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Evaluation of Micellar Electrokinetic Capillary Chromatography for the Separation and Detection of Normal and Modified Deoxyribonucleosides and Deoxyribonucleotides

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EVALUATION OF MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY FOR THE SEPARATION AND DETECTION OF NORMAL AND MODIFIED DEOXYRIBONUCLEOSIDES AND DEOXYRIBONUCLEOTIDES*

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ABSTRACT

Micellar electrokinetic capillary chromatography allows high resolution separations with very small sample volume requirements. Deoxyribonucleotide oligomers can be resolved on the basis of chain length, nucleic base sequence, and base modification. Sensitivity for dideoxyribonucleosides in the presence of a 1,000-fold excess of deoxyribonucleosides is ca. 12 pg with on-column UV absorbance detection.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC), introduced by Terabe (1) in 1984, is an electrophoretic separation method which can resolve both neutral and also charged solutes using partitioning and electrophoresis mechanisms. MECC and the related method of capillary zone electrophoresis (essentially, MECC without the detergent micelles) offer very high separations efficiency (2), very small sample volume requirements (3,4), and reasonably good sensitivity (5) with the UV absorbance detector.

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We are evaluating the utility of MECC for the separation and determination of modified and adducted nucleic acid components. In our previous paper (2), we examined the effects of detergent concentration, injection voltage and time, and separation voltage upon the efficiency and resolution of nucleic acid constituents. Optimized conditions were identified which allow highly efficient (up to 370,000 theoretical plates) separations of picogram quantities of normal and modified deoxyribonucleosides and deoxyribonucleotides. This paper reports the extension of MECC to the separation of oligodeoxyribonucleotides and therapeutic dideoxyribonucleosides, and the determination of the limits of detection for the lastter using an on-column UV absorbance detector.

EXPERIMENTAL

Reagents and Materials

The deoxyribonucleosides used in this study were obtained from the Sigma Chemical Co. (St. Louis, MO). The oligodeoxyribonucleotides were purchased from the Pharmacia Co. (Piscataway, NJ) and the dideoxyribonucleosides were kindly provided by Samuel Broder of the National Cancer Instutute (Bethesda, MD). Their abbreviations are 2'-deoxyadenosine (dA), 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG), 2'-deoxythymidine (dT), 2',3'-dideoxyadenosine (ddA), 2',3'-dideoxycytidine (ddC), 2'-deoxyadenosine-5'monophosphate (dAMP), dephospho-oligo dA_2 (dA_2), dephospho-oligo dA_4 (dA_4) and dephospho-oligo dA_6 (dA_6). The five tetradeoxyribonucleotides are 5'-d(CCGG)-3', 5'-d(AGCT)-3', 5'-d(CGCG)-3', 5'-d(GCGC)-3' and 5'-d(GGCC)-3', and the four hexadeoxyribonucleotides are 5'-d(N^6MeATGCAT)-3', 5'-d(ATGUAT)3', 5'-d(ATGMe^{5}CAT)3', and 5'-d(ITGCAT)3'. Samples were dissolved in distilled water. The concentrations of the deoxyadenosine monophosphate oligomers dAMP, dA_2, dA_4, and dA_6 were 1-2 absorbance unit/100 μL, where an absorbance unit of oligomer is the amount of oligomer which produces an optical absorbance of 1.0 (at its maximum absorbance wavelength) when dissolved in 1.0 ml of water and examined in a 1 cm path length cell. For the tetra- and hexadeoxyribonucleotides, the concentrations of each mixture were prepared to 1.0 absorbance unit/50 μL of distilled water and 1.0 absorbance unit/10 μL of distilled water, respectively. The concentration of four deoxyribonucleosides was set at 25 mmol/mL, while those of the dideoxyribonucleosides was varied from 4000 to 25 nmol/mL. Sodium dodecylsulfate (SDS) and the buffer components (sodium tetraborate and disodium hydrogen phosphate) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). The two mobile phases were 0.075 M and 0.008 M SDS in phosphate/borate buffer (0.01 M Na_2HPO_4 and 0.006 M $Na_2B_4O_7$).

Apparatus and Method

The experimental apparatus was the same as that described previously by Row et al. (2). The capillary column was 1 m x 60 μm ID fused silica tubing purchased from SGE, Inc. (Austin, TX). The column length from the high voltage reservoir tip to the UV absorbance detector was 70.0 cm. Regulated DC high voltage was delivered by an R30B Hippotronics (Brewster, NY) power supply. The separated components were detected on-column using a Jasco (Japan Spectroscopic Co., Inc., Tokyo, Japan) UVIDEC-100-III spectrophotometric detector set at 256 nm. The detector signal was displayed on a model 355 strip chart recorder (Linear Instruments, Reno, NV), with a span varied from 2 to 10 mv depending upon the sample concentrations. The operator was protected from contact with high voltages by two Plexiglass boxes equipped with line power cut-offs on the doors.

New capillary tubing was rinsed with 0.1 M HCl, distilled water, and the mobile phase for several hours using ca. 1,380 kPa (200 psig) of nitrogen pressure. Electroinjection was used to introduce samples into the column, as described by Burton et al. (3). To maintain reproducibility, the column was rinsed briefly with the mobile phase between each experimental run, and the current was checked at a given separation voltage.

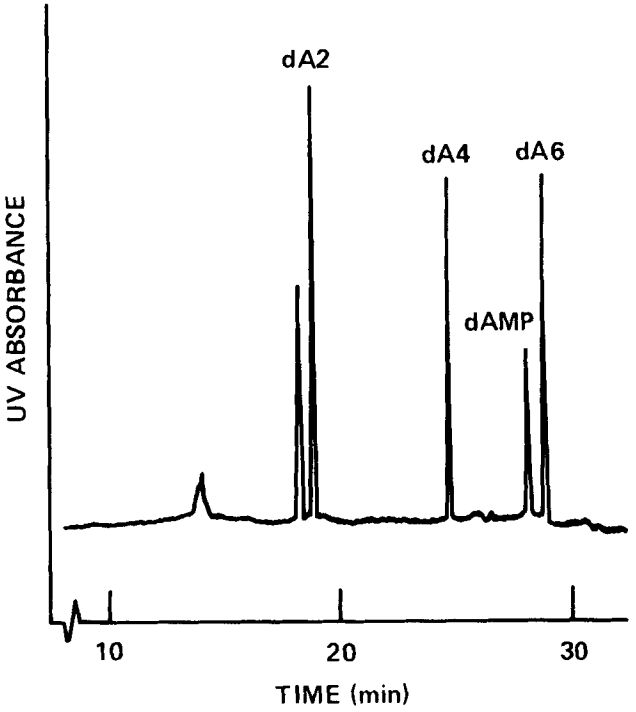
The number of theoretical plates (N) for component i was calculated from the peak width (W_i, min) and the retention time (T_i, min) by

$$N = 16\ (T_i/W_i)^2 \qquad (1)$$

The number of plates per meter of effective column length or min of retention time were calculated by dividing N by 0.7 m or the retention time in min.

RESULTS AND DISCUSSION

Previously, we demonstrated (2) that MECC can readily separate normal and modified deoxyribonucleosides and deoxyribonucleotides with efficiencies of ca. 100,000 theoretical plates. At low solute concentrations corresponding to 10-20 pg injected on-column, efficiencies up to 370,000 theoretical plates (ca. 500,000 plates/m) can be realized. At higher solute concentrations, efficiency degrades, particularly at μmol/mL levels. Using detergent concentrations above the critical micelle concentration (ca. 0.0083 M for SDS at 25°C in pure water, reference 6), optimized efficiencies were found at an SDS concentration of 0.075M and separation voltages in the range of 8 to 12 kV and an injection voltage and time of 5 kV and 5 sec, respectively. These conditions were used as a starting point for the studies of oligonucleotide separations.



1. Resolution of dAMP Oligomers by MECC. (Conditions: 5 kV injection voltage, 15 sec injection time, 15 kV separation voltage, 0.075 M SDS)

A highly efficient separation of dAMP oligomers is illustrated in Figure 1. The order of retention, dA_6>dAMP>dA_4>dA_2, is consistent with the charge/mass ratios of the molecules (z/m of -2.63,-6.04, -2.42, and -1.73 [x10^{-3}], respectively, if all POH groups are ionized at pH 8.4), except for dAMP. A higher negative molecular charge increases the electrophoretic velocity of the oligomer, which opposes the electroosmotic velocity of the mobile phase and retards the elution of the oligomer. The reason for the smaller than expected retention of dAMP is not clear. It is not the result of an increased partitioning of this molecule into the micelles versus that of the higher oligomers, because the same order of elution is observed when the SDS is replaced with 0.5% (vol/vol) i-propanol. The second POH (pK_a of ca.6) should be ionized at this pH (8.4). The fact that the electrophoretic velocity is greater than

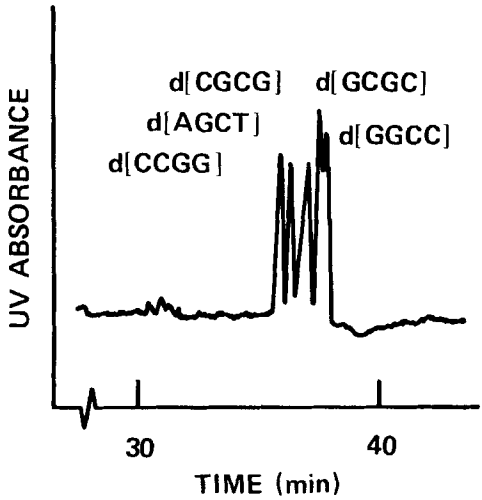
Table 1. Total Plates and Plates per Minute for the Separation of Deoxyadenosine Monophosphate Oligomers[a]

	Separation Voltage, kV					
	10		15		23	
Compound	N	N/T	N	N/T	N	N/T
dA_2	155,000	5,250	97,700	5,210	82,900	7,680
dA_4	137,000	3,520	109,000	4,400	100,000	7,020
dAmP	154,000	3,490	141,000	5,010	130,000	8,000
dA_6	161,000	3,550	148,000	5,130	135,000	8,150

[a]Conditions: 5 kV injection voltage, 15 sec injection time, 0.075 M SDS.

expected suggests that the second POH may not be fully ionized in the presence of micelles. As indicated in Table 1, the efficiencies generally exceeded 100,000 theoretical plates (150,000 plates/m). The increasing efficiencies with increasing oligomer size are expected (7) from their decreasing diffusion coefficients and increasing electrophoretic mobilities. For dA_6, 161,000 theoretical plates (235,000 plates/m) were generated at 10 kV, the lowest of the three separation voltages tested. We observed previously (2) that the efficiency for deoxyribonucleosides and deoxyribomononucleotides was maximized at voltages in the range of 8 to 12 kV. Although an increased number of theoretical plates is generated at decreased separation voltages, an increasing number of plates per minute is achieved by increasing the voltage. Rapid separations at high voltages are therefore feasible.

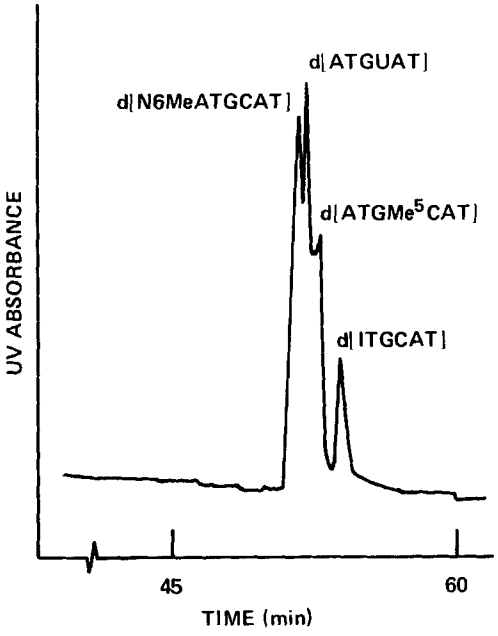
Application of MECC to the separation of isomeric oligodeoxyribonucleotides is shown in Figure 2. This is a much more difficult separation to achieve in that the structures and charges of these tetramers are much more similar to each other than they are for the dAMP oligomers. Four of the five tetramers are isomers having two dG and two dC arranged in different orders, while the remaining tetramer contains a dA and a dT in place of one dG and one dC. As expected from the more subtle molecular differences among the tetramers, the separation is not as good as for the dAMP oligomers. However, there is a partial resolution of all five tetramers which suggests that this separation method could be used to resolve DNA fragments according to base sequence.



2. Nucleic Base Sequence-Selective Separation Using MECC. (Conditions: 5 kV injection voltage, 15 sec injection time, 10 kV separation voltage, 0.075 M SDS.)

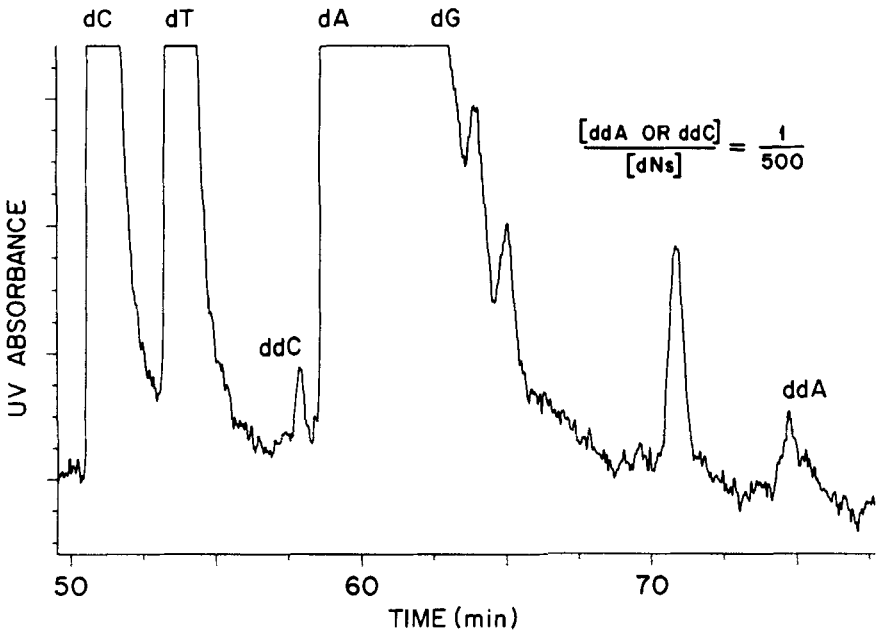
There is more band broadening evident in this separation than for the dAMP oligomers. Considering that guanidine and cytosine are complementary bases (8), association of complementary tetramers could be a major contributor to dispersion, particularly during the early stages of the MECC when the solute bands are incompletely separated. Cohen et al. (9) included 7M urea in the mobile phase to disrupt association and improve efficiency in the MECC separation of ribonucleotide octamers. It also is possible that the use of a mobile phase modifier to extend the elution range would improve the separation as reported for uncharged solutes by Balchunas and Sepaniak (10). These mobile phase modifiers have not yet been tested, and certainly the separation would benefit from a careful optimization of conditions.

The potential of MECC for separation of oligonucleotides on the basis of nucleic base modification is suggested by the chromatogram in Figure 3. These four hexamers have the same backbone sequence of purine and pyrimidine deoxyribonucleotides, and they differ only in the base substituents or modifications. For example, the first- and third-eluting species, d[N^6MeATGCAT] and d[ATGMe^{5}CAT], differ only in which base is modified with a methyl group. As for the tetramers, the structural and electronic differences are subtle, and the separation is difficult. The



3. Nucleic Base Modification-Selective Separation of Oligomers. (Conditions: 5 kV injection voltage, 10 sec injection time, 23 kV separation voltage, 0.008 M SDS.)

mobile phase used in this separation was 0.008 M SDS. This lower SDS concentration was tested for the hexamers when it was noted that the resolution of the tetramers was not changed appreciably when the SDS concentration was decreased from 0.075 M to 0.008 M. This observation suggests that the oligonucleotides do not partition effectively with the micelles. This behavior might be expected from their large negative charges and coulombic repulsion from the negatively charged polar head groups of the micelles. Although some of the same mobile phase changes as noted immediately above may improve the separation, affinity interactions may offer a greater promise for increasing the resolution of species differing mainly in nucleic base modification. Substituents on the purine base could alter the electronic structures of the bases sufficiently to alter their interactions with complexation agents. Cohen et al. (9) utilized transition metal ions in their separation of unmodified ribonucleotide octamers, but it is not clear that the increased



4. Separation of ddC and ddA from 500-Fold Excess of Deoxyribonucleosides Near their Limit of Detection. (Conditions: 5 kV injection voltage, 5 sec injection time, 5 kV separation voltage, 0.075 M SDS.)

resolution resulted solely from complexation. The relative order of elution was changed by only one nucleotide when a metal ion was included in the mobile phase.

Separations on the basis of the pentose structure also are possible. The ribonucleosides (Ns), deoxyribonucleosides (dNs), and dideoxyribonucleosides (ddNs) differ in the number of hydroxyl groups at the 2'- and 3'- carbons, with the dNs lacking the 2'-hydroxyl, and the ddNs lacking both the 2'- and 3'-hydroxyl groups. The retention should increase with decreasing aqueous solubility because of increased partitioning into the slower-moving micelles in the mobile phase. The predicted order of elution is Ns<dNs<ddNs whereas the actual order of elution was found to be dNs<ddNs<Ns. The Ns elute much later than expected from their relative aqueous solubilities. Deletion of borate from the mobile phase altered the order of elution to the expected order, but resulted in a less stable current during the chromatography. The former indicates that borate complexed or

reacted with the 2'- and 3'-hydroxyl groups of the ribose moiety, rendering the Ns less water soluble and more favorable for partitioning into the micelles. Such sugar-borate reactions are well known, and have been used for many years (11) in liquid chromatographic separations of nucleosides.

Separations of modified nucleic acid components and their normal counterparts from in-vivo or in-vitro systems involve resolution of small quantities of the former from large excesses of the latter. This separation is important because ddC and ddA have shown (12) considerable potential as a therapeutic agent for the treatment of patients afflicted with AIDS. Figure 4 shows the resolution of trace concentrations of these two ddNs from 500-fold excesses of four dNs. Under these conditions, the limit of detection at a signal to noise ratio of four was estimated to be 40 pg of ddC and 28 pg of ddA. Because of the 1 to 2 nL injection volumes achieved by the electroinjection process (2), these detection limits correspond to sample concentrations of 58 and 49 nmol/mL, respectively. These are quite similar to the detection limits of 26 pg (64 nmol/mL) for deoxyadenosine, 18 pg (42 nmol/mL) for deoxyguanosine, and 14 pg (36 nmol/mL) for deoxyinosine determined (2) under very similar conditions. However, sample volume requirements are very small; 3 μL is a sufficient volume in which to dip the end of the column and high voltage electrode during electroinjection, and even smaller volumes can be accomodated using microinjectors (13). The separation in Figure 4 was achieved with a 5 sec injection at 5 kV and a separation voltage of 5 kV. Increasing the separation voltage to 15 kV sharpened the peaks and increased the sensitivity for ddA to ca. 12 pg, or 20 nmol/mL in the presence of a 1,000-fold excess of the deoxyribonucleotides. However, the ddC was not detectable between the more closely-eluting dT and dA.

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