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RETENTION MODIFICATION OF NUCLEIC ACID CONSTITUENTS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

R. S. RAMSEY*
Oak Ridge National Laboratory, Analytical Chemistry Division, Post Office Box 2008, Oak Ridge, TN 37831-6120 (U.S.A.)
V. W. CHAN
College of Wooster, Wooster, OH 44691 (U.S.A.)
B. M. DITTMAR
Duke University, Durham, NC 27706 (U.S.A.)
and
K. H. ROW
University of Tennessee, Department of Chemistry, Knoxville, TN 37996 (U.S.A.)

SUMMARY

Secondary equilibria in reversed-phase liquid chromatography have been investigated as a means of enhancing selectivity and optimizing separations of nucleic acid constituents. The retention behavior of various nucleotides, nucleosides and modified compounds has been examined as a function of five different metal ion additives in the mobile phase: K^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}. Complexation of the solute molecules with the metal ions changes the electronic structure and alters solute–solvent interactions. Alkali and alkaline earth metals bind primarily to phosphate groups while transition metals also interact with the N^7 of purine bases. All nucleotides were found to be eluted very close to the void volume of the high-performance liquid chromatographic column without any metal additive, but retention increased as the concentration of a given cation increased. The transition metals were found to have the greatest effect, with affinities for nucleotide monophosphates on the order of 100 times greater than potassium, and 10 times that of magnesium. Differences in affinity based upon phosphate structure (*i.e.*, cyclic *vs.* linear), phosphate position (*e.g.*, 2′- *vs.* 3′-monophosphates), and base modification were also noted. The retention of most nucleosides, unlike the charged compounds, remained relatively constant as the ionic strength or type of cation was varied. Also, improvements were obtained in the resolution of some oligonucleotides with the addition of divalent ions to a potassium buffer mobile phase.

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INTRODUCTION

The basic parameters that affect retention behavior in reversed-phase high-performance liquid chromatography (HPLC) (*i.e.*, pH, type and amount of organic modifier, ionic strength, and temperature) have been investigated in detail for nucleotides, nucleosides, and bases[1–6]. Since these compounds are ionizable, some secondary equilibria effects using oppositely charged mobile phase additives have also been examined[1,7–11]. In this case, pairing reagents, such as tetraalkylammonium or alkylsulfonate ions may be added, resulting in one type of interaction, or small, charged inorganic constituents may be used for other types of interactions. It is assumed that large hydrophobic ions are adsorbed on the non-polar stationary phase and that ion-pair formation then occurs at the mobile phase–stationary phase interface[8,12]. With the small inorganic ions the pairing interaction occurs primarily in the mobile phase, although the exact retention mechanism may depend upon the actual operating conditions[1,9,12].

Nucleotides are known to complex with metal ions, alkali and alkaline earth metals binding to the phosphate groups, and transition metals also interacting with the N^7 of the purine bases[1,11,13–15]. This complexation changes the electronic structure and the conformation of the compounds, and this in turn alters solute–solvent interaction and retention behavior. Horváth *et al.*[9] demonstrated that the addition of Mg or Zn ions to the mobile phase resulted in increased retention of some nucleoside di- and triphosphates. The chromatographic data showed a dependence of the capacity factors on the metal ion concentration and allowed the determination of apparent stability constants. The increase in capacity factors was attributed to the reduction of electronic charge in the molecules, allowing for stronger interactions on the non-polar stationary phase. Cohen and Grushka[10] also examined the effects of some divalent metal ions on the retention of nucleotides, nucleosides, and bases. Ni and Zn at low levels reduced k' values for nucleotides, while higher concentrations slightly increased the values. Mg at all levels was found to decrease retention while Cu provided an increase. Braumann and Jastorff[7] on the other hand demonstrated that Mg and K increased the retention of some nucleoside mono- and cyclicphosphates. Their data also indicate that differences in complexation (and retention) may be related to the structure of the phosphate group and to the nature of the cation. The discrepancies in data in these studies likely pertain to differences in buffer composition and ionic strengths[1,7]. The retention will be influenced by all the cations present in the mobile phase and if the concentration of the metal ion under investigation is low relative to other ions, the effect may appear negligible.

In this study, the effects of five different metal ions (K^+, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}) on the retention behavior of a variety of nucleotides and nucleosides differing in base modification, phosphate structure, or phosphate position have been examined. Mobile phases containing only the one cation under investigation were used to eliminate the confounding effects other metal ions may have. The data were generated to indicate how metal ion interaction may be utilized to enhance selectivity and optimize separations. Results for some oligonucleotides are also reported.

EXPERIMENTAL

Instrumentation

The oligonucleotides were analyzed on a Waters liquid chromatographic system, equipped with a Model 600E multisolvent delivery system, a Model U6K injector, and a Model 490 programmable multiwavelength detector (Waters Chromatography Division, Millipore, Millford, MA, U.S.A.). Data acquisition was performed by a Maxima 820 chromatography workstation (Dynamic Solutions, Millipore, Ventura, CA, U.S.A.). The other compounds were analyzed on a Beckman LC system consisting of two Model 112 solvent delivery modules and a Model 421 system controller (Beckman Instruments, Altex Division, San Ramon, CA, U.S.A.). Injections were made with a Rheodyne Model 7125 injector, using a 20-μl sample loop (Rheodyne, Cocatati, CA, U.S.A.). The UV absorbance was monitored at 254 nm.

Materials and methods

The oligonucleotides were separated on an Alltech 250 × 4.6 mm C_{18} HS column (7 μm), while other nucleic acid constituents were analyzed on a Vydac 250 × 4.6 mm C_{18} HS column (10 μm), (both from Alltech Assoc., Deerfield, IL, U.S.A.). Potassium dihydrogenphosphate or chloride salts of Mg, Mn, Zn and Ni were used for preparing the mobile phase. The pH of these solutions was adjusted to 6.6, using Tris–HCl or Tris, with the exception of the KH_2PO_4 solution for which KOH was used. Methanol (Burdick & Jackson, Muskegon, MI, U.S.A.) was then added to provide a 20% (v/v) concentration and the final solutions were filtered through 0.45-μm Nylon 66 membranes (Supelco, Bellefonte, PA, U.S.A.). Data were obtained at a flow-rate of 1.5 ml/min at ambient temperatures. Retention times were measured from the start of the injection and all analyses were performed in duplicate.

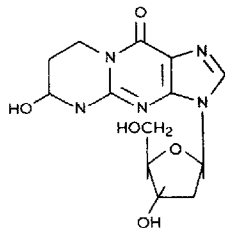
KH_2PO_4 was obtained from Mallinckrodt (St. Louis, MO, U.S.A.), $MgCl_2$ from J. T. Baker (Phillipsburg, NJ, U.S.A.), $MnCl_2$ and $NiCl_2$ from Tridom/Fluka (Hauppauge, NY, U.S.A.), Tris–HCl and Tris from Eastman Kodak (Rochester, NY, U.S.A.), KOH from Fisher Scientific (Pittsburgh, PA, U.S.A.), and $ZnCl_2$ from Aldrich (Milwaukee, WI, U.S.A.). Only certified or analyzed purity grade reagents were used. Over 35 different nucleic acid constituents were examined for changes in retention behavior as a function of metal ion additive (type and concentration) to the mobile phase. These compounds are listed in Table I. They were obtained from Schwarz Labs. (Orangeburg, NY, U.S.A.), Aldrich, Sigma (St. Louis, MO, U.S.A.), P-L Biochemicals (Milwaukee, WI, U.S.A.), Calbiochem (Los Angeles, CA, U.S.A.), or Tridom/Fluka. The compounds differed in their total charge (*e.g.*, deoxyadenosine diphosphate, deoxyadenosine monophosphate and deoxyadenosine), the location of charge (*e.g.*, guanosine 2′-monophosphate and guanosine 3′-monophosphate), or in the structure of the base (*e.g.*, 8-bromoadenosine 5′-monophosphate and adenosine 5′-monophosphate). The oligonucleotides were purchased from Pharmacia (Piscataway, NJ, U.S.A.). In addition to the commercially available compounds, two deoxyguanosine derivatives (6- and 8-hydroxyacrolein–deoxyguanosine adducts), synthesized according to the procedure by Chung *et al.*[16], were examined. Stock solutions (10 m*M*) of all the standards were prepared and diluted 1:50 for working standards, using HPLC-grade water (Burdick & Jackson).

TABLE I

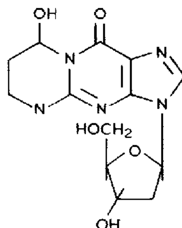
NUCLEIC ACID CONSTITUENTS EXAMINED AS A FUNCTION OF METAL ION ADDITIVE

Compound	*Abbreviation*
Adenosine 5′-monophosphate	5′-AMP
Adenosine 3′-monophosphate	3′-AMP
Adenosine 2′-monophosphate	2′-AMP
8-Bromoadenosine 5′-monophosphate	8-Br-5′-AMP
Adenosine 2′,3′-monophosphate	2′,3′-cAMP
Adenosine 3′,5′-monophosphate	3′,5′-cAMP
8-Bromoadenosine 3′,5′-monophosphate	8-Br-3′,5′-cAMP
Guanosine 2′-monophosphate	2′-GMP
Guanosine 3′-monophosphate	3′-GMP
Guanosine 3′,5′-monophosphate	3′,5′-cGMP
Guanosine 2′,3′-monophosphate	2′,3′-cGMP
2′-Deoxycytidine 5′-monophosphate	dC-5′-MP
2′-Deoxycytidine 3′-monophosphate	dC-3′-MP
2′-Deoxycytidine 3′,5′-diphosphate	dC-3′,5′-DP
Cytidine 5′-monophosphate	5′-CMP
2′-Deoxyadenosine 5′-monophosphate	dA-5′-MP
2′-Deoxyadenosine 5′-diphosphate	dADP
2′-Deoxyadenosine	2′-dA
2′-Deoxycytidine	2′-dC
5-Chloro 2′-deoxycytidine	5-Cl-2′-dC
5-Methyl 2′-deoxycytidine	5-Me-2′-dC
2′-Deoxyuridine	2′-dU
5-Bromo 2′-deoxyuridine	5-Br-2′-dU
2′-Deoxyguanosine	2′-dG
8-Bromodeoxyguanosine	8-BrdG
6-Hydroxyacrolein deoxyguanosine adduct[a]	6-OHAdG
8-Hydroxyacrolein deoxyguanosine adduct[b]	8-OHAdG
Adenosine	A
Guanosine	G
8-Bromoguanosine	8-BrG
Thymidine	T
3-Methylthymidine	3-MeT
Uridine	U
5-Aminouridine	5NHU-s
5-Aminouracil	5NHU
Cytidine	C

[a]



[b]



RESULTS

Data for all compounds were obtained without the addition of any metal ions, using only 20% aq. methanol as the mobile phase. Under these conditions, most charged compounds were found to be eluted close to the void time of the column. However, as metal ions were added, the retention times were found to increase significantly. Fig. 1 shows results for various adenosine nucleotides as the K^+ concentration is increased from 1 to 100 m*M*. The two cyclic compounds, adenosine 3',5'-monophosphate and adenosine 2',3'-monophosphate, and deoxyadenosine diphosphate show an almost linear increase over the entire range. The measurable increases between no additive and 1 m*M* K^+ indicate that these compounds have a strong affinity for the metal ion. Increases in retention for adenosine 3'-monophosphate, adenosine 5'-monophosphate, and deoxyadenosine 5'-monophosphate on the other hand are more pronounced above 1 m*M* K^+. Overall, the singly charged cyclic nucleotides showed the greatest change in retention, followed by the doubly charged compounds, and finally deoxyadenosine diphosphate. These results are in agreement with the work of Braumann and Jastorff[7], who also suggested that the resulting cyclic nucleotide–metal complexes may attain electrical neutrality, as

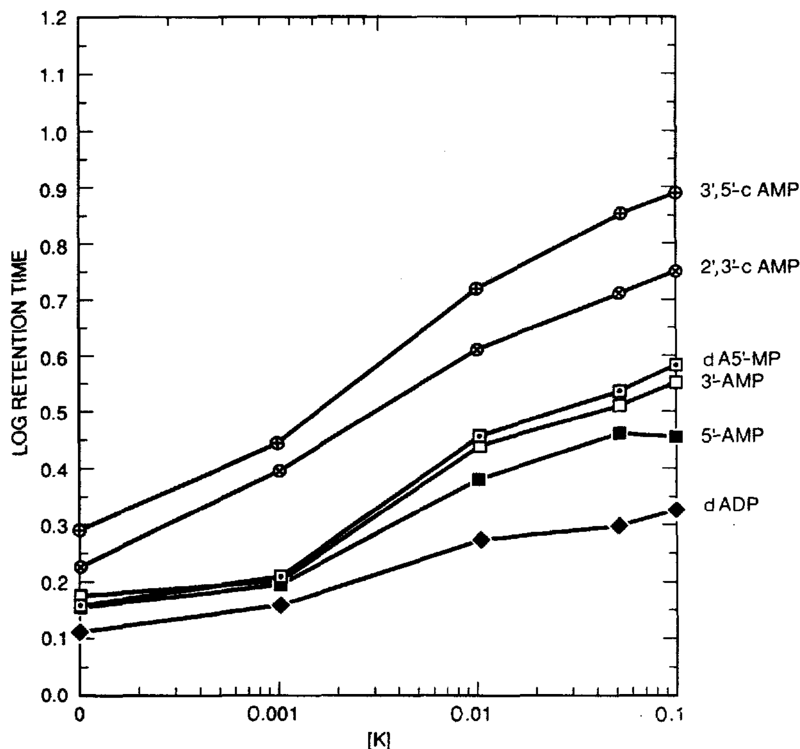


Fig. 1. Retention behavior of various adenosine nucleotides as a function of K^+ levels in mobile phases containing different molar concentrations of KH_2PO_4 in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

evidenced by their capacity factors, which are similar to uncharged adenosine (see Fig. 6).

The effect of magnesium ion on charged nucleotides was more marked, as indicated by the increases in retention at low cation concentration (*i.e.*, 1 m*M* Mg^{2+}). Since magnesium has a higher affinity for phosphate[7], these results may be expected. From Fig. 2 it can also be concluded that retention behavior is not significantly changed above 10 m*M* Mg^{2+}, and this indicates that the effects of complexation are saturated at that level. Results for the other nucleotides (*i.e.*, GMP and dCMP compounds) were similar to the AMP compounds, with retention increasing up to about 10 m*M* Mg^{2+} and then leveling off. However, there was no preferential binding of the Mg to the guanosine cyclic monophosphates over 2′-GMP or 3′-GMP. The retention of all four GMP compounds was about the same at each Mg level. Both the GMP and dCMP nucleotides had capacity factors approaching their corresponding nucleosides at cation saturation (*i.e.*, 10 m*M* Mg^{2+} or above). From the data in Fig. 2 it is also interesting to note that the base-modified 8-Br-3′,5′-cAMP and 8-Br-5′-AMP exhibited sufficiently different retention behavior from their corresponding parent compounds (*i.e.*, 3′,5′-cAMP and 5′-AMP) with the addition of Mg ion to the mobile phase that they may be resolved. At 0.1 *M* Mg^{2+} the resolution for 8-Br-3′,5′-cAMP and 3′,5′-cAMP is 5.6 and for 8-Br-5′-AMP and 5′-AMP is 0.9 ($R = 2\Delta t/W_{b1} + W_{b2}$, where t = retention time and W_b = base width of the peak).

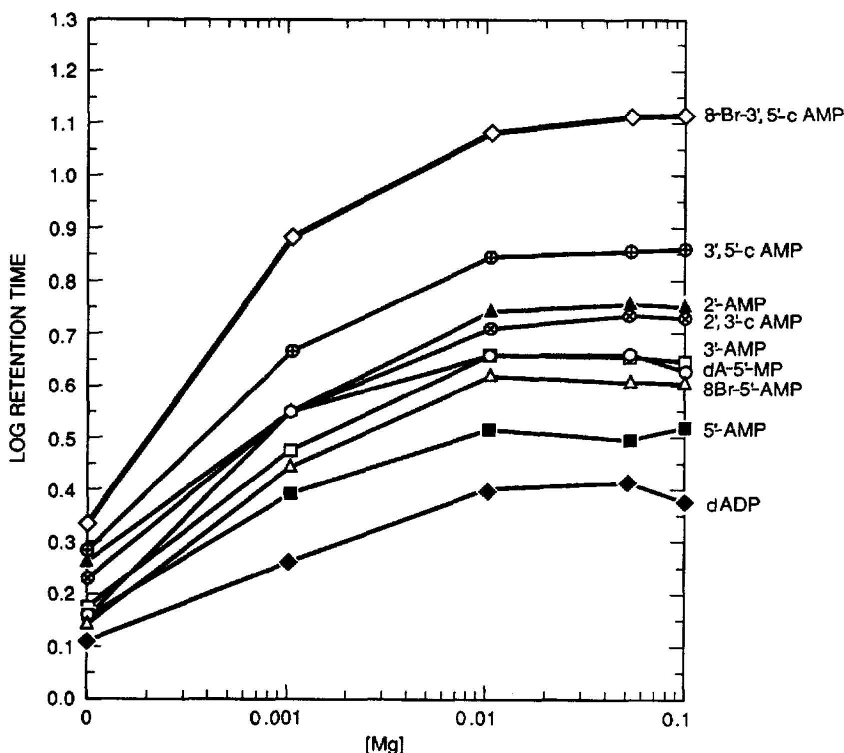


Fig. 2. Retention of adenosine nucleotides as a function of Mg^{2+} levels in mobile phases containing different molar concentrations of $MgCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

Qualitatively, the results for Mn were similar to those for Mg. The major difference is that Mn appears to have a higher affinity for the nucleotides (*i.e.*, the capacity factors for most compounds at 1 m*M* Mn were similar to those at 10 m*M* Mg). Ni and Zn were also found to have very high affinities for the charged compounds. Data for adenosine mononucleotides are shown in Figs. 3 and 4. It should be noted that nucleotides carrying a negative charge of 2 or more were not eluted from the column with 5 m*M* or 10 m*M* Zn^{2+} or with 10 m*M* Ni^{2+} ion added. To determine whether these compounds may be precipitating on the column, 100-ppm solutions of the standards in the mobile phase (10 m*M* $ZnCl_2$ or $NiCl_2$ in 20% aq. methanol) were prepared and examined for any cloudiness. Deoxyadenosine diphosphate, 3′,5′-dCDP, 2′-GMP, 3′-GMP, 5′-dCMP, and 3′-dCMP were found to precipitate with the $ZnCl_2$ and 2′-GMP and 3′-GMP with the $NiCl_2$ solution. After the analytical column had been exposed to 10 m*M* Zn^{2+} for several days and after the analysis of various nucleotides had been attempted, the retention factors were also found to fluctuate for both charged and uncharged compounds. As efforts to reactivate the column by flushing it with water were unsuccessful, the column was replaced. The new column was characterized by analyzing all the standards with the 10 m*M* $MgCl_2$ mobile phase, and the capacity factors were found to be within 5% of the old column. Studies with Ni and Zn cations then proceeded, starting with 0.5 m*M* concentrations and carefully washing the column before introducing the next higher concentration. Compounds

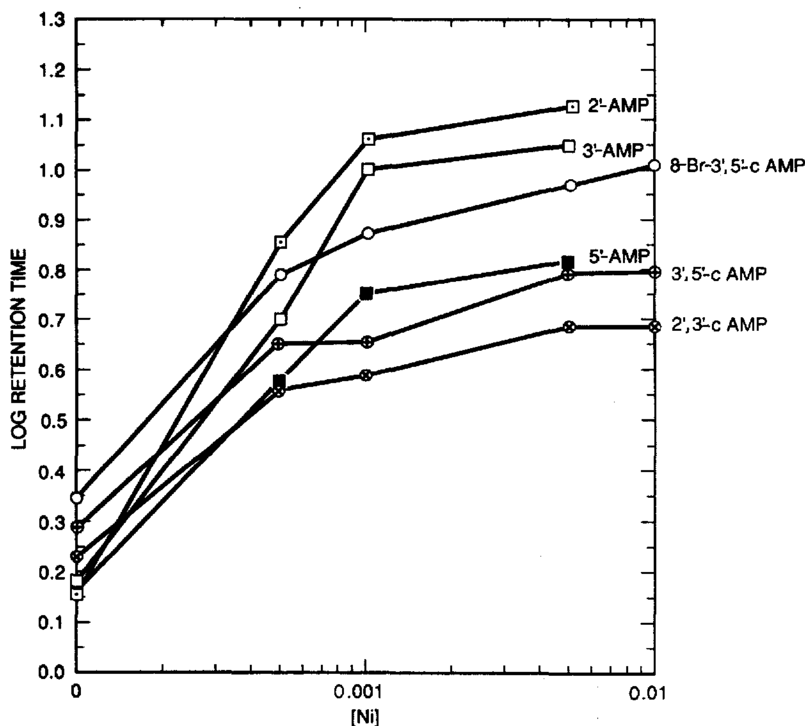


Fig. 3. Retention of adenosine nucleotides as a function of Ni^{2+} levels in mobile phases containing different molar concentrations of $NiCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

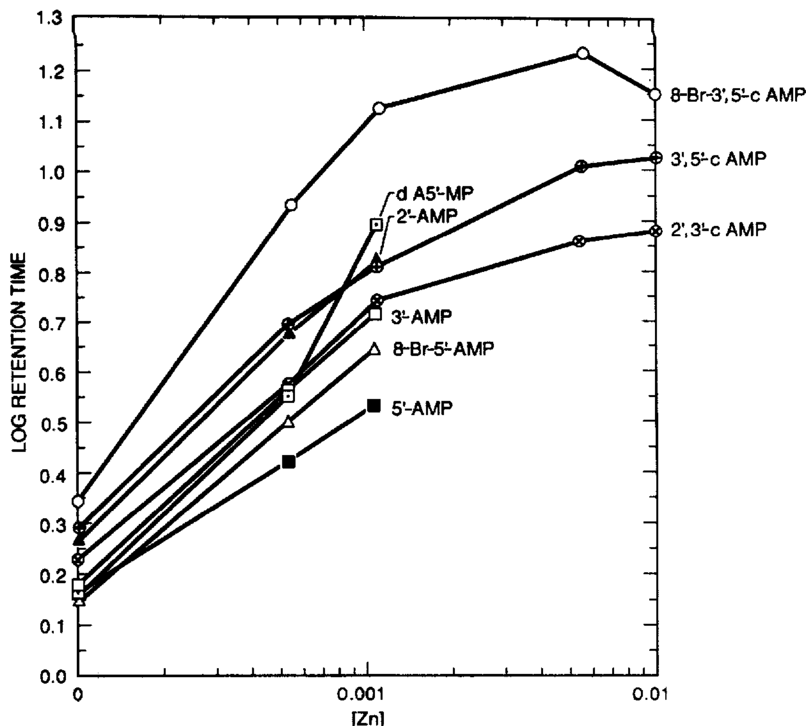


Fig. 4. Retention of adenosine nucleotides as a function of Zn^{2+} levels in mobile phases containing different molar concentrations of $ZnCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

that were not eluted from the old column at a given cation concentration were not reexamined on the new column under the same conditions. Comparing the Ni, Mn and Zn data, Ni was shown to have a much stronger affinity for the monophosphates than for the cyclic monophosphates. In fact, differences in retention between 2'- or 3'-GMP and 2',3'-cGMP or 3',5'-cGMP at 1 m*M* Ni^{2+} exceeded 10 min.

Unlike the charged species, the retention times of most uncharged compounds remained fairly constant as the concentration or the type of metal ion was varied. Fig. 5, which shows the results of various nucleosides with Ni^{2+} ion, is representative of the data obtained for these compounds with K^+, Mg^{2+}, Mn^{2+}, or Zn^{2+}. The only compound found to shift retention as a function of the type of metal ion added was 8-aminoguanosine. With 10 m*M* Zn^{2+} the retention time more than doubled from that obtained using 10 m*M* of any of the other metal cations. Fig. 6 shows data for this compound and other purine nucleosides as a function of the Zn^{2+} concentration. The addition of the amine group to guanosine results in a compound that may have multiple conjugated forms of varying stability. At different ionic strengths forms that are more or less amenable to complexation or aggregation may predominate, leading to differences in retention. The reason for the increase in retention of this compound with Zn^{2+} only is not understood at this time. It is also interesting to note that the 6- and 8-hydroxyacrolein derivatives of deoxyguanosine exhibited different retention

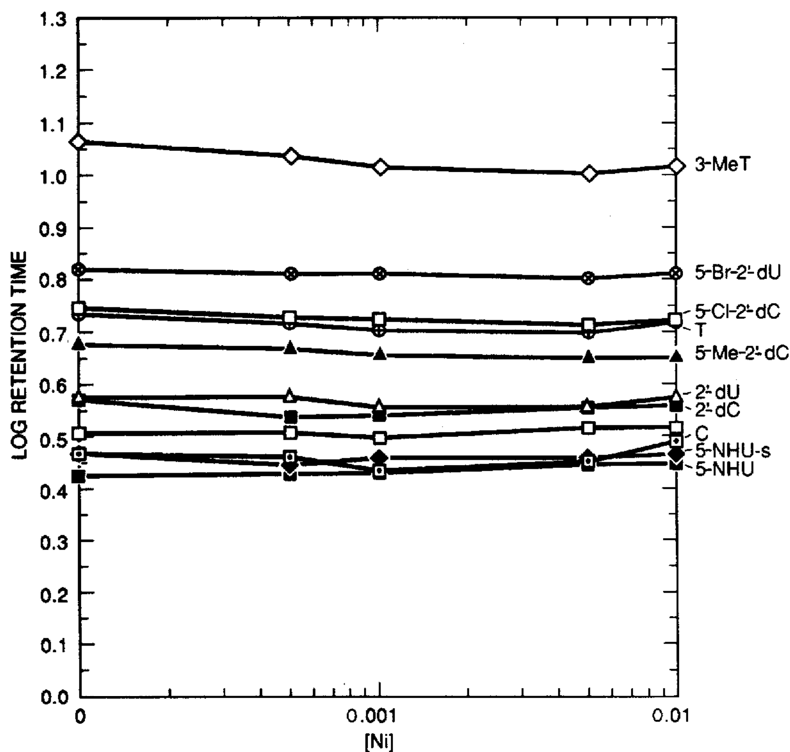


Fig. 5. Retention of various nucleosides and modified nucleosides as a function of Ni^{2+} concentration in the mobile phase. Abbreviations identified in Table I.

properties, despite similarities in their structure. The retention between the two isomers at 10 m*M* Zn^{2+} is 1.7.

The effects of metal cations on the separation of some oligonucleotides were also briefly investigated. A mixture of tetramers containing 5'-d(GGCC)-3', 5'-d(CCGG)-3', 5'-d(CGCG)-3', 5'-d(GCGC)-3', and 5'-d(AGCT)-3' was examined, using 0.01, 0.1, and 0.2 *M* KH_2PO_4 at pH 6.6 with 20% (v/v) aq. methanol (see Fig. 7). At 10 m*M* K^+ only d(AGCT) was separated from the remaining compounds. However, as the K^+ ion concentration increased, increasing complexation resulted in greater differences in retention among the five compounds. The best isocratic separation was thus obtained with 0.2 *M* K^+, where four peaks and a shoulder were evident. Methanol gradient conditions which would optimize the separations at 0.1 *M* and 0.2 *M* K^+, were also examined, with the results shown in Fig. 8. Attempts to resolve these compounds by using only divalent metal ions were unsuccessful, since the oligonucleotides were found to be eluted as broad, tailing peaks. Peak asymmetry and tailing have been reported as the most significant problem in ion-pair HPLC, and have been attributed to association and dissociation processes[12]. However, we determined that the addition of divalent ions to the K^+ buffer would improve the overall resolution and maintain the efficiency. Figs. 9 and 10 show separations of the tetramers and hexamers, respectively, when KH_2PO_4 doped with $NiCl_2$, was used as the mobile phase. In the case of the

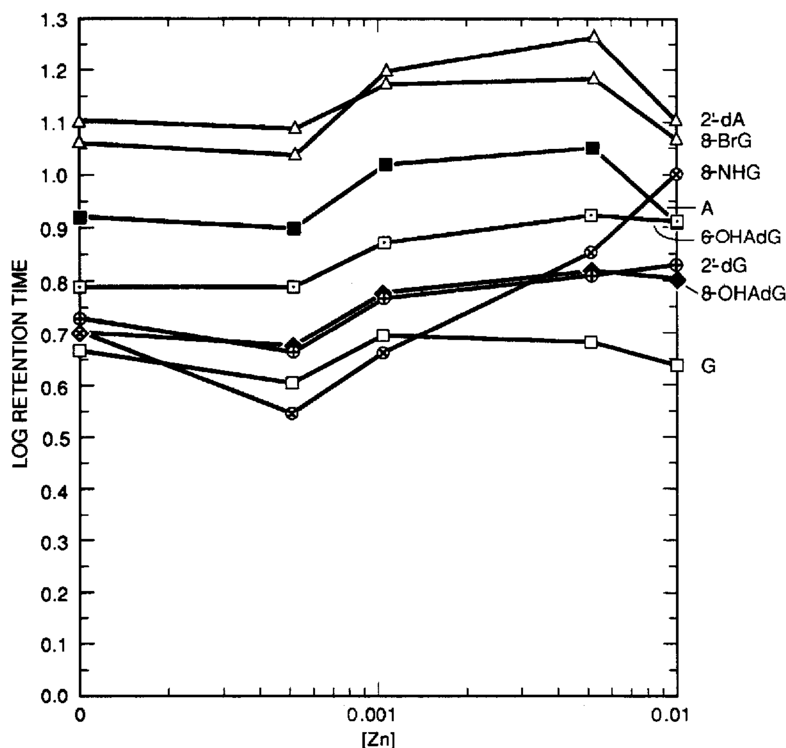


Fig. 6. Retention of nucleosides and modified nucleosides as a function of Zn^{2+} concentration in the mobile phase. Abbreviations identified in Table I.

hexamers, increasing the Ni^{2+} concentration from 1.2 to 3 m*M* altered the selectivity in such a way that 5'-d(ITGCAT)-3', and 5'-d(ATG5MeCAT)-3' were found to be resolved. When only K^{+} buffer was used under similar gradient conditions, only four peaks were observed for the hexamers, in contrast to the six peaks observed when Ni^{2+} was added.

DISCUSSION

This study was undertaken to determine how secondary equilibria through metal-ion complexation may be used to control selectivity and optimize separations of various nucleic acid constituents. Other investigators have examined cation binding for the purpose of determining stability constants or relating chromatographic behavior to known physico-chemical properties of the complex[7,9,10]. The data in the literature also appear to be conflicting, both increasing and decreasing retention with the addition of a given metal ion to the mobile phase having been reported[7,10]. We have shown that as the concentration of the cation is increased, the retention of nucleotides also increases up to the level where the effects of complexation are saturated.

The transition metals were found to have a *ca.* ten times greater affinity for the

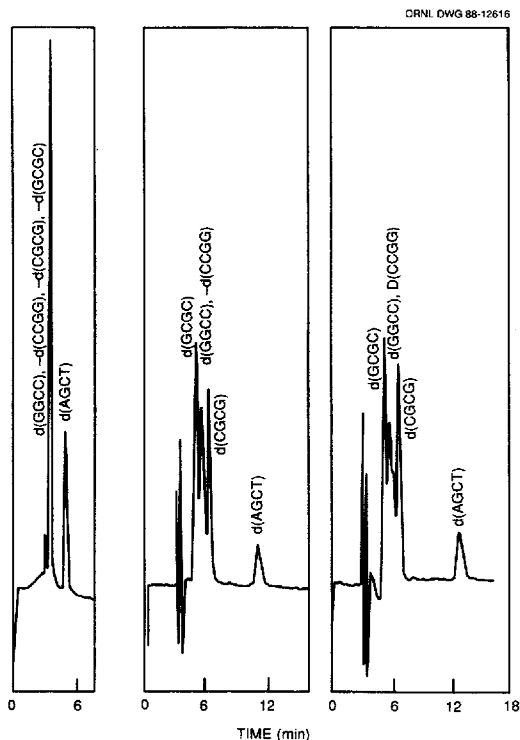


Fig. 7. Isocratic separation of tetramers with (a) 0.01 *M*, (b) 0.1 *M*, and (c) 0.2 *M* KH_2PO_4 (pH 6.6) in 20% (v/v) aq. methanol at a flow-rate of 1 ml/min.

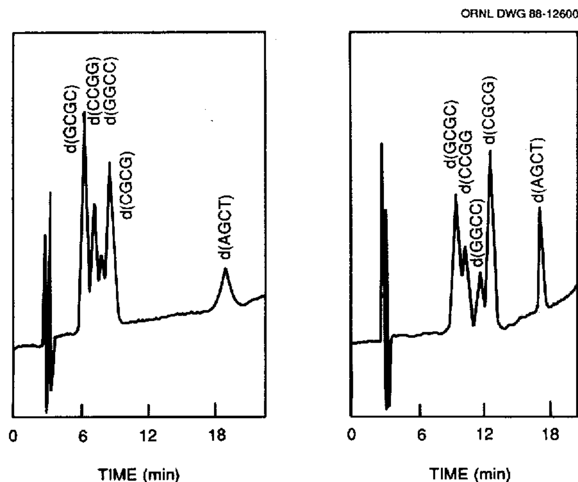


Fig. 8. Gradient separation of five tetramers. (a) Eluent A, 0.1 *M* K H_2PO_4 (pH 6.6) in 10% aq. methanol; eluent B, 0.1 *M* KH_2PO_4 (pH 6.6) in 20% aq. methanol; linear program at 5 min from 80 to 100% B in a 10-min interval; flow-rate 1.0 ml/min. (b) Eluent A, 0.2 *M* KH_2PO_4 (pH 6.6) in 10% aq. methanol; eluent B, 0.2 *M* KH_2PO_4 (pH 6.6) in 30% aq. methanol; linear program at 6 min from 35 to 90% B in a 10-min interval; flow-rate, 1.0 ml/min.

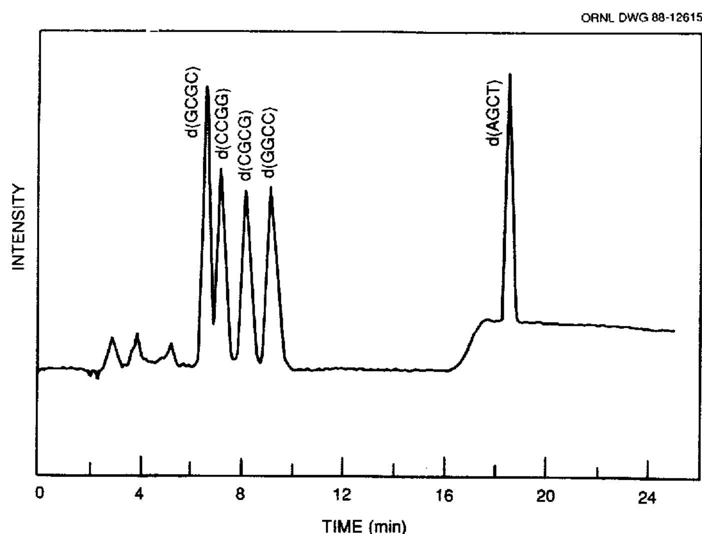


Fig. 9. Separation of five tetramers with the addition of $NiCl_2$ to the mobile phase. Eluent: 0.14 *M* KH_2PO_4, pH 6.6, 1.2 m*M* $NiCl_2$, 17% aq. methanol increasing to 25% aq. methanol after 5 min; flow-rate 1.0 ml/min.

nucleotides than magnesium and 100 times that of potassium, as determined by the differences in concentration at which the effects become saturated. These results on relative affinities follow the order of reported stability constants for nucleotides (*i.e.*, $K^+ < Mg^{2+} < Mn^{2+} < Ni^{2+} < Zn^{2+}$) in coordinating ability[15]. Differences in retention behavior, based upon total charge, charge position, and base modification, were also noted.

Although the divalent metal ions significantly enhanced the separation of charged compounds, some peak broadening for nucleoside monophosphates (linear as

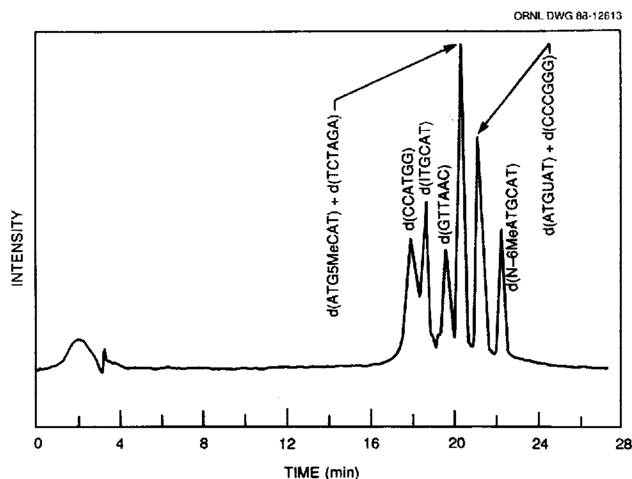


Fig. 10. Separation of hexamers. Eluent: 0.14 *M* KH_2PO_4 (pH 6.6) 1.2 m*M* $NiCl_2$, in 18% aq. methanol increasing to 28% aq. methanol after 5 min; flow-rate 1.0 ml/min.

opposed to cyclic) was observed when Ni^{2+} or Zn^{2+} was used. More importantly, the column performance was found to degenerate after relatively high levels of these ions had been introduced. This may have been due to the precipitation of some complexes in the column or to an exchange of ionic sites, leading to the incorporation of the metal cations. Horváth *et al.*[9] have also reported that Zn^{2+} at relatively high levels (20 m*M*) irreversibly changed the nature of a reversed-phase column they were working with. They reasoned that the changes may have resulted from interaction of the metal ion with surface silanols in the packing material. However, lower concentrations may not pose a problem in terms of column degeneration. Since the separations attainable for the mononucleotides may be effected at low concentrations with these ions, there may be no need to use higher levels. For example, the resolution of guanosine monophosphates from guanosine cyclic monophosphates at 1 m*M* Ni^{2+} may be the same as at 5 m*M* Ni^{2+}.

The nucleosides were largely unaffected by the type or concentration of metal additives. This was the case for both purine- and pyrimidine-type compounds. Notably, however, it was determined that the resolution of oligonucleotides may be improved if low concentrations of divalent cations are added to a K^+ mobile phase. Since complexation occurs mainly in the aqueous phase, rapid rates of formation and dissociation of the complex should result in sharp, symmerical peaks[12]. This is observed when only K^+, or K^+ doped with other metals, is used, but not when only divalent ions are used. In the latter case, the metal ion may complex with more than one phosphate group in the oligonucleotide chain, leading to prolonged interactions, in contrast to K^+, which may couple to only one phosphate at any given time.

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