines have been found in chick embryo retina proliferation and development [11,12] and in malignant breast tissues, whereas they are not detectable in normal tissues [13]. N^1-Acetylspermidine has also been identified as an excretion product of cultured cells [14], in which acetylation seems to be a prerequisite for excretion [15,16].

The availability of a sensitive assay for the simultaneous detection of polyamines and their conjugated forms is important to detect the fluctuations of the levels of these metabolites related to the regulation of cell proliferation and differentiation.

Several studies have been performed on the HPLC

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separation of derivatized polyamines but they were often related to classical polyamines (putrescine, cadaverine, spermidine and spermine) [17–20]. When more polyamines were separated, methods were developed for special application [21,22]. Only few methods were suitable for separation and quantitation of polyamines and acetylpolyamines in a single HPLC run [23,24].

Here we report a modification of our previous RP-HPLC procedure for the determination of the best-known natural polyamines and acetylpolyamines.

2. Experimental

2.1. Chemicals

Benzoic acid, benzoic anhydride, polyamines and their monoacetyl-derivatives (hydrochloride forms) were purchased from Sigma Chimica (Milan, Italy). All commercial reagents (A-grade or for chromatographic use) were supplied by Carlo Erba Reagenti (Milan, Italy). Fertilized Warren Breed chicken eggs were supplied by Centro Avicolo Mediterraneo of Palermo.

2.2. Instruments

A Gilson gradient analytical liquid chromatograph was used throughout this work. The HPLC apparatus consisted of two model 306 pumps, an 811B dynamic mixer, a model 234 auto injector equipped with 20 μl injection loop, and a 116 model variable-wavelength UV detector. Data acquisition and elaboration were performed by a UniPoint (v1.71) HPLC System Controller Software. The separation of polyamines was performed using a Waters Spherisorb C_{18} S3 ODS2 column (15×0.46 cm I.D.; 3 μm particles), protected by a guard column (3×0.46 cm I.D.; 5 μm particles) (Waters Corporation, Myles Standish Industrial Park, Tauton, MA 02780, USA).

2.3. Biological sample preparation

Eggs of uniform size and weight were placed in an automatic incubator (Victoria) at 38°C and 60% relative humidity. Chick embryos (10-day-old) were killed by decapitation, and their eyeballs bisected near the equatorial plane. Four neural retinas were accurately separated from the pigment epithelium and placed into a polypropylene tube containing sterile saline at 4°C. After centrifugation at 500 *g* for 5 min, the retinas were suspended in one volume of 1 *M* perchloric acid (80 mg wet weight/ml) containing 40 μ*M* 1,7-diaminoheptane as internal standard (I.S.). These samples were homogenized by sonication at 10 s bursts (10 μm amplitude), in a Soniprep 150 (MSE), centrifuged at 4°C (20 000 *g*) for 15 min and their supernatants were collected and stored in a polypropylene tube at −80°C.

2.4. Derivatization procedure

Samples containing 1–50 nmol of standard polyamines (1.0 ml) were treated with 2 *M* sodium hydroxide (1.0 ml) and 10 μl benzoyl chloride solution (benzoyl chloride–methanol, 1:1, v/v). The mixture was briefly vortex-mixed and, after 20 min, extracted with chloroform (2.0 ml). This solution was then vortex-mixed twice for 1 min, centrifuged at 2000 *g* for 5 min, after which the upper aqueous phase was discarded. The lower organic phase was vortex-mixed twice for 5 min with 0.1 *M* sodium hydroxide (2.0 ml). This treatment was repeated twice and allowed to transform about 50% of benzoic anhydride to benzoic acid. This latter is then drawn together with benzoylated amino acids and the other discarded water-soluble compounds. At this stage, 1.0 ml of the lower organic phase was withdrawn and evaporated to dryness under a stream of nitrogen. Before injection and HPLC analysis, this residue was dissolved in 0.5 ml of 60% methanol. Retina specimens were subjected to the same treatment.

2.5. Chromatographic separation

Prior to use, eluent components were degassed by bubbling with helium. Samples were filtered through a Millex-FG_{13} filter (0.2 μm pore size) and 20 μl aliquots injected onto a Waters Spherisorb S3 ODS2 column (15×0.46 cm I.D.; 3 μm). The mobile phase was a mixture of solvent A (water) and B (methanol). Polyamines and acetylpolyamines were eluted from 61% of B (0 to 3 min) to 67% of B (4.5 to 14 min) at room temperature. The gradient was then immediately returned to 61% of solvent B (14 to 15 min)

and the initial conditions restored in 5 min. The flow-rate was 0.8 ml/min and the detection was performed at 229 nm and 254 nm (detector sensitivity 0.01 a.u.f.s.).

Each polyamine was quantitated using calibration curves previously generated from standard solutions. Recovery of the single compound was determined by the percent recovery of the internal standard (see Section 2.3). Each determination was performed in quadruplicate.

2.6. Kinetic study on benzoic anhydride degradation

26.74 μmol of benzoic anhydride, corresponding to the concentration of benzoyl chloride added to a sample for polyamine derivatization (see Section 2.4), were solubilized in 1 ml of chloroform. Next, 2.0 ml of 0.1 *M* sodium hydroxide were added to the organic phase and the mixture was vortex-mixed for 5 min. This treatment was repeated three times. The organic phase was then evaporated to dryness under a stream of nitrogen, the residue was dissolved in 1 ml of methanol–water (60:40, v/v) and an aliquot was immediately analyzed. The remaining sample was incubated at 30°C and an aliquot was withdrawn every hour for 4 h and analyzed by RP-HPLC.

2.7. Peak identification of benzoic anhydride products

In order to check the retention time of benzoic acid and methylbenzoate, obtained from benzoic anhydride degradation, 7.541 mg of benzoic anhydride were dissolved in 1 ml of pure methanol. After stirring, 0.6 ml of this solution were added to 0.4 ml of deionized water, thereby reaching the final concentration of 20 μ*M*. After mixing, the solution was incubated at 30°C for 1 h and then analyzed by RP-HPLC. The retention times obtained were the same as those calculated with the standards.

3. Results and discussion

Most of HPLC methods that have been developed for polyamine determinations require a programmed gradient elution system, elution times between 20 and 50 min, thermostated columns and post-column derivatization [18–20]; this implies additional costs as well as a lengthy procedure. On the other hand, the HPLC method reported in an extensive review on polyamines [25] has some drawbacks such as high retention times and band broadening. We consider they are caused by the length of column which also determines an excessive sample dilution and inability to detect *N*-acetylputrescine and *N*-acetylcadaverine, since they feature a lower extinction coefficient than the other polyamines. Moreover, the sample washing with water does not seem to eliminate benzoyl chloride and benzoic anhydride remaining after derivatization.

In the method here reported, a two-step gradient elution (61% and 67% methanol in water) was performed instead of the isocratic elution with 62% methanol that we adopted in our previous method [23]. This modification allowed the perfect separation of ten polyamines, namely N^1-acetylputrescine, N^1-acetylcadaverine, N^1-acetylspermidine, putrescine, 1,3-diaminopropane, cadaverine, N^1-acetylspermine, spermidine, 1,7-diaminoheptane and spermine in very narrow towering peaks (Fig.1). With this eluent system, vicinal *N*-acetylputrescine and *N*-acetylcadaverine as well as putrescine, 1,3-diaminopropane and cadaverine were well resolved; furthermore, a net separation of the degradation products of benzoyl chloride, as benzoic acid, methylbenzoate and benzoic anhydride, was obtained. Their detection in traces in polyamine chromatogram was the result of accurate clean up of chloroform extract with NaOH that reduced the contaminant aliquot of benzoic anhydride favouring its conversion in benzoic acid, discarded in water phase. As reported by Verkolen et al. [26], the use of chloroform instead of diethyl ether [23] proved to be an additive device for a better recovery of the polyamine acetyl forms, *N*-acetylputrescine and *N*-acetylcadaverine in particular. In our previous study [23] a method of separation of nine polyamines was developed, with detection at 254 nm. The choice of the column (15×0.46 cm I.D., 3 μm particle diameter) concurred to achieving a high peak resolution and a short running time. This method received a negative evaluation by Schenkel et al. [27]. In their opinion, the chromatographic peaks of contaminant compounds, methylbenzoate and benzoic anhydride, would superimpose on N^1-acetylspermine and spermine. They also affirmed that our procedure was not

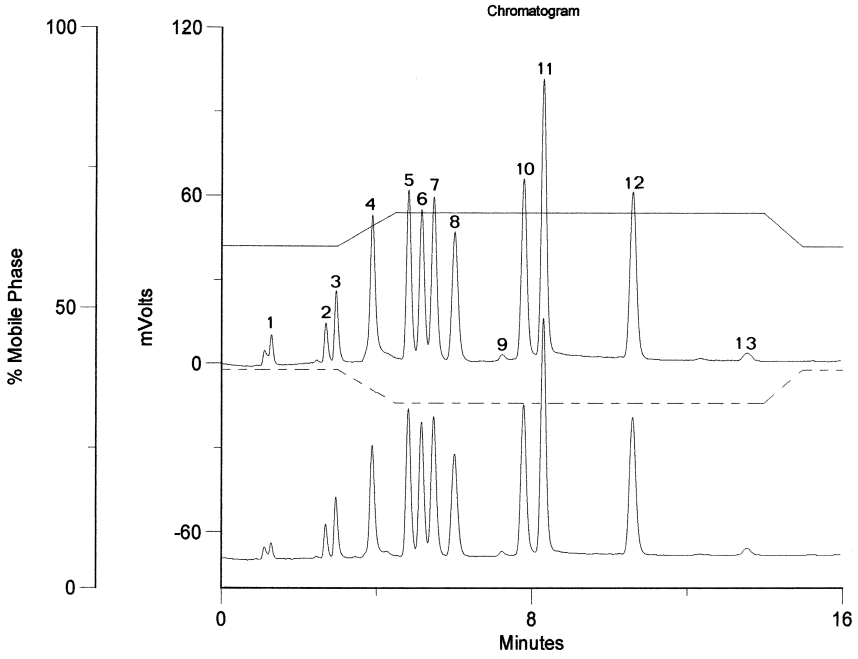

Fig. 1. HPLC elution profile of benzoylated polyamines and acetylpolyamines. Test mixture injected contained 250 pmol of each compound. Peaks: 1=benzoic acid; 2=*N*-acetylputrescine; 3=*N*-acetylcadaverine; 4=N^1-acetylspermidine; 5=putrescine (1,4-diaminobutane); 6=1,3-diaminopropane; 7=cadaverine (1,5-diaminopentane); 8=N^1-acetylspermine; 9=methylbenzoate; 10=spermidine [N^1-(3-aminopropyl)-putrescine]; 11=1,7-diaminoheptane; 12=spermine [N^1,N^4-bis(3-aminopropyl)-putrescine]; 13=benzoic anhydride. Eluent composition was reported as percentage of water (dashed line) and methanol (continous line). Detection was performed at 229 nm (upper chromatogram) and 254 nm (lower chromatogram).

suitable for the extraction of acetyl derivatives. It is our view that their remarks arise from analysis observations performed in different conditions of the chromatography system as column size (25×0.46 cm I.D., 5 μm particle size) and UV detection (234 nm). On this basis, their criticism [27] appears to be quite unfounded. Thus, in the RP-HPLC method here presented, kinetics of benzoic anhydride transformation and its products was studied to find a definitive solution to the problem of contaminants.

3.1. Analysis of benzoyl chloride products

Benzoyl chloride is decomposed by water to benzoic acid. Benzoyl chloride remaining after reaction with the aminated compounds reacts with benzoic acid to form benzoic anhydride. The latter is the only contaminant compound extractable by chloroform [27,28], since benzoic acid is insoluble in the organic phase. In order to reduce the amount of benzoic anhydride, the chloroform phase was washed with NaOH solution to enhance the transformation of benzoic anhydride to benzoic acid. As expected, the content of the residual benzoic anhydride in chloroform was halved by this treatment (data not shown). But, with suspension of the sample in methanol–water, benzoic anhydride reacts with water, forming benzoic acid (stoichiometric ratio 1:2) and, by reaction with methanol, benzoic acid and methylbenzoate; thus, there exist three contaminant products in the solution ready for injection. Their absorbance was measured at 229 nm and 254 nm (Fig. 2). The detection at 254 nm proved to be advantageous because the extinction coefficients of benzoic acid and methylbenzoate at this wavelength are very much lower than those of polyamines, so it is possible to single out contaminants in the chromatogram.

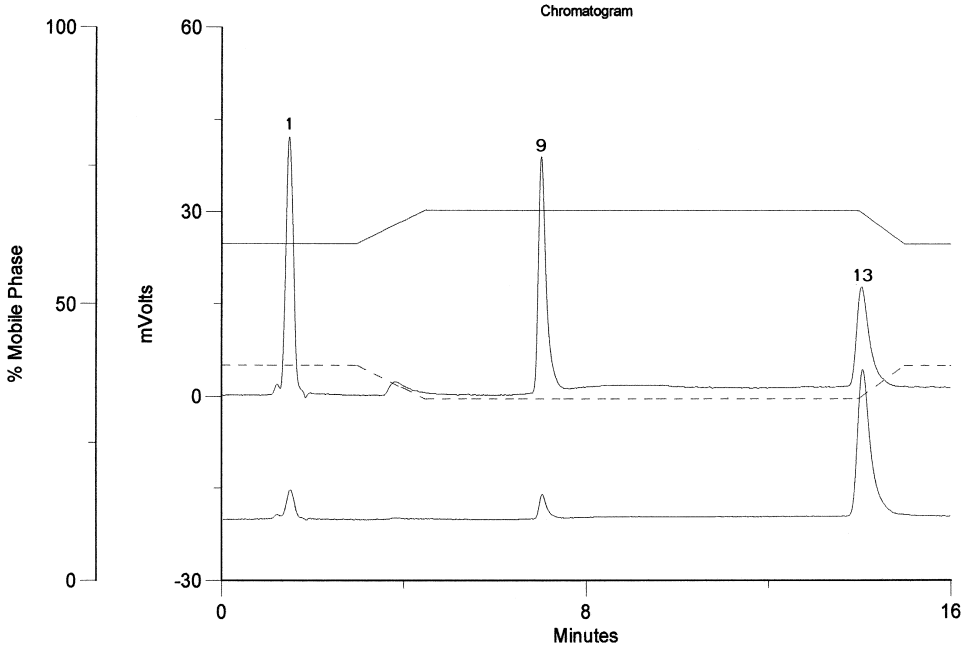

Fig. 2. Analysis of benzoic anhydride products. 20 μl of a blank sample of benzoic anhydride dissolved in methanol–water incubated for 1 h at 30°C were injected (see Section 2.7). Peaks: 1=benzoic acid; 9=methylbenzoate; 13=benzoic anhydride. Eluent composition was reported as percentage of water (dashed line) and methanol (continous line). Detection was performed at 229 nm (upper chromatogram) and 254 nm (lower chromatogram).

3.2. Statistic parameters for polyamine standard curves

The procedure was first checked using a mixture of the standards. A linear relationship was established between polyamine concentration and peak height in the concentration range 1.0–40 μ*M*. For each concentration four samples were analyzed and linear regression parameters (estimate intercept=b_0; estimate slope=b_1) and coefficients of correlation (r) were calculated. 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; 1:2; 1:4 and 1:8. For each dilution four samples were analyzed. The same run was repeated for a period of three weeks, and intra- and inter-day coefficients of variation (C.V.) were calculated. Data reported in Table 1 show that the calibration curves exhibited excellent linearity for all the polyamines. The correlation coefficients were greater than 0.9980 for the concentration range investigated (0.02–1 nmol). As shown in Fig. 1, spermine was eluted from the column at 10.2 min, but analysis was usually stopped at 15 min to permit the elution of benzoic anhydride, if present. An additional time of 5 min was required for column and detector stabilization.

3.3. Recovery of endogenous polyamines extracted from retina explants

The optimized method, here reported, has been used for the determination of acetylated and non conjugated polyamines in chick embryo retina explants. Five endogenous polyamines were identified and determined in 10-day-old chick embryo retinas: *N*-acetylputrescine, putrescine, cadaverine, spermidine and spermine (Fig. 3 and Table 2). The recoveries and the precision of the extraction were estimated from the peak height ratio of the poly-

Table 1
Linear regression parameters and intra-day and inter-day mean coefficients of variation for HPLC assay of polyamines and their monoacetylderivatives[a]

Compound	Slope (mean±SD)	Intercept (mean±SD)	Correlation coefficient (mean±SD)	Intra-day mean C.V. (%)	Inter-day mean C.V. (%)
N-Acetylputrescine	0.005±0.0004	−0.07±0.04	0.9980±0.0003	2.93	5.35
N-Acetylcadaverine	0.007±0.0006	0.014±0.008	0.9997±0.0006	2.75	4.96
N^1-Acetylspermidine	0.011±0.001	0.11±0.02	0.9996±0.0008	3.13	6.17
Putrescine	0.03±0.003	0.085±0.02	0.9992±0.0002	1.64	3.35
1,3-Diaminopropane	0.012±0.002	−0.011±0.01	0.9994±0.0003	2.8	4.6
Cadaverine	0.019±0.002	−0.052±0.01	0.9999±0.0005	1.25	3.15
N^1-Acetylspermine	0.012±0.001	0.012±0.004	0.9996±0.0003	2.18	5.42
Spermidine	0.017±0.002	0.001±0.001	0.9999±0.0004	1.42	4.31
Spermine	0.017±0.003	0.063±0.01	0.9998±0.0005	1.51	3.60

[a] Intra-day C.V. was calculated from four different concentrations of standard polyamine mixture. For each concentration four samples were analysed. The same run was repeated for a period of three weeks, and inter-day C.V. was calculated.

amine to the I.S. (1,7-diaminoheptane). The results obtained are consistent with those reported in our previous paper [23]; only *N*-acetylputrescine content is higher than previous data because of the chloroform-extraction procedure.

4. Conclusion

A sensitive and reproducible RP-HPLC method has been developed to determine ten polyamines in a single run. The analysis method of polyamines and

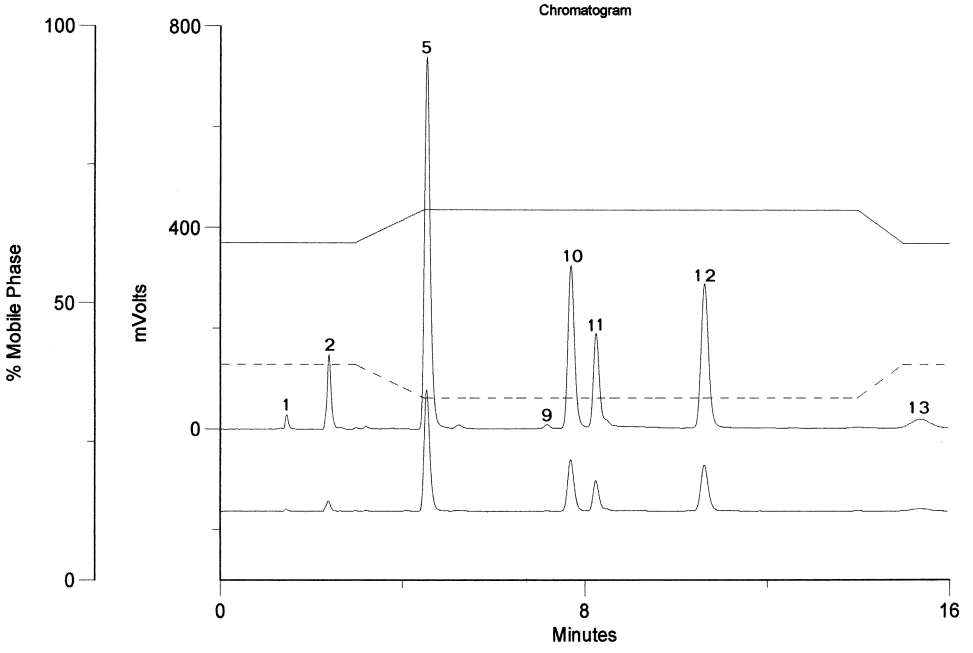

Fig. 3. Representative HPLC elution profile of benzoylated polyamines in a 10-day-old chick embryo retina. A volume of 20 μl of retina extract (1.5 mg of wet tissue) was analyzed. Eluent composition was reported as percentage of water (dashed line) and methanol (continous line). Detection was performed at 229 nm (upper chromatogram) and 254 nm (lower chromatogram). Peaks of polyamines were numbered as in Fig. 1.

Table 2
Polyamine contents in 10-day-old chick embryo retina ($n=5$)

Compound	Concentration (mean±SD) (nmol/mg wet weight)
N-Acetylputrescine	0.9±0.12
Putrescine	0.39±0.03
Cadaverine	0.008±0.002
Spermidine	0.23±0.03
Spermine	0.19±0.04

N-acetylpolyamines here reported is an improved procedure on the one reported in our previous paper [23]. The selectivity of this method was considerably improved by a simple extraction, clean up and double detection of derivatized polyamine solution. The differences in the procedure now adopted consist in the use of chloroform in the extraction procedure instead of diethyl ether, resulting in a better recovery of the acetylated forms of polyamines [26], *N*-acetylputrescine and *N*-acetylcadaverine especially. However, not less important was the choice of the two steps gradient elution procedure permitting a better resolution of benzoic acid, *N*-acetylputrescine and *N*-acetylcadaverine as well as putrescine, 1,3-diaminopropane and cadaverine. All these simple experimental devices concurred to a perfect separation of polyamines and acetylated polyamines in standard samples and in biological specimens.

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