Journal of Chromatography, 602 (1992) 189–195
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2557

Separation of nucleotides and nucleosides by gradient macrocycle-based ion chromatography

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ABSTRACT

Adsorption of the macrocyclic cryptand *n*-decyl-2.2.2 (D2.2.2) to the matrix of the reversed-phase polystyrene Dionex MPIC column generates a novel mixed-mode chromatographic column on which both ionic and hydrophobic interactions can occur. To the aqueous eluent are added cations that bind dynamically to the adsorbed macrocycle, forming positively charged ion-exchange sites. The hydrophobic tail and the MPIC column matrix provide the basis for hydrophobic interactions. Experiments have been carried out to characterize the use of this column in separating both nucleotides and nucleosides. The influence of eluent cation concentration, type of eluent cation and anion, eluent pH and organic solvent are demonstrated. Excellent resolution of nucleotides and nucleosides was achieved under different conditions, due to the respective differences in the mode of retention between the two compound types. A chromatographic gradient was designed which facilitates the simultaneous determination of both species.

INTRODUCTION

The qualitative and quantitative determination of nucleotides and nucleosides in biological samples is very important for research in nucleic acid biochemistry. The need for separating and quantifying hydrolysates of nucleic acids and free nucleotides has spurred the development of high-performance liquid chromatography (HPLC) for this application. The most commonly used separation techniques involved in the analysis of nucleosides and nucleotides are reversed-phase[1–9], ion-pair[10–15] and ion-exchange[16–21] chromatography.

The conventional ion-exchange method usually allows for the determination of the nucleotides. But this method is not effective for the separation of nucleosides and nucleobases [22]. On the other hand, reversed-phase chromatography is useful for the separation of these molecules, although there are problems in resolving the weakly retained nucleotides [23,24]. Thus, the column-switching technique has been used to separate nucleotides and nucleosides simultaneously in the same analysis [25]. First, the nucleotides are separated on an anion-exchange column. Then, after all the nucleotides are eluted, the C_{18} hydrophobic column is switched into line for separation of the nucleosides and nucleobases. Bischoff and McLaughlin [26–31] developed a mixed-mode chromatography for this purpose. In their experiments a chromatographic matrix which contains sites for both ionic and hydrophobic interactions was used for the separation of the nucleic acid compounds. The mixed-mode matrix could be produced by the addition of hydrophobic moieties to an anion-exchange resin, or the introduction of sites for ionic interactions onto a hydrophobic support. Using this column, oligonucleotides and tRNA molecules were separated with high resolution.

Capillary electrophoresis is another powerful technique for the separation of nucleotides, especially for the separation of oligonucleotides [32]. Nguyen *et al.* [33] developed a method for the separation and quantitation of nucleotides in fish tissues using capillary eletrophoresis. The analysis of three major nucleotides was completed within 15 min. Because there is no negative charge on nucleosides, these are not directly separated by capillary

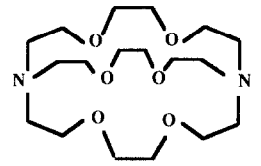
0021-9673/92/$05.00

electrophoresis. Cohen *et al.* [34] solved this problem by using micellar solutions and metal additives to partition nucleosides within the micelles.

Macrocyclic ligands, such as crown ethers, have been used to separate cations [35–38], based on the size-selective binding of metal and other cations [39,40]. Anions have also been separated using bis- and polymeric crown ethers covalently bonded or polymerically coated on silia [41–43]. In our laboratory, we have developed a novel method for employing macrocyclic ligands as exchange sites in the analysis of inorganic anions using chemically suppressed ion chromatography [44]. The column was prepared by coating macrocycles on commercially available C_{18}-derivatized silica or polystyrene columns. The aqueous eluent contains a cation that has an affinity for the immobilized macrocycle, causing the formation of stationary positively charged cation-macrocycle complex exchange sites. Inorganic anions were eluted from the column by OH^- eluent.

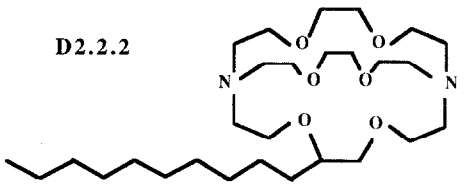
Josic and Reutter [45] developed a stationary phase with crown ether for the separation of nucleic acids and proteins. The chromatographic column sorbent was prepared by immobilization of the crown ether 1,10-diaza-18-crown-6 to different porous and non-porous epoxy activated supports. In the presence of potassium ions, the column could be used for the separation of both nucleic acids and proteins. In experiments with standard proteins the influence of pH and the role of loading the column with potassium ions were demonstrated. The retention time is dependent on the size of the nucleic acids. Nucleotides were not retained by this column.

We report here a novel method for the separation of nucleotides and nucleosides by macrocycle-based chromatography. The macrocyclic ligand which we used for the experiment is *n*-decyl-2.2.2 (D2.2.2) cryptand. A Dionex MPIC column was loaded with D2.2.2, yielding a stationary phase having a mixed-mode chromatographic matrix on which both ionic and hydrophobic interactions can occur. The D2.2.2 complexes with a metal ion, forming the desired sites for electrostatic interactions. The hydrophobic tail of the D2.2.2 molecule and the MPIC column matrix provide the sites for hydrophobic interactions. Nucleotides and nucleosides were separated simultaneously on this column. Both ionic and hydrophobic interactions were demonstrated, and excellent resolution for nucleotides and nucleosides in one analysis was achieved.



2.2.2

D2.2.2



EXPERIMENTAL

Materials

Macrocyclic ligand D2.2.2 was purchased from EM Science. Reagent-grade nucleotide and nucleoside compounds were obtained from Sigma. HPLC-grade methanol was obtained from Fisher Scientific. Eluent water was purified to 18 MΩ-cm resistivity using a Milli-Q purification system (Millipore). Eluents were degassed by helium purging or sonication. All the other chemicals used to prepare eluents were of analytical grade.

Methods

All the chromatographic separations were performed on a Dionex 2000i (isocratic) or 4000i (gradient) liquid ion chromatograph equipped with a Dionex variable-wavelength UV–VIS detector set at 254 nm. Chromatograms were plotted on a Dionex 4270 integrator and collected using the Spectra-Physics Labnet computer system. A Hewlett-Packard Deskjet Plus printer and 7470A plotter were used for hard copy data presentation. Dionex Autoion 400 software was also used to collect data.

The following columns were used: Dionex MPIC IonPak NS1 (25 cm × 4.6 mm I.D.), polystyrene–divinylbenzene; Dionex OmniPak PAX-500 (25 cm × 10 mm I.D.); Spherisorb 10-μm ODS-2 (25 cm × 4.6 mm I.D.), C_{18} on silica.

The eluents used to separate the nucleotides and nucleosides were aqueous solutions containing varying amounts of salts, acid or base to control

pH, and methanol. Unless otherwise indicated, all chromatograms were made at a flow rate of 1.0 ml/min.

Preparation of D2.2.2 columns

A 0.1 ml solution of D2.2.2 [50% (w/v) D2.2.2 in toluene] was put into 50 ml of methanol–water (55:45, v/v) which was degassed by sonication for 10 min. The column had been rinsed first with methanol–water (90:10, v/v) for 2 h. Then the column was rinsed with methanol–water (55:45, v/v) for 30 min. The D2.2.2 was loaded onto the column by recycling the D2.2.2 solution through the column for 3 h.

RESULTS AND DISCUSSON

Comparison of column performance with and without D2.2.2

The retention of nucleotides of the MPIC column without macrocyclic ligand was poor. All the nucleotides eluted immediately after the dead volume of the column with poor resolution even when using pure water as the eluent. In this case, the repulsion effect of the negative charges located at the phosphate moiety must predominate over hydrophobic attraction to the stationary phase. When potassium chloride solution was used as the eluent instead of water, the retention of nucleotides was increased, and some resolution of the three nucleotides used to test the system was achieved when the concentration of KCl in the eluent reached 1 *M*. The peak shapes were poor and the column efficiency was not high. This effect of the addition of salt to the eluent can be explained by formation of weak ion pairs between the metal ion and the ionic nucleotides, which reduces the net negative charges of the solutes, and enhances their retention [46,47].

When the macrocyclic ligand D2.2.2 was adsorbed to the same stationary phase, a dramatic improvement in nucleotide separation was achieved, as shown in Fig. 1. After the column loaded with D2.2.2, the nucleotides do not elute at all with pure water as the mobile phase. Since higher concentrations of salt in the eluent were necessary to reduce the retention of the nucleotides on this column, it was concluded that an ion-exchange mechanism applies, as demonstrated previously for inorganic anions.

Effect of salt

The effects of variation in the eluent potassium (KCl) or lithium (LiCl) concentrations on the nucleotide capacity factor using the MPIC/D2.2.2 col-

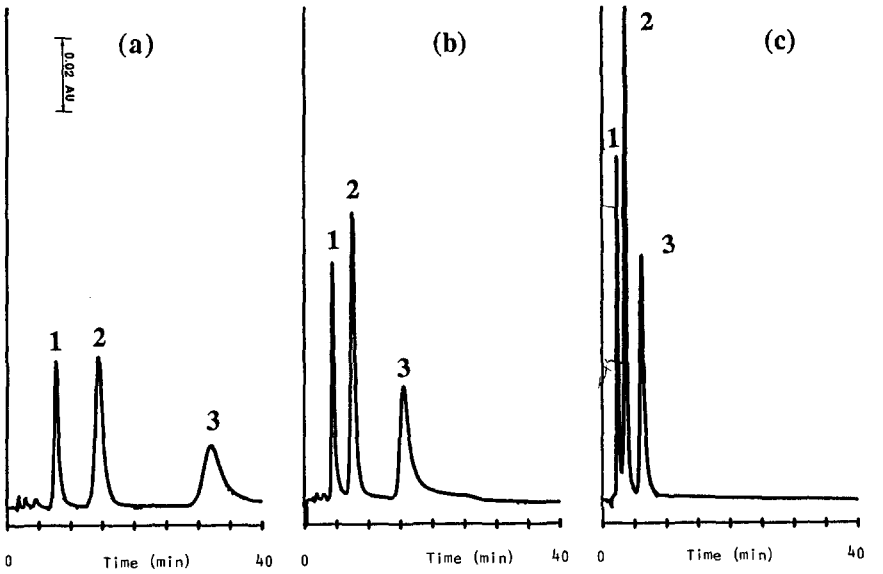


Fig. 1. Chromatograms of three nucleotides (1 = CMP; 2 = TMP; 3 = AMP; concentration 10.0 μM, 20-μl injection loop) on MPIC column loaded with D2.2.2. Conditions: UV, 254 nm, flow-rate, 1.0 ml/min. Eluent: (a) 40 mM KCl; (b) 60 mM KCl; (c) 100 mM KCl.

umn was investigated. Retention was consistently higher with K^+ than with Li^+, in keeping with the much stronger affinity of D2.2.2 for K^+. When the concentration of metal ions in the mobile phase was low, the retention of nucleotides was high, especially for the purine nucleotides and the di- and triphosphate nucleotides. The capacity factors (k') for the nucleotides were dramatically decreased when the potassium concentration in the eluent was increased. This result corresponds to conventional anion-exchange chromatography, which adds veracity to the concept of an ion-exchange mechanism for the separation of nucleotides on this macrocycle-loaded column. If ion exchange were the only mechanism for nucleotide retention, nucleotides would not be significantly retained or separated in the absence of a cation which binds with the macrocyle, as we observed with inorganic anions [44]. However, when AMP, CMP, TMP and GMP samples (made free of cation by treatment with H^+-loaded ion-exchange resin) were injected into the macrocycle column using pure water eluent, significant retention was still observed. Thus, it is clear that another retention mechanism is at work, as described below.

The population of ion-exchange sites on the stationary phase of the column can be altered simply by changing the mobile phase cation. The higher the binding constant between D2.2.2 and the cation, the higher the column capacity. Fig. 2 shows the effect of changing the cation in the mobile phase on the retention of four nucleotides. Based on this result, we postulated that a cation gradient for the separation of organic anions such as nucleotides was possible, just as was previously achieved for inorganic anions. However, other factors also needed to be explored for this system. Specifically, the presence of hydrophobic interactions with the column, albeit relatively weak, offered possibilities of varying eluent organic content. Furthermore, UV detection in this case made it possible to use anions other than OH^- ion, which is necessary for suppressed conductimetric detection.

The choice of eluent anion has a great influence on the separation of the nucleotides. For eluent halide anions, the order of decreasing retention of nucleotides is $F^- > Cl^- > Br^- > I^-$, which results from the order of affinity of the K^+–D2.2.2/MPIC column among these eluent anions, I^- being most

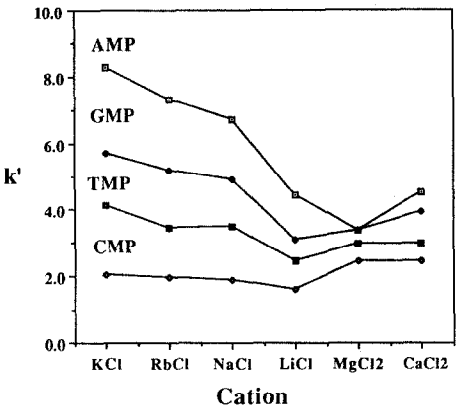


Fig. 2. Variation of nucleotide capacity factor with different eluent cations. Column D2.2.2 on MPIC, eluent 100 mM chloride.

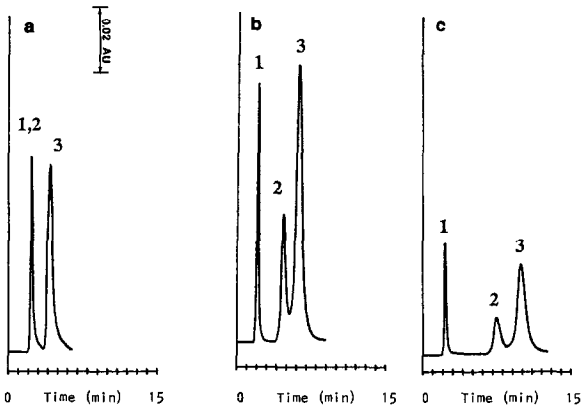
strongly attracted to the column. This result is not surprising, since competition of the mobile phase anion with sample anions is the main mechanism for elution of the anionic nucleotides.

Effect of pH

The capacity factor of nucleotide retention increases with the pH of the eluent. The pH was varied while holding $[K^+]$ constant by adding variable amounts of KCl, KOH, and/or HCl to the eluent. This result can be explained by the variation with pH of the charges on the acid and base sites of the nucleotide molecules. The phosphate groups increase in negative charge at higher pH, while the amino groups on the nucleobases are neutralized. Capacity increases with pH because the net negative charge of the molecule increases. The other factor influenced by pH is the loading of the macrocyclic compounds with potassium ions [46], since these molecules are weak bases and at low pH, K^+ must compete with H^+. However, since both the H^+–D2.2.2 complex and the K^+D2.2.2 complex carry the same charge, this effect must be small.

Effect of organic solvent

Addition of an organic solvent to the mobile phase can be used to vary the retention of nucleotides based on the hydrophobic interaction. Indeed, varying the percentage of organic solvent in the eluent illustrates the relative strength of hydro-



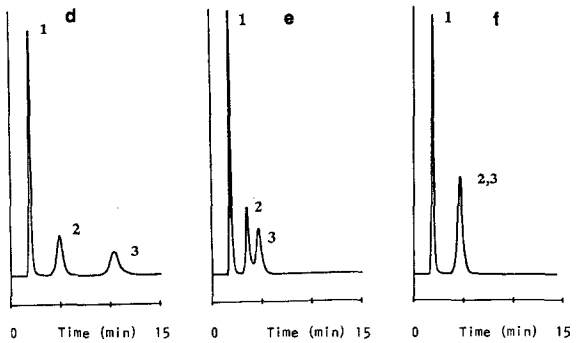


Fig. 3. (a–c) Separation of nucleotides (1 = CMP; 2 = AMP; 3 = TMP; concentration 10.0 μM, 20 μl) on D2.2.2/MPIC column. Eluent: 20 mM KCl with (a) 20%, (b) 10% or (c) 5% methanol. (d–f) Separation of nucleosides (1 = cytidine, 2 = thymidine, 3 = adenosine; concentration 10.0 μM, 10 μl) on D2.2.2/MPIC column. Eluent: (d) water; (e) water–methanol (90:10); (f) water–methanol (80:20).

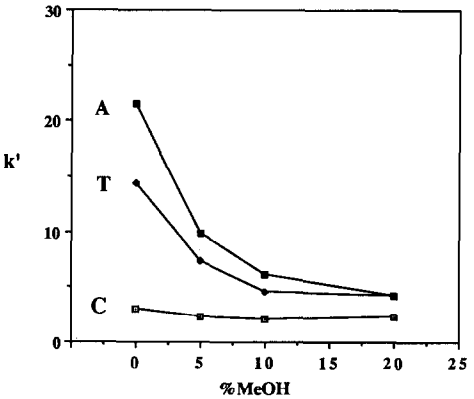


Fig. 4. Variation of nucleoside capacity factor with eluent methanol (MeOH) concentration. Column: D2.2.2 on MPIC. Eluent: water–methanol.

phobic interactions with the column. Fig. 3 shows the influence of organic solvent on nucleotide separations on the MPIC/D2.2.2 column. It is interesting to compare this effect on nucleotide retention with the effect on nucleoside retention, since the corresponding nucleoside constitutes the organic moiety of each nucleotide which is prone to hydrophobic interaction with the column. The capacity factors for both nucleotides and nucleosides decrease with increasing percentage of methanol in the mobile phase. This result implies that hydrophobic interactions between nucleosides or nucleotides and the stationary phase do take place, and that two mechanisms of retention, *i.e.*, a combination of electrostatic and hydrophobic interactions, come into play with nucleotides. The effect on nuclosides is larger than that of nucleotides, because of the different electron charge in the molecules.

Separation of nucleosides

Nucleosides are commonly separated on reversed-phase columns. Thus, as expected, the macrocycle-loaded column is also very good for the separation of nucleosides since hydrophobic interactions are possible. Excellent resolution of three test nucleosided was achieved, as shown in Fig. 4. The effect of salts and organic solvent in the eluent is different for nucleosides than for nucleotides, since there is no phosphate group in these molecules.

The effect of salt concentration in the eluent for nucleosides is much smaller than that for nucleotides because the ion-exchange separation mechanism does not apply.

pH is an important factor which influences the retention of nucleosides because of the variation of net electron charge in the molecules. Higher pH enhances the retention of nucleosides, as is true for nucleotides. Hydrophobic interaction is the predominant separation mechanism. The hydrophobic tail of the cryptand and column matrix provides the site for these interactions. Thus, it is observed that the MPIC/D2.2.2 mixed-mode is very good for the separation of negatively charged or neutral compounds in one analysis.

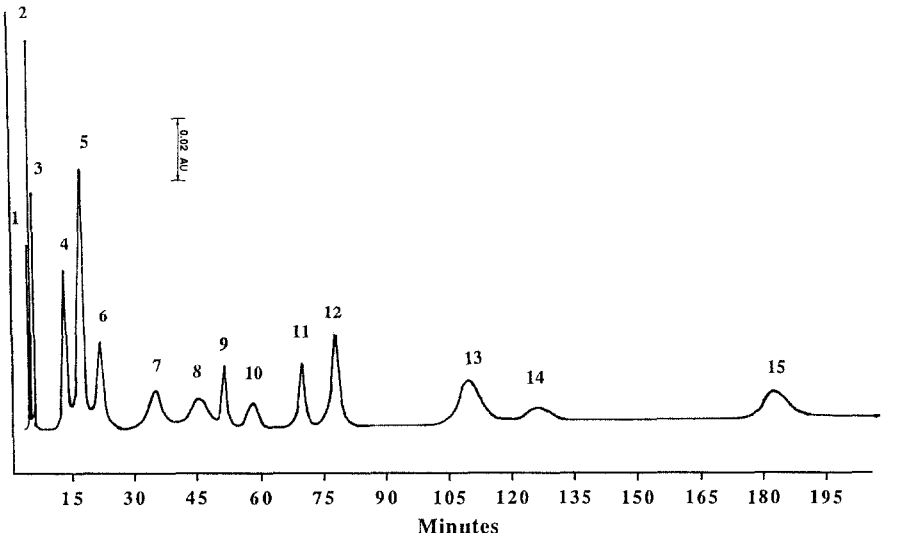


Fig. 5. Gradient chromatogram showing separation of 15 nucleotides and nucleosides. Conditions: UV 254 nm; flow-rate, 1.0 ml/min; concentration of KCl solution 100 m*M*; all concentrations are 10.0 μM, injection volume, 20 μl; [KCl] programs: 0–5.0 min: 100% water; 5.0–20 min: 5% KCl solution; 20–150 min: 100% KCl solution. Peaks: 1 = Cytidine; 2 = deoxycytidine; 3 = thymidine; 4 = cytidine-5′-monophosphate; 5 = deoxycytidine-5′-monophosphate; 6 = thymidine-5′-monophosphate; 7 = guanosine; 8 = adenosine + deoxyguanosine; 9 = uridine-5′-monophosphate; 10 = deoxyadenosine; 11 = inosine-5′-monophosphate; 12 = guanosine-5′-monophosphate; 13 = adenosine-5′-monophosphate; 14 = deoxyguanosine-5′-monophosphate; 15 = deoxyadenoside-5′-monophosphate.

Simultaneous separations of nucleotides and nucleosides

When the macrocycle columns are combined to separate both nucleosides and nucleotides simultaneously, the resolutions and the peak shape are not very good, especially for the long retented nucleotides. Thus, a gradient was adopted, yielding good resolution for 16 nucleotides and nucleosides as shown in Fig. 5. A KCl concentration gradient program was used to achieve this excellent separation. The chromatogram shows that the gradient is very important for good resolution, especially for the 2-deoxy and oxy nucleotides and nucleosides. Pure water as eluent is good enough to separate nucleosides, but different concentrations of potassium chloride in the eluent are necessary for the separation of the nucleotides and the achievement of good peak shape.

It is conceivable that not only salt concentration gradients can be used to achieve these separations, but also the pH and temperature. The cation gradient described here is a novel gradient separation method we can use which is based on the macrocycle-loaded column because of the change in column ion-exchange capacity when cation is changed in the eluent. So, only macrocycle-based chromatography has cation gradient for the separation of compounds. The cation gradient is one of the advantages of macrocycle-based chromatography.

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