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Capillary electrophoretic separation of nucleotide isomers via complexation with cyclodextrin and borate

Tanya Tadey, William C. Purdy*

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, Quebec H3A 2K6, Canada

Abstract

The electrophoretic behaviour of monophosphorylated nucleotide isomers can be manipulated using complex-forming reactions with ß-cyclodextrin (ß-CD) and borate. Resolution of the 2'- and 3'-isomers of nucleotides is possible when the electrophoresis buffer contains 10 m*M* CD. The effect of ß-CD concentration on electrophoretic mobility is used to calculate the formation constant, *K*, of ß-CD–nucleotide complexes. The 3'-isomer of adenosine monophosphate (AMP) forms the strongest complex with ß-CD probably as a result of hydrogen bonding between the phosphate group of AMP and hydroxyls of ß-CD. In addition, complexation of 5'-nucleotides with borate increases the migration time window and leads to better separation. Complex-forming reactions of guanosine monophosphate and uridine monophosphate are shown to be strongly dependent on buffer pH. A mixture of 12 monophosphorylated nucleotides can be separated in less than 15 min using a buffer of 20 m*M* borate–10 m*M* ß-CD.

1. Introduction

Nucleotides in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are linked by phosphodiester bonds at the 3'- and 5'-positions of the sugar moiety. This is despite evidence suggesting that the 2'-5' linkage predominated during prebiotic evolutionary development [1]. The reason for natural selection of the 3'-5' linkage is not completely understood. Currently much research is being directed at the synthesis and properties of 2'-5' nucleotide oligomers [2,3]. The relative reactivities of 2'- and 3'-hydroxyl groups in nucleotides are also being studied [4]. Recently, oligonucleotides containing 2'-5' linkages have demonstrated potential as antisense chemotherapy agents [5]. It is increasingly important, therefore, to have reliable techniques for nucleotide analysis. Specifically, these developments call for methods capable of high speed separation of 2'-, 3'- and 5'-phosphorylated nucleotide isomers.

Chromatographic methods for nucleotide analysis are well-established. Separations employing the ion-exchange [6–8], reversed-phase [9,10] and ion-pair [11,12] modes of HPLC have been reported. Comparison revealed superior resolution and reproducibility using ion-pair reversed-phase HPLC [13]. Despite the large volume of work describing nucleotide separation, there are relatively few reports concerning separation of monophosphorylated nucleotide isomers. Presently, ion-pair reversed-phase HPLC gives best resolution of 2'-, 3'- and 5'-nucleoside monophosphate isomers [11]. However, the analysis generally takes more than 30 min for completion.

* Corresponding author.

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More recently, there have been numerous reports employing capillary electrophoresis (CE), for separation of nucleosides [14] and nucleotides [15–17]. In the last decade CE has emerged as a powerful separation technique. The use of narrow bore capillaries (25–50 μm I.D.) and high electric fields results in separation efficiencies which are much higher than those obtained with conventional chromatographic methods. Shortened analysis times and improved resolving power are also among the advantages of CE over HPLC.

To date, there is only one report of CE separation of monophosphorylated nucleotide isomers. Huang *et al.* [18] used low pH buffers to resolve the various 2'-, 3'- and 5'-nucleotide isomers [18]. However, this method exhibited poor resolution and reproducibility.

Separation of nucleotide isomers is poor because neutral molecules or those with similar charge-to-size ratios are generally difficult to resolve by CE. In order to increase selectivity for these compounds buffer additives must be employed. These additives often impart a chromatographic character to the CE separation by acting as pseudo-stationary phases. Micellar electrokinetic chromatography (MEKC) which employs detergent micelles as buffer additives has illustrated impressive results for separation of neutral compounds [19]. Host–guest complexation with cyclodextrin [20] and crown-ether [21] additives has been used to resolve chiral isomers. Borate ions, which form complexes with *cis*-diols, are used as buffer additives for separation of catechols [22] and carbohydrates [23].

The purpose of the present work is to study the effect of two complexing agents, ß-cyclodextrin (ß-CD) and borate, on nucleotide separation by CE. Both compounds are routinely used to enhance selectivity in CE. However, this is the first report which employs complexation with both CD and borate to achieve resolution. The effect of complexing agent concentration and pH on the resolution of 12 mononucleotide isomers is discussed. Furthermore, we illustrate that interaction between CD and nucleotide is severely reduced in the presence of SDS micelles.

2. Instrumental

2.1. Apparatus

The capillary electrophoresis system used a Bertan Model 230R (Bertan Assoc., Hicksville, NY, USA) power supply and a Isco CV^4 (Isco, Lincoln, NE, USA) detector. The output of the power supply was connected to the buffer reservoir via platinum electrodes (Bioanalytical Systems, West Lafayette, IN, USA). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 375 μm O.D. were used. Electropherograms were collected using the Waters System Interface Module (Millipore, Milford, MA, USA) and then further processed on the Waters Maxima 820 Chromatography Workstation.

2.2. Chemicals

Distilled, deionized water was used for all experiments. Ultra-pure sodium dodecyl sulfate (SDS) and boric acid were obtained from ICN Biochemicals (Montreal, Canada). Sodium tetraborate (borax) and ß-cyclodextrin hydrate were from Aldrich (Milwaukee, WI, USA). Sodium hydoxide and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Glycine and mononucleotide standards were purchased from Sigma (St. Louis, MO, USA).

2.3. CE procedures

All CE separations were performed at ambient temperature. The capillaries had a total length of 80 cm and a separation length of 60 cm. A wavelength of 254 nm was used for detection. A field strength of 312 V/cm was used for all separations. The pH of sodium tetraborate buffers was adjusted by titrating with appropriate amounts of boric acid, hydrochloric acid or NaOH. Bare silica capillaries were conditioned between runs by two-minute rinses of 0.1 *M* NaOH and water. Samples were injected hydrodynamically at a height of 15 cm and an injection time of 8–10 s.

3. Results and discussion

Nucleotides are the phosphorylated esters of nucleosides. The structures and ring numbering system of nucleosides based on ribose sugar is shown in Fig. 1. Phosphorylation at the 2'-, 3-' or 5'-position of the sugar moiety yields the corresponding nucleotide.

Studies by UV spectroscopy demonstrated that adenosine monophosphates (AMP) are able to form inclusion complexes with ß-CDs [24]. Later, the more sensitive technique of circular dichroism was used to show that cytidine monophosphate (CMP) and uridine monophosphate (UMP) also form inclusion complexes with CD [25]. Hoffman was able to separate all three isomers of AMP by chromatography on CD gels [26]. However, resolution of the other nucleotide isomers was poor and the analysis took hours to perform.

By taking advantage of the high efficiencies and short analysis times available with CE, we expected a significant improvement in nucleotide separations based on inclusion complex formation with CD. Glycine buffers at pH 9 were used to study the effect of ß-CD concentration on nucleoside monophosphate resolution. No borate was added to buffers in these experiments in order to study the effect of ß-CD independently of other complexation effects. The mobility difference of 2'- and 3'-nucleotide isomers as a function of ß-CD concentration is illustrated in Fig. 2. The mobility difference for each pair of isomers increases with increasing CD concen-

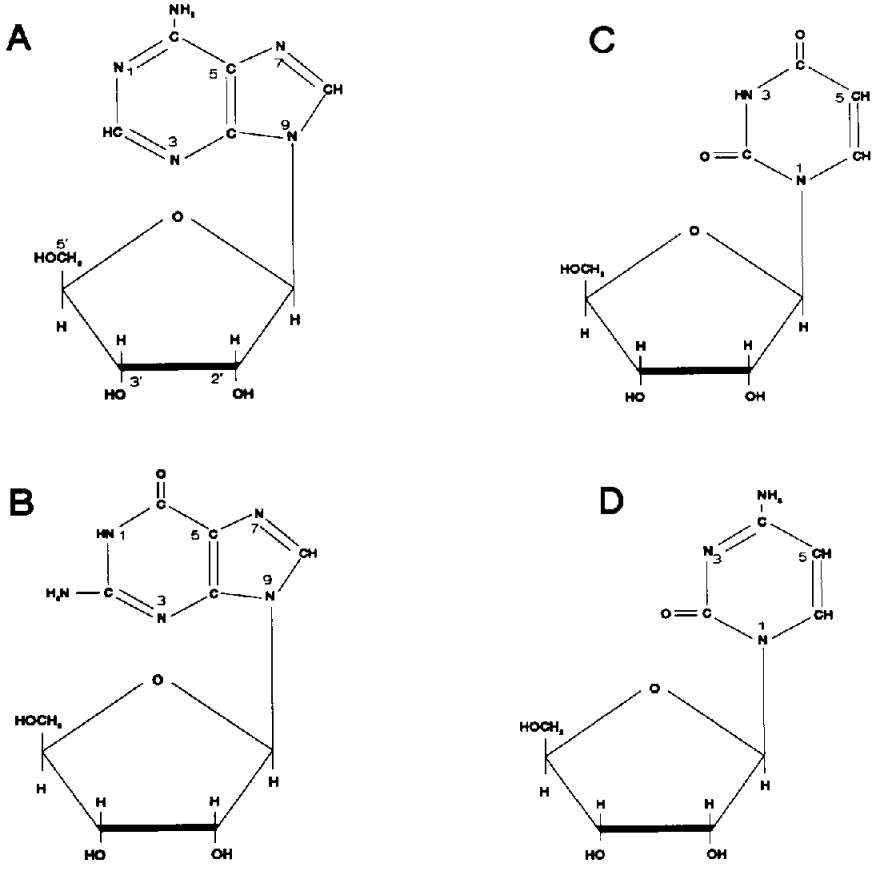

Fig. 1. Structures of nucleosides. A = Adenosine; B = guanosine; C = uridine; D = cytidine.

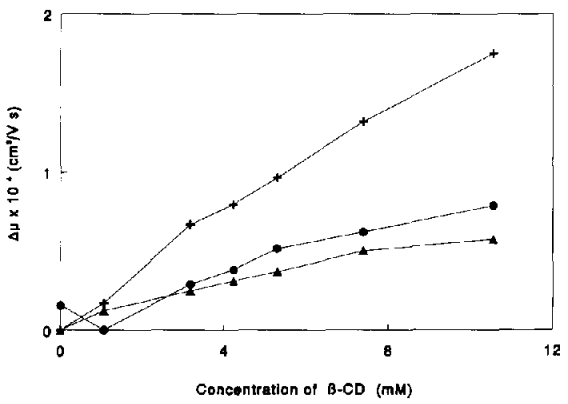

Fig. 2. Effect of ß-CD concentration on mobility difference of 2'- and 3'-nucleotide isomers. Conditions: 50 m*M* glycine, 15 m*M* NaCl, pH 9. + = GMP; ● = UMP; ▲ = CMP.

tration due to varying degrees of complexation. At a CD concentration of 10 m*M* all four pairs of isomers are baseline separated with a resolution factor greater than one. The plots in Fig. 2 indicate that the effect of CD is more pronounced for the two purine nucleotides, GMP and AMP. This is expected since the smaller pyrimidine bases of CMP and UMP pass more easily into and out of the CD cavity and consequently inclusion complexation is weak.

The change in electrophoretic mobility on addition of ß-CD to the running buffer can be used to estimate the complex formation constant, K. The observed mobility is related to K by the following equation:

$$\mu_{obs} = \frac{1}{1 + K[CD]}\mu_f + \frac{K[CD]}{1 + K[CD]}\mu_c + \mu_{eo} \qquad (1)$$

where μ_{obs} is the observed electrophoretic mobility, μ_f is the mobility of the free, uncomplexed nucleotide, μ_c is the mobility of the nucleotide–cyclodextrin complex, μ_{eo} is the mobility of electroosmotic flow and [CD] is the concentration of CD. In previous papers describing the relationship between CD and electrophoretic mobility, the effect of electroosmotic flow was ignored [26]. This was because the work was performed under conditions of negligible electroosmotic flow. However, at pH 9 electroosmotic flow is large and must be included in Eqn. 1.

Rearrangement of Eqn. 1 yields the following relationship:

$$K[CD] = \frac{\mu_f - \mu_e}{\mu_e - \mu_c} \qquad (2)$$

where μ_e is the electrophoretic mobility of the nucleotide and is equal to $\mu_{obs} - \mu_{eo}$. The value of K can be obtained therefore by plotting the ratio of mobilities on the right side of Eqn. 2 as a function of CD concentration.

Since μ_c is not directly measurable from the electropherogram, its value can be estimated from the following equation for electrophoretic mobility [27]:

$$\mu = k'QM^{-2/3} \qquad (3)$$

where k′ is a constant, Q is the charge of the molecule, M is the molecular mass. The value of Q is assumed to be the same for the free and complexed nucleotide since CD is uncharged. Therefore, μ_c is obtained from the ratio of free and complex molecular masses and the mobility of the free uncomplexed nucleotide:

$$\mu_c = \frac{M_c^{-2/3}}{M_f^{2/3}}\mu_f \qquad (4)$$

A plot of $(\mu_f - \mu_e)/(\mu_e - \mu_c)$ *vs.* [CD] for the various AMP isomers is illustrated in Fig. 3. The slope of these plots represents the complex formation constant, K. The 3'-isomer has the

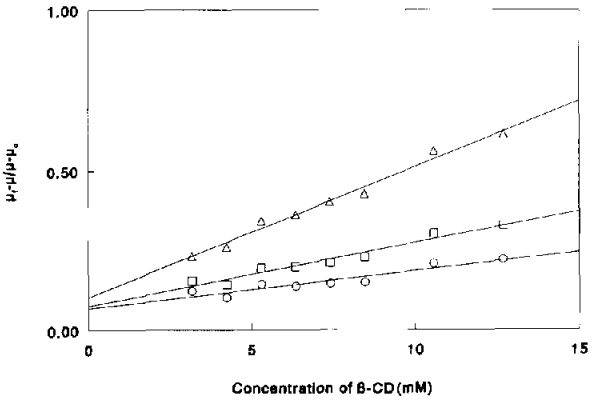

Fig. 3. Determination of K for AMP isomers: △ = 3'-AMP, $K = 41\ M^{-1}$; ○ = 2'-AMP, $K = 12\ M^{-1}$; □ = 5'-AMP, $K = 20\ M^{-1}$. Conditions as in Fig. 2.

strongest interaction with CD as evidenced by its large value of K. This is expected because hydrogen bonding between the phosphate group of the nucleotide and the hydroxyls of CD stabilizes the inclusion complex. The 2'-isomer of AMP exhibited the lowest value of K. Probably inclusion of the base into the CD cavity is hindered by having the phosphate group and base on adjacent carbons of the ribose moiety. Finally, the 5'-isomer represents the intermediate case since the phosphate group is not in a position to either sterically hinder complex formation or to stabilize the complex once it is formed.

Electropherograms of the separation of all 12 nucleotides in glycine buffer with and without CD are shown in Fig. 4. Resolution improves when the buffer contains 10 mM ß-CD. However, while all isomers of individual nucleotides can be separated in the presence of CD, Fig. 4 illustrates that resolution of a complex mixture of all possible nucleotide isomers is not possible.

3.1. Interaction of nucleotides with borate

To improve resolution of nucleotide mixtures a second complex-forming reaction, employing borate ions, was used. It is well known that 1,2-*cis* diols interact with borate ion. Böeseken [28] studied the interaction of various sugars with borate and suggested a two-stage equilibrium representing formation of 1:1 and 1:2 borate (B^-)–diol ligand (L) complexes according to Eqn. 5:

$$B^- + L \rightleftharpoons BL^- + L \rightleftharpoons BL_2^- \quad (5)$$

Interaction of 5'-ribonucleotides, which contain hydroxyl groups at the 2'- and 3'-positions of the sugar moiety, with borate has been used to separate these compounds by chromatography. Weith and co-workers [29] used dihydroxyboryl derivatives of cellulose to separate 5'-ribonucleotides from the corresponding deoxyribonucleotides. Recently, borate complexation was used to resolve cytidine and deoxycytidine by CE [30].

Representative plots of the effect of borate concentration on the migration time of nucleotides are shown in Fig. 5. In each case the buffer also contains 10 mM ß-CD. The migration time of all the nucleotides increases with borate concentration. This effect is observed because the electroosmotic flow decreases at higher ionic strength due to lower zeta potentials at the buffer capillary interface. Although the current in the capillary increased from 7 μA at 10 mM

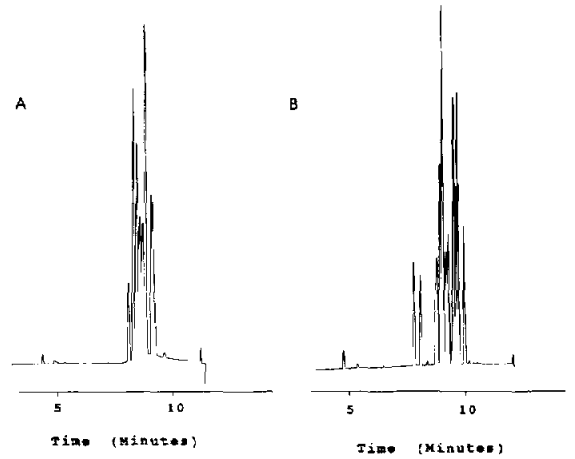

Fig. 4. Separation of 12 monophosphorylated nucleotides. (A) No ß-CD in buffer; (B) buffer containing 10 mM ß-CD. Other conditions as in Fig. 2.

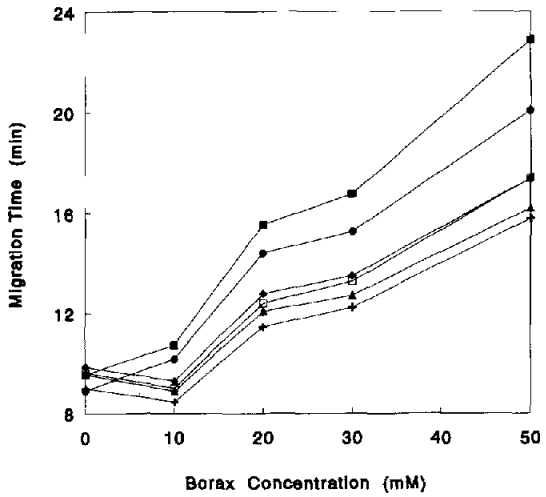

Fig. 5. Effect of borate concentration on migration time. Conditions: 10 mM ß-CD, pH 9. ■ = 5'-UMP; ● = 5'-GMP; ◆ = 2'-UMP; □ = 3'-UMP; ▲ = 2'-GMP; + = 3'GMP.

borate to 45 μA at 50 mM borate, Joule heating is not a major factor since there is no significant loss in efficiency at higher buffer concentrations. The most significant aspect of Fig. 5 is the change in migration order when borate is a component in the buffer. In glycine buffers, the 5'-isomer of all nucleotides migrates past the detector before the 2'-isomer. However, in all of the borate buffers the 5'-isomer has the longest migration time and is affected to a greater extent than the other isomers. This is expected since the 5'-isomer becomes more negative when it complexes with borate and is thus repelled to a greater extent from the cathode at the detection end of the capillary.

It should be noted that the migration time of 5'-nucleotides is governed by several equilibria when both CD and borate are buffer constituents. They may interact with borate as the free nucleotide or as the CD complex. The various reactions of 5'-nucleotides (N), are summarized below:

$$N + CD \rightleftharpoons NCD \quad (6)$$

$$N + B^- \rightleftharpoons NB^- \quad (7)$$

$$NCD + B^- \rightleftharpoons NCDB^- \quad (8)$$

$$NB^- + CD \rightleftharpoons NCDB^- \quad (9)$$

Eqns. 6–9 are simplified by assuming only 1:1 nucleotide–borate complexes are formed. However, they illustrate the complex equilibria involved in determining the mobility of 5'-nucleotide isomers.

Aside from their ability to form complexes with 5'-nucleotides, borate buffers also lead to improved separation of the other isomers. The glycine buffer used to study the effect of ß-CD and the 20 mM borate buffer give rise to the same current during electrophoresis. However, migration times in 20 mM borate are higher than in glycine buffer which represents zero borate concentration in Fig. 5. It may be that borate ions can also interact with the hydroxyl groups on CD thereby imparting a negative charge to the molecule and increasing the migration time of CD complexes. It is also possible that borate ions interact with the silanol groups of fused silica and alter the charge density of the capillary wall.

3.2. Effect of pH

The effect of pH on electrophoretic velocity of cytidine and guanosine nucleotides is illustrated in Fig. 6. In the range pH 8 to 9, CMP and GMP nucleotides exhibit similar behaviour. However, above pH 9, selectivity of the system changes dramatically. The electrophoretic velocity increases for GMP isomers and decreases for CMP isomers. The presence of an amide-like nitrogen at position 1 of guanosine imparts an acidic character to GMP (pK_a of 5'-GMP is 9.4) [31]. This means that at pH 9.6 GMP isomers have an additional negative charge and consequently experience a significant increase in electrophoretic mobility toward the cathode. Conversely, the bases of CMP isomers remain neutral in the pH range studied here since all of the ring nitrogens are basic (pK_b of 5'-CMP is 4.5) [31]. A similar change in selectivity was observed for acidic UMP isomers and basic AMP isomers.

In general, the migration time of the whole mixture increased with increasing pH. At higher pH the migration time window is larger for two reasons. First, 5'-nucleotide–borate complexes exhibit a larger negative charge due to ionization of free hydroxyl groups of borate ($pKa = 9.14$)

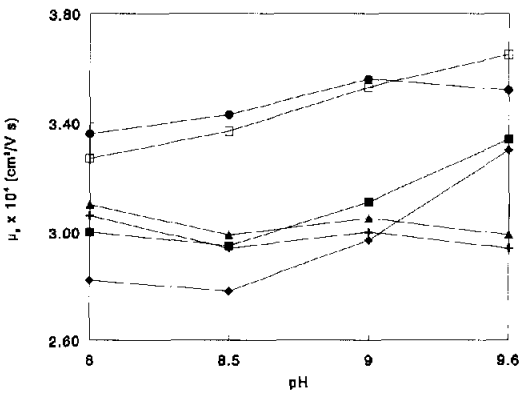

Fig. 6. Effect of pH on electrophoretic mobility. Conditions: 20 mM borax buffer containing 10 mM ß-CD, NaCl was added to maintain constant current. ■ = 2'-GMP; ● = 5'-CMP; ◆ = 3'-GMP; □ = 5'-GMP; ▲ = 2'-CMP; + = 3'CMP.

[32]. This results in a stronger attraction to the anode and therefore an increase in migration time for the 5'-isomers. Also, the two acidic nucleotides, GMP and UMP, exhibit increased migration times due to partial ionization of their amide groups.

Fig. 7 shows the effect of pH on resolution of individual 2'-, 3'-nucleotide isomers. Resolution of the basic nucleotides, CMP and AMP, is not affected significantly by pH. Conversely, both UMP and GMP show a large decrease in resolution with increasing pH. This suggests that partial ionization of the purine or pyrimidine ring results in a weaker nucleotide–CD complex. This can be explained by the fact that the strength of the inclusion complex is generally proportional to the hydrophobicity of the guest molecule.

3.3. Optimal separation

The separation of the complete nucleotide mixture in 20 m*M* borate buffer containing 10 m*M* ß-CD is shown in Fig. 8. There is a dramatic improvement over separations performed in borate-free buffer (Fig. 4b). Using complexation with both borate and CD all 12 nucleotides are resolved in less than 15 min.

Buffers containing SDS micelles in addition to optimal concentrations of CD and borate were also tested. Previous work indicated that separation of negatively charged molecules, based on complexation with CDs, could be improved by using micellar solutions [33]. In this case, the opposite appears to be true. Separations in borate buffers containing both ß-CD and 15 m*M* SDS exhibited the same migration order and selectivity as those in borate buffers without ß-CD. The main difference was that migration times in the micellar system were about 15% higher. This indicates that partitioning of nucleotides into the micellar phase is preferred over the CD phase. Thus, even though the migration time window is increased resolution actually deteriorates.

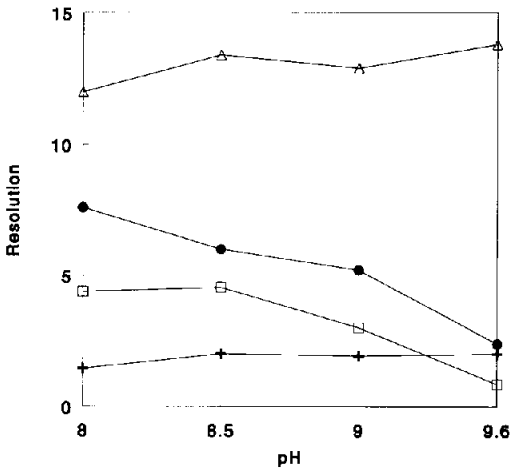

Fig. 7. Effect of pH on resolution of 2'- and 3'-nucleotides, conditions as in Fig. 6. △ = AMP; ● = GMP; □ = UMP; + = CMP.

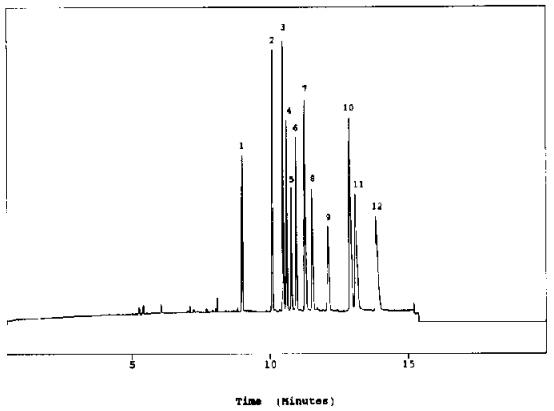

Fig. 8. CE separation of 12 nucleotides based on complexation with borate and ß-CD. Conditions: 20 m*M* borate, 10 m*M* ß-CD, pH 9. Peaks: 1 = 3'-AMP; 2 = 2'-AMP; 3 = 3'-GMP; 4 = 3'-CMP; 5 = 2'-CMP; 6 = 2'-GMP; 7 = 3'-UMP; 8 = 2'-UMP; 9 = 5'-AMP; 10 = 5'-GMP; 11 = 5'-CMP; 12 = 5'-UMP.

4. Conclusions

Capillary electrophoresis employing complex-forming reactions with cyclodextrin and borate appears to be very effective for resolution of various nucleotide isomers. The method described here is much faster than HPLC separations for resolving nucleotide isomers. The high efficiencies available with CE allow molecules whose migration times are very similar to

be resolved. Therefore, purine nucleotides which formed weak complexes with CD could easily be separated in a short period. CE separations are also simpler and less expensive than HPLC since unmodified fused-silica capillaries and readily available reagents are used. The possibility of using ß-CD and borate complexation to separate oligonucleotides is being studied.

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