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NOTE

SEPARATION OF OLIGONUCLEOTIDES BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Kyung Ho ROW

Separation Process Lab.,
Division of Chemical Engineering, Korea Institute of Science and Technology,
P.O. Box 131, Cheongyang, Seoul, Korea

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Abstract—The use of Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) was investigated for the separation of oligonucleotides. By the gradient elution with the mobile phase of methanol, 0.1 M KH_2PO_4 and 0.02 M $MaCl_2$, the mixtures could be separated in a 250 mm×4.6 mm I.D. column. The number of theoretical plates and resolution were compared between this technique and Micellar Electrokinetic Capillary Chromatography (MECC). The charged nucleic acid constituents in the RP-HPLC were relatively better separated than in the MECC.

INTRODUCTION

Reversed-phase high performance liquid chromatography (RP-HPLC) has proven very useful for the analysis of bases, nucleosides, and nucleotides because of its high sensitivity, accuracy, and reproducibility[1].

The separation of the oligonucleotides by this technique is determined by the base composition and chain length of the compounds to be separated, which in turn determine their polarity and size. This system has been utilized to investigate the presence of modified nucleotides in hydrolysates of RNA, DNA, and physiological fluids such as urine and blood serum[2]. Extensive studies have been done on the effects of column temperature, methanol concentration and the pH of the mobile phase on the retention of modified nucleosides[3].

Nucleosides and nucleotides are known to complex with metal ions, alkali, and alkaline earth metals binding to the phosphate groups and transition metals which complex with the ^7N of purine moieties[5]. This complexation changes the electronic structure which alters the solute-solvent interactions and thus retention behavior. In this study, the oligonucleotides of five tetramers and four hexamers have been separated by an addition of a metal ion. The column efficiency and the resolution between RP-HPLC and MECC (micellar electrokinetic capillary chromatography) has also been compared.

EXPERIMENTAL

A liquid chromatograph of waters was used with a Model 600E multisolvent delivery system, a Model U6K injector, and a Model 490 programmable multiwavelength detector. The column was an Alltech 250 mm (length)×4.6 mm (inner diameter) C18 HS column (0.07 mm packings). UV absorbance was monitored at 254 nm.

Special grade HPLC water and methanol were obtained from American Burdick and Jackson (Muskegon, MI) and were used without further purification. Reagent grade KH_2PO_4 was obtained from Mallinckrodt, Inc. (St. Louis, MO), and $MgCl_2$ from J.T. Baker, Inc. (Phillipsburg, NJ). The five tetradeoxyribonucleotides of 5'-d(GCGC)-3', 5-d(CCGG)-3', 5'-d(CGCG)-3', 5'-d(GGCC)-3', and 5'-d(AGCT)-3' and the four hexadeoxyribonucleotides of d[ITGCAT], d[ATGMe5CAT], d[ATGUAT], and d[N-6MeATGCAT] were purchased from Pharmacia (Piscataway, NJ).

Potassium dihydrogen phosphate and chloride salt of Mg were used preparing the mobile phase. The pH of the solution was adjusted to 6.6 using Tris HCl or Tris base. After the addition of methanol, the solvent blends were filtered through 0.0045 mm Nylon 66 membranes (Supelco, Bellefonte, PA) and degassed by bubbling helium through at 30 ml/min. In series of experiments, data were acquired by a Maxima 820 chromatography workstation.

RESULTS AND DISCUSSION

Generally all the charged compounds elute close to the column void volume in absence of metal ion additive in the mobile phase of water/20% methanol. However, their retention times increase significantly with increase in metal ion concentration. In the previous paper[6], five different metal ion additives of K^+, Mg^{+2}, Mn^{+2}, Ni^{+2}, and Zn^{+2} were compared and it showed that the effect of the magnesium ion on charged nucleotides was most marked and their retention behaviors were significantly changed with the magnesium ion concentrations. Since magnesium has a higher affinity for the phosphate [7], this result may be expected.

A mixture of isomeric oligonucleotides containing 5'(GCGC)-3', 5'-d(CCGG)-3', 5'-d(CGCG)-3', 5'-d(GGCC)-3', and 5'-d(AGCT)-3' was examined using 0.1 M KH_2PO_4 and 0.02 M $MgCl_2$ at pH 6.6 (see Figure 1). Four of the five tetramers are isomers having two deoxyguanosines and two deoxycytidines. This is a difficult separation to achieve in that the structures and charges of these tetramers are very similar to each other. The separation is as good as for the neutral nucleosides reported in Ref. [3].

Application of RP-HPLC to the separation of hexadeoxyribonucleotides is shown in Figure 2. These four hexamers have the same backbone sequence of purine and pyrimidine deoxyribonucleotides, and they differ only in the base substituents or modifications. As in the tetramers, methanol gradient conditions were examined with the results shown in the two figures. The decrease in KH_2PO_4 concentration and the increase in methanol concentration resulted in greater difference in retention of the oligonucleotides. These figures also show that the retention times of the hexamers are longer than those of the tetramers, even though the two experimental conditions are slightly different. A higher negative molecular charge increases the complexation of the hexamer with the metal ion, which retards the elution of the oligomer.

Comparisons of the number of theoretical plates and resolution of between RP-HPLC and MECC are listed in Table 1. The experimental data of the MECC were taken from Ref.[8]. In the tetramers, the number of theoretical plates and resolution of the MECC is better than those of the RP-HPLC except 5'-d(AGCT)-3'. The MECC (68 cm of column length) has the more performance to separate the four isomers of subtle molecular differences. This is mainly due to the narrower peak width from an addition of the surfactant of sodium dedecyl sulfate[9] and much smaller column diameter (0.06 mm). It is interesting to note that in the hexamers which have the six phosphate groups in the oligonucleotide chain, such the trend is reversed. This suggests that as the number of the phosphate group increases, the magnesium ion in the mobile phase of the RP-HPLC may complex vigorously with the phosphate group, leading to stronger interaction and better resolution. It may be expected in the MECC, negative charges of micelles and coulombic repulsion do not effectively separate the hexamers, even though in the nucleosides which do not contain the phosphate group, efficiencies up to 500,000 theoretical plates/m and better resolution were realized[9]. The neutral nucleosides are resolved based on their differential partitions between an aqueous mobile phase by the electroosmotic flow and a hydrophobic interior of the

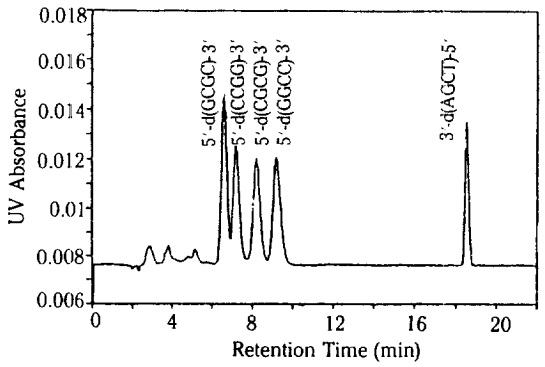

Fig. 1. Nucleic base sequence-selective separation using RP-HPLC.
(1 ml/min flow rate, initial composition of mobile phase: 17% (by vol.) MeOH, 80% 0.1 M KH_2PO_4, 3% 0.02 M $MgCl_2$, final: after 5 min, step increase to 25% MeOH, 72% 0.1M KH_2PO_4, 3% 0.02M $MgCl_2$)

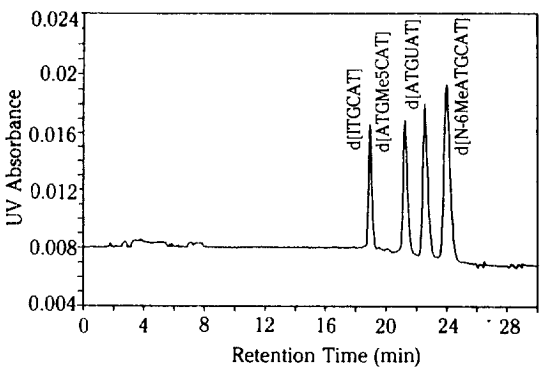

Fig. 2. Nucleic base sequence-selective separation using RP-HPLC.
(1 ml/min flow rate, initial composition of mobile phase: 18.2% (by vol.) MeOH, 75.8% 0.1M KH_2PO_4, 6% 0.02M $MgCl_2$, final: after 4 min, step increase to 27.8% MeOH, 66.2% 0.1M KH_2PO_4, 6% 0.02M $MgCl_2$)

Table 1. Comparison of number of theoretical plates and resolution betwen RP-HPLC and MECC

	RP-HPLC	MECC*
(Number of Theoretical Plates)		
5'-d(GCGC)-3'	2000	5400
5'-d(CCGG)-3'	1800	6100
5'-d(CGCG)-3'	2000	2200
5'-d(GGCC)-3'	1800	8800
5'-d(AGCT)-3'	25300	5200
d(ITGCAT)	19800	4100
d(ATGMe5CAT)	13300	5000
d(ATGUAT)	13800	2500
d(N-6MeATGCAT)	9200	1100
(Resolution)		
5'-d(GCGC)-3' and 5'-d(CCGG)-3'	0.93	3.11
5'-d(CCGG)-3' and 5'-d(CGCG)-3'	1.40	1.69
5'-d(CGCG)-3' and 5'-d(GGCC)-3'	1.25	1.13
5'-d(GGCC)-3' and 5'-d(AGCT)-3'	14.00	2.99
d(ITGCAT) and d(ATGMe5CAT)	3.61	1.16
d(ATGMe5CAT) and d(ATGUAT)	1.71	0.69
d(ATGUAT) and d(N-6MeATGCAT)	1.58	0.32

*Capillary column: 0.06 mm (diameter) and 68 cm (length)
Mobile phase: 0.075 M sodium dodecyl sulfate, 0.01M Na_2HPO_4, 0.006M $Na_2B_4O_7$
Separation voltage: 10 kv (tetramers), 23 kv (hexamers)

micelles[10]. However, it must be mentioned that MECC and RP-HPLC are equally suitable for separations of bases and nucleosides, but for oligonucleotides, the RP-HPLC is superior to the MECC. And for the preparative work, there is as yet no alternative to the RP-HPLC, since the MECC is limited to the microanalytical technique.

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