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Journal of Chromatography A, 921 (2001) 323–329

JOURNAL OF CHROMATOGRAPHY A

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Micellar electrokinetic chromatography of polyamines and monoacetylpolyamines

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Received 7 June 2000; received in revised form 19 January 2001; accepted 6 April 2001

Abstract

A selective procedure for qualitative and quantitative analysis of ten polyamines by micellar electrokinetic chromatography (MEKC) was developed. Benzoylated polyamines and acetylpolyamines in micellar phase of SDS (10 m*M*) were separated at 25°C by 20 m*M* borate buffer pH 8.5, containing 8% ethanol, with an applied voltage of 25 kV (5 μA) and then detected at 198 nm. The experimental factors and operational parameters were optimized by performing analysis at different surfactant concentrations, pH, voltage and temperature with and without ethanol. The repeatibility of migration times and peak heights is a peculiarity of the method here described.

Keywords: Micellar electrokinetic chromatography; Polyamines; Monoacetylpolyamines

1. Introduction

Polyamines are important cellular components able to regulate many biological functions [1–4]. No less important is the biological role of monoacetylpolyamines whose concentrations change significantly in response to various cellular signals [5–8]. Although many studies have been performed on polyamines, methods for their separation are largely confined to high-performance liquid chromatography and thin-layer chromatography [9–14]. HPLC methods for polyamine analysis are reported in an extensive review [15] but only a few methods were suitable for separation and quantitation of polyamines and acetylpolyamines in a single HPLC run [16,17]. Capillary zone electrophoresis (CZE) with indirect photometric detection has proved to be a powerful technique, although only the separation of putrescine, spermidine and spermine has been performed to date [18–20]. In this work, using uncoated capillary column, we set up a fine MEKC method that permits the separation of benzoylated polyamines and *N*-acetylpolyamines as SDS micelles.

2. Experimental

2.1. Chemicals

Sodium tetraborate decahydrate, boric acid, sodium dodecyl sulfate (SDS), SDS washing solution, benzyl alcohol, benzoic acid, benzoic anhydride, polyamines and acetylpolyamines (hydrochloride forms) were purchased from Sigma Aldrich s.r.l. (Milan, Italy). All commercial reagents (A-grade or for chromatographic use) were supplied by Carlo

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00861-5

Erba Reagenti (Milan, Italy). Deionized water was prepared with a Milli-Q system (Millipore S.p.A. 20090, Vimodrone, Milan, Italy).

2.2. Instruments

A model 270A-HT High Throughput Capillary Electrophoresis System (Applied Biosystem) equipped with a HETO cooling bath was used. Data acquisition and elaboration were performed by a UniPoint (v1.71) HPLC System Controller Software. A positive voltage was applied to the capillary by keeping the injection end at a positive potential, while the cathodic end was held at ground potential. Capillary column (Thermo Quest Italia) was 100 cm (60 cm to detection system)×50 μm I.D. fused-silica. The polyimide coating was burned off 40 cm from the cathodic end of the capillary to form the detection window.

2.3. Column treatment

A new capillary column was conditioned by washing the interior surface for 30 min with 1 *M* sodium hydroxide and then with 0.1 *M* sodium hydroxide for 10 min, followed by washes with deionized water and running buffer for 10 min. After every four runs the capillary was washed with SDS washing solution (5 min), 0.1 *M* sodium hydroxide (5 min), deionized water (5 min) and running buffer (5 min).

2.4. Buffer solutions

Sodium tetraborate decahydrate (19.07 g) and boric acid (6.18 g) were dissolved in deionized boiling water and volumes were brought to 100 ml obtaining a 0.5 *M* sodium tetraborate decahydrate solution (*sol. A*) and a 1.0 *M* boric acid solution (*sol. B*).

2.4.1. Borate buffer solutions (sol. a–e)

Borate buffers solutions in the pH range 8–9 were prepared from sodium tetraborate decahydrate (0.5 *M*) and boric acid (1.0 *M*) solutions and mixed as shown in Table 1.

These volumes were completed at 100 ml with water to obtain 200 m*M* borate buffer solutions.

Table 1
Borate buffer composition

pH	Buffer solution	Solution *A* ml	Solution *B* ml
8.0	*a*	2.18	18.91
8.2	*b*	3.34	18.33
8.5	*c*	6.16	16.92
8.7	*d*	8.96	15.52
9.0	*e*	14.61	12.69

2.4.2. SDS solution (sol. 2)

Sodium dodecyl sulfate (57.68 g) was dissolved in 1 l of deionized water by stirring for 10 min at 30°C and volume brought to 2 l. The final concentration of SDS solution was 100 m*M*.

2.4.3. Resuspension buffers

They were prepared by using 10 ml of the *a–e* buffer solutions (pH range of 8–9), 30 ml of *sol. 2* and 40 ml of deionized water; after stirring for 5 min volumes were brought to 100 ml. The final concentration of resuspension buffers was 20 m*M* sodium tetraborate–boric acid and 30 m*M* SDS.

2.4.4. Running buffers

A total of 10 ml of *a–e* buffer solutions were added to 10–60 ml of *sol. 2* and after stirring volumes were brought to 100 ml with deionized water. The concentration of sodium tetraborate–boric acid was 20 m*M*, that of SDS was variable from 10 to 60 m*M*.

The preparation of running buffers with ethanol was performed by adding 5, 8, 10 or 15 ml of absolute ethanol to 10 ml of *c* solution (pH 8.5). After addition of 10 ml of *sol. 2*, volumes were brought to 100 ml. Buffers were prepared and degassed immediately prior to use.

2.5. Derivatization procedure

Mixture containing 0.5–50 nmoles of standard polyamines (1.0 ml) was treated with 2 *M* sodium hydroxide (1.0 ml) and 10 μl benzoyl chloride in methanol (1:1, v/v). It 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 to facilitate the transformation of benzoic anhydride to benzoic acid [17], the latter was then drawn together with benzoylated amino acids and other discarded water–soluble compounds. At this stage, 1.0 ml of the lower organic phase was withdrawn and evaporated. The dry residue was dissolved in 0.5 ml of resuspension buffer, vortex-mixed for 5 min, held at 25°C for 1 h and then analyzed by MEKC. Each determination was performed in quadruplicate.

2.6. Standard stocking

A total of 0.5 μmoles of benzoylated polyamines were dissolved in 10 ml of resuspension buffer. This solution was stable at room temperature for several weeks.

2.7. Loading, separation and detection

Samples were injected by hydrodynamic injection i.e. by applying a highly regulated vacuum (5″ Hg) to the outlet of the capillary for 1 s; separation was carried out at 25°C and 25 kV (5 μA) for 20 min. Benzoylated derivatives were detected at 198 nm (detector sensitivity 0.001 a.u.f.s.). Each determination was performed in quadruplicate.

2.8. Retention factor calculation

Retention factors (*k*) were calculated by the following equation: $k = (t_m - t_{eo})/t_{eo}(1 - t_m/t_{mc})$ [18].

Parameters t_{eo} and t_{mc} were experimentally determined by injecting benzyl alcohol, which is assumed not to interact with micelles, and benzoic anhydride, which is assumed to be fully solubilized into micellar phase. The choice of benzoic anhydride as t_{mc} marker is based on the following considerations: it is practically insoluble in 7–15% ethanol in water, it always remains in traces in analysis samples of derivatized polyamines and its separation in the electropherogram occurs after that of polyamines. This latter behaviour is indicative of a stronger association of benzoic anhydride with SDS micelles compared to benzoylated polyamines and hence makes it a good t_{mc} marker in this MEKC separation.

3. Results and discussion

CZE methods with indirect photometric detection have been set up for the determination of polyamines in biological samples such as serum and PC 12 cells [19–22], although acetylated forms of polyamines were not detected. Moreover, high ion concentrations in samples, as Na^+, K^+ and denaturating TCA, caused some interferences in the analysis [19,20]. As polyamines and their monoacetyl derivatives are usually present in very small concentrations in tissues and biological fluids, a good analysis may require to eliminate these as well as others contaminating compounds. Since these contaminants can be almost totally removed during derivatization procedure of polyamines with benzoyl chloride, we developed a MEKC procedure for the separation of benzoylated polyamines. It proved to be more efficient than CZE methods, avoiding the presence of any contaminating compound and also permitting to detect *N*-acetyl derivatives of polyamines.

A representative electropherogram of a standard mixture of benzoylated polyamines and acetylpolyamines ($5*10^{-5}$ moles/l) analyzed by our MEKC procedure is shown in Fig. 1. Such a very selective separation was obtained by an applied voltage of 25 kV (5 μA) at 25°C using a surfactant solution of 10 m*M* SDS in 20 m*M* borate buffer pH 8.5, containing 8% ethanol. Nine natural polyamines and 1,7-diaminoheptane were separated in less than 14 min.

3.1. Statistic parameters for polyamine standard curves

The linearity of polyamine detection was investigated and a linear response over two orders of magnitude ($5*10^{-7}$–$5*10^{-5}$ moles/l) for each polyamine was obtained except for *N*-acetylputrescine and *N*-acetylcadaverine; for these analytes the range of linearity was $1*10^{-6}$–$1*10^{-4}$ moles/l. For each concentration four samples were analyzed and linear regression parameters (estimate intercept = b_0; estimate slope = b_1) and coefficients of correlation (*r*)

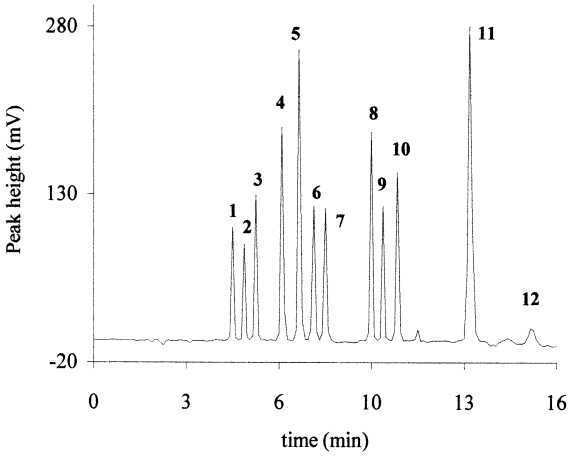

Fig. 1. Representative electropherogram of a standard mixture of benzoylated polyamines. The derivatized samples, dissolved in 0.5 ml of resuspension buffer, were vortex-mixed for 5 min, held at 25°C for 1 h and then analyzed by MEKC. A total of 150 fmoles of each sample were injected by hydrodynamic injection for 1 s; benzoylated derivatives were detected at 198 nm and separation carried out at 25°C and 25 kV (5 μA) for 20 min, using the running buffer pH 8.5, containing 8% ethanol (see in Section 2.4.4). Peaks: 1=Benzyl alcohol; 2=*N*-Acetylputrescine; 3=*N*-Acetylcadaverine; 4=Putrescine (1,4-diaminobutane); 5=1,3-Diaminopropane; 6=Cadaverine (1,5-diaminopentane); 7=N^1-Acetylspermidine; 8=N^1-Acetylspermine; 9=Spermidine [N^1-(3-aminopropyl)-putrescine]; 10=1,7-Diaminoheptane (I.S.); 11=Spermine [N^1-N^4-bis(3-aminopropyl)-putrescine]; 12=Benzoic anhydride. Each determination was performed in quadruplicate.

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 here described, a standard mixture of derivatized polyamines containing $1*10^{-5}$ moles/l was diluted 1:1, 1:2, 1:4 and 1:8, and for each dilution four samples were analyzed. The same run was repeated for a period of 3 weeks, and intra- and inter-day coefficients of variation (C.V.) were calculated. The calibration curves exhibited excellent linearity for all the polyamines (Table 2).

3.2. Optimization procedure

The optimization of the here reported MEKC method was achieved by investigation of the influence of various SDS concentrations (10, 20, 30, 40, 50 and 60 m*M*) at variable voltage (20, 22, 25 and 30 kV) using borate buffer at pH varying from 8.0 to 9.0, in the temperature range of 25 to 40°C. Such analysis led to establish a first-trial set of optimal parameters, namely 10 m*M* SDS–20 m*M* sodium borate, pH 8.5, a voltage of 25 kV (5 μA) and temperature of 25°C. However, such conditions did not allow to obtain the full resolution of all polyamines assayed, as shown in Fig. 2: peaks of putrescine and 1,3-diaminopropane and those of N^1-acetylspermine and spermidine were overlapped. In fact, their retention factors (*k*), calculated on dependence of SDS concentration and voltage (Figs. 3 and 4), were similar. We observed that retention factors undergo negligible changes on dependence of pH,

Table 2
Linear regression parameters and intra-day and inter-day mean coefficients of variation for MEKC analysis of benzoylated polyamines and monoacetylpolyamines

Compound	Slope (mean±SD)	Intercept (mean±SD)	Correlation coefficient (mean±SD)	Intra-day C.V. (%)	Inter-day C.V. (%)
N-Acetylputrescine	0.005±0.0004	−0.07±0.04	0.9980±0.0003	2.93	4.85
N-Acetylcadaverine	0.006±0.0005	0.013±0.007	0.9997±0.0005	2.75	4.56
Putrescine	0.04±0.004	0.085±0.02	0.9994±0.0002	1.84	3.35
1,3-Diaminopropane	0.015±0.002	−0.012±0.01	0.9993±0.0003	0.95	2.15
Cadaverine	0.017±0.002	−0.048±0.001	0.999±0.0005	1.25	3.18
N^1-Acetylspermidine	0.010±0.001	0.10±0.02	0.9997±0.0007	3.13	5.17
N^1-Acetylspermine	0.012±0.001	0.012±0.004	0.9995±0.0003	2.18	5.42
Spermidine	0.018±0.002	0.001±0.001	0.9999±0.0005	1.45	3.91
1,7-Diaminoheptane	0.003±0.0004	0.002±0.001	0.9997±0.0004	2.11	4.59
Spermine	0.018±0.003	0.064±0.01	0.9999±0.0006	1.58	3.40

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 3 weeks, and inter-day C.V. was calculated.

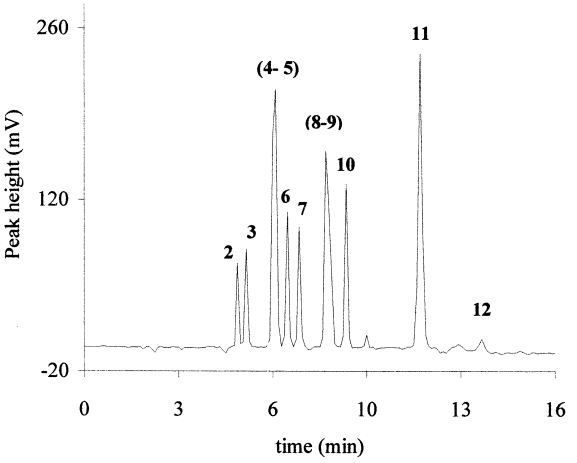

Fig. 2. MEKC separation of benzoylated polyamines in absence of ethanol. The MEKC conditions were those reported in legend of Fig. 1 except the running buffer which was 10 m*M* SDS in 20 m*M* sodium tetraborate–boric acid, pH 8.5. Peaks of polyamines were numbered as in Fig. 1.

when it ranges from 8 to 9, that temperatures over 25°C have no influence and finally that voltage is only able to modify the analysis time without altering the time window. Such evidences suggested to consider other parameters besides electroosmotic flow (EOF) such as partitioning coefficient (P_{wm}) and phase ratio (β) to modify retention factor values. When applied to neutral solutes, the MEKC separation is based on a partitionig mechanism, defined by P_{wm}, and on β. Both parameters are related to retention factors by the following equation: $K = P^{*}_{wm}\beta$ [23]. Since in MEKC the addition of organic modifier alters P_{wm} and β, ethanol was added to the running buffer to modify k values [23]. Concentrations of 5, 8, 10 and 15% were tested and the best separation was achieved at 8% of ethanol (v/v) as demonstrated in Fig. 5, where the plot of k vs. ethanol concentration is shown; ethanol decreases EOF, as demonstrated by the increment of t_{mc}/t_{eo} ratio, alters P_{wm} and β, and is therefore capable to change the retention factors values. Ethanol was used instead of methanol in order to prevent production of methylbenzoate after benzoyl chloride derivatization [17,24]. The change in the chemical nature of the separation system with the achieved increase of the time window resulted in a net separation of putrescine and 1,3-diaminopropane, and N^1-acetylspermine and spermidine in spite of their similar molecular mass and their similar charge/mass ratio.

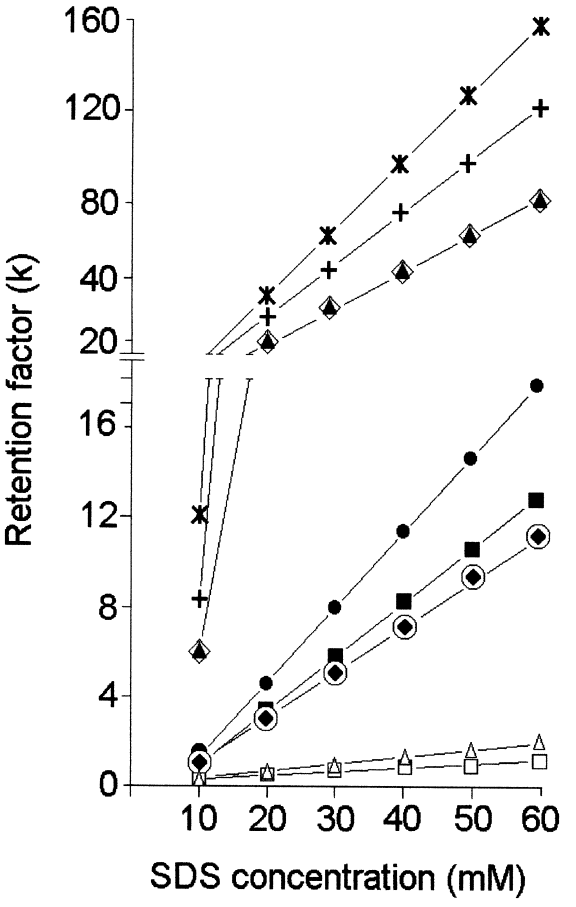

Fig. 3. Dependence of retention factor of benzoylated polyamines on SDS concentration. Symbols in the plot represent: (□) *N*-acetylputrescine; (△) *N*-acetylcadaverine; (♦) Putrescine; (○) 1,3-Diaminopropane; (■) Cadaverine; (●) N^1-acetylspermidine; (▲) N^1-acetylspermine; (◇) Spermidine; (+) 1,7-Diaminoheptane; (*) Spermine. Running buffer were 10–60 m*M* SDS in 20 m*M* borate buffer, pH 8.5. Electropherogram was performed at 25°C with an applied voltage of 25 kV (5 μA).

4. Conclusion

The MEKC procedure here introduced gave an excellent resolution of a mixture of ten benzoylated polyamines in SDS micelles. The addition of ethanol to the separation system contributed to optimize the conditions of analysis. To this aim, the check of the

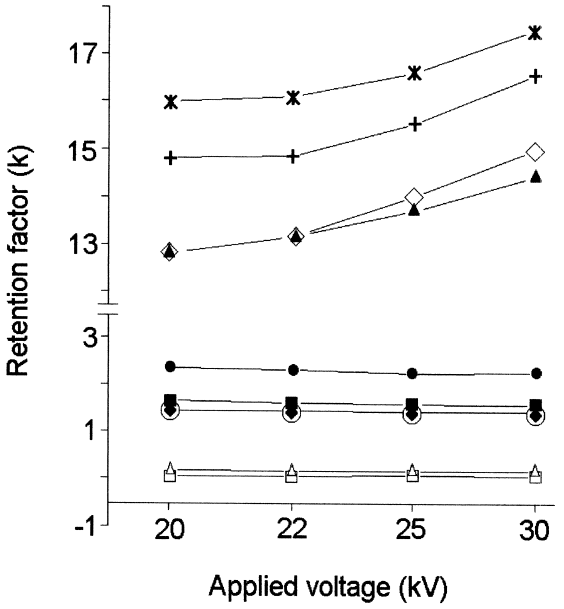

Fig. 4. Plot of retention factor versus the applied voltage. Symbols in the plot represent: (□) *N*-acetylputrescine; (△) *N*-acetylcadaverine; (♦) Putrescine; (○) 1,3-Diaminopropane; (■) Cadaverine; (●) N^1-acetylspermidine; (▲) N^1-acetylspermine; (◇) Spermidine; (+) 1,7-Diaminoheptane; (*) Spermine. Running buffer was 10 m*M* SDS in 20 m*M* borate, pH 8.5.

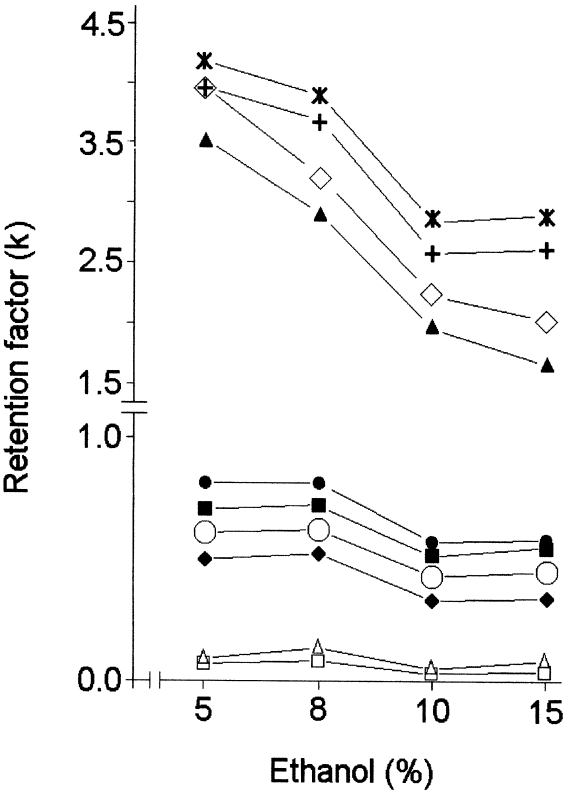

Fig. 5. Dependence of retention factor on the concentration of ethanol. Experimental conditions are from Fig. 1, except the concentration of ethanol in the running buffer. Symbols in the plot represent: (□) *N*-acetylputrescine; (△) *N*-acetylcadaverine; (♦) Putrescine; (○) 1,3-Diaminopropane; (■) Cadaverine; (●) N^1-acetylspermidine; (▲) N^1-acetylspermine; (◇) Spermidine; (+) 1,7-Diaminoheptane; (*) Spermine.

most important variables of the electrokinetic system and the determination of retention factor for each polyamines was carried out, thus allowing to achieve an efficient separation for those polyamines whose mass/charge ratio were similar. In addition, pre-derivatization of the polyamine mixture gave the advantage to eliminate ions and undesidered contaminants. Therefore, a method of high sensitivity is now available permitting the separation and measurement of femtomole amounts of polyamines in standard samples and in biological specimens.

Acknowledgements

The authors wish to thank Dr. F. Paternò and Dr. G. Cangelosi for their technical assistance. This work was supported by Research Grant (60%) from the ''Ministero dell'Università e della Ricerca Scientifica'' (MURST).

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