ION CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES USING A DUAL-DETECTION SYSTEM

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(First received June 13th, 1983; revised manuscript received July 28th, 1983)

SUMMARY

The retention behaviour of nucleobases, nucleosides, and nucleotides has been systematically investigated using ion chromatography with a hollow fibre ion-exchange membrane suppressor. A low-capacity anion-exchange resin column and sodium carbonate buffer or sodium phosphate buffer eluents were employed for the separation of these compounds. The effects of concentration and pH of the mobile phase on the retention behaviour of twelve nucleotides was studied in detail. A conductivity detector was used for the detection of inorganic anions as well as organic compounds along with a UV detector. Optimum conditions for the rapid separation of nucleobases, nucleosides, nucleotides and their mixtures using carbonate and phosphate buffers are reported.

INTRODUCTION

Anion-exchange chromatography is a useful technique for the separation and identification of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and their constituents. Although more recent methods applied to a variety of biological samples include reversed-phase high-performance liquid chromatography[1-5] and ion-pair chromatography[6-9], ion-exchange chromatography is used extensively for the separation of nucleobases, nucleosides, nucleotides and their mixtures, and anion-exchange resins are becoming increasingly popular owing to their high efficiency and stability and the reproducibility of the results[10-16].

Ion chromatography with a combination of separator and suppressor columns was introduced in 1975 by Small *et al.*[17]. It has since been used for analysis of ionic as well as neutral compounds with the help of a dual-detector system comprising a UV detector and a conductivity detector[18-21].

Quantitative identification of certain individual constituents of DNA and RNA has great importance in biomedical studies[22-24], such as diagnosis of a particular disease as well as studies of the effect of chemotherapy by analysing physiological body fluids[25,26]. For these studies, simultaneous separation of all the constituents of DNA and RNA may not be required, but rapid and complete separation of a particular group of compounds[24] is necessary for routine analyses.

The ion chromatographic method reported from our laboratory[18] for separation of carboxylic and keto acids is now being extended to the separation of nucleobases, nucleosides and nucleotides. The purpose of this report is to present a detailed study of the retention behaviour of these compounds in carbonate and phosphate eluents. Optimum conditions for their separation are reported and discussed.

MATERIALS AND METHODS

Nucleobases, nucleosides, and mononucleotides for standards were purchased from Kohjin (Tokyo, Japan) or Sigma (St. Louis, MO, U.S.A.). Dodecylbenzene sulphonic acid was from Lion (Tokyo, Japan). Other reagents were purchased from Nakarai Chemicals (Kyoto, Japan).

Ion chromatographic analyzer Model IC 100 (Yokogawa-Hokushin Electric Work, Tokyo, Japan) was used. A Jasco UV 254 detector (Japan Spectroscopic, Tokyo, Japan) was used with a condictivity detector. The UV detector was connected between an analytical column and a suppressor tube. A precolumn (50 × 4.6 mm I.D.) and a separation column (250 × 4.6 mm I.D.) were packed with a low-capacity surface agglomerated and bonded anion-exchange resin YEW AX-1 (Yokogawa-Hokushin) with an ion-exchange capacity of 25 μequiv./g and a particle diameter of 10 μm. The suppressor was constructed from drawn Nafion 811 X tubing (hollow fibre perfluorosulphonic acid cation-exchange membrane, DuPont), which was inserted coaxially in PTFE tubing of 1 mm I.D.[21].

A standard stock solution of each compound was prepared and kept in a freezer when not in use. Working standard solutions were prepared by appropriate dilution of the stock solutions. The pH of the eluent was adjusted at ambient temperature. The carbonate eluent of pH 8.6–11.3 was obtained by appropriate addition of 8 mM sodium carbonate solution to 8 mM sodium bicarbonate solution. pH 11.3–13.6 was obtained by addition of 0.5 M sodium hydroxide solution to the 8 mM sodium carbonate solution. Carbonate eluents of higher concentration of (40 mM) with different pH values were obtained in a similar way.

When the conductivity detector was used, 0.05 M dodecylbenzene sulphonic acid (DBS) was employed as scavenger at a flow-rate of 2 ml/min for eluents of lower concentration and 5 ml/min for eluents of higher concentration.

A phosphate eluent of pH 5–8.5 was obtained by appropriate addition of an equimolar solution of disodium hydrogen phosphate to the sodium dihydrogen phosphate. Chromatography was carried out at 40°C and a flow-rate of 1.6 ml/min. The UV detector was used for all identifications, whereas the conductivity detector was used only for carbonate buffer eluent.

RESULTS AND DISCUSSION

The importance of anion chromatography with carbonate buffer is increasing steadily owing to its wide applicability in the analysis of inorganic anions as well as organic compounds using a dual-detector system. Inorganic anions are usually separated on an anion-exchange resin column using alkaline eluents such as carbonate buffer, and detected conductometrically[18–21].

To find the optimum conditions for the separation of nucleobases, nucleosides,

and nucleotides along with the inorganic anions, the retention behaviour of individual sample components was studied by varying the concentration and the pH of the mobile phase. Even though the separation of inorganic anions is possible at any pH in the range 10–10.5, the eluent pH has a profound effect on the separation of nucleobases, nucleosides, and nucleotides. At pH 10.45, fourteen compounds can be eluted within 30 min (Fig. 1).

Conductometric response is associated with the ability of the compound to dissociate. Fig. 1 shows that nucleobases and nucleosides do not produce a response in the conductivity detector, whereas nucleotides can be detected to some extent. This indicates that dissociation of nucleobases under these conditions is very poor, but the phosphate group in nucleotides dissociated at pH *ca.* 4.5, which is the usual pH value of the effluent after the suppressor, and could be detected by the conductivity detector. After the elution of inorganic standards at this pH, it was confirmed that peaks I, II, and III in Fig. 1 correspond to Cl^-, PO_4^{3-}, and SO_4^{2-}, respectively. The UV detector has better sensitivity for these compounds than the conductivity detector.

A mixture of cytosine (Cyt), cytidine (Cyd) and cytidilic acids (CMPs) was chromatographed; Cyt, Cyd, and cytidine 5′-monophosphate (5′-CMP) were separated completely, whereas 2′- and 3′-isomers of CMP were difficult to separate from
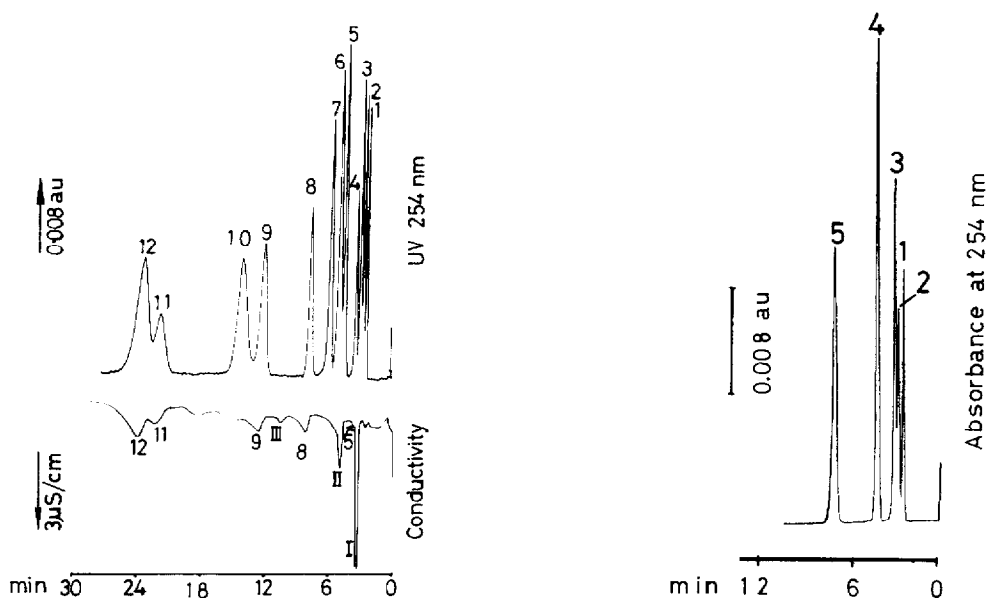

Fig. 1. Chromatogram of nucleobases, nucleosides, and nucleotides recorded with a dual-detection system. Column, YEW AX-1 250 × 4.6 mm I.D. (precolumn 50 × 4.6 mm I.D.); eluent, 8 mM carbonate buffer pH 10.45; eluent flow-rate, 1.6 ml/min; scavenger, 0.05 M dodecylbenzene sulphonic acid; scavenger flow-rate, 2 ml/min; temperature, 40°C; sample volume, 5 μl; sample concentration, 8 mM of each compound. Peaks: 1 = Cyt; 2 = Ura + Urd; 3 = Cyd; 4 = Gua; 5 = 5′-CMP; 6 = Guo; 7 = Ade; 8 = 2′ (3′)-CMP; 9 = 5′-AMP; 10 = Ado; 11 = 2′-AMP; 12 = 3′-AMP; I = Cl^-; II = PO_4^{3-}; III = SO_4^{2-}.

Fig. 2. Chromatogram of cytosine, cytidine, and cytidilic acid isomers. Eluent, 8 mM carbonate buffer pH 10.25. Other conditions as in Fig. 1. Peaks: 1 = Cyt; 2 = 2′,3′-cCMP; 3 = Cyd; 4 = 5′-CMP; 5 = 2′-CMP.

each other under these conditions. Cytidine 2′,3′-cyclic monophosphate (2′,3′-cCMP) was eluted close to Cyt and Cyd, and the retention was strongly affected by the pH of the eluent. At pH between 8.6 and 10 the retention times of these compounds are in the order Cyt < Cyd < 2′,3′-cCMP; at pH higher than 10 the order is Cyt < 2′,3′-cCMP < Cyd. These three peaks can be separated completely at lower pH, whereas CMP isomers require too much retention time and the optimum separation of Cyt and its nucleoside and nucleotides can be obtained within 8 min at pH 10.25 using carbonate buffer with low concentration (Fig. 2).

It was observed that the retention times of pyrimidine nucleobases and nucleosides were not affected by the eluent pH, whereas the retention times of purine nucleobases and nucleosides decreased with increasing pH of the mobile phase. The separation of nucleobases as well as nucleosides using a lower concentration of mobile phase was achieved at lower pH (8.6), as shown in Fig. 3. Because uracil (Ura) cannot be separated from uridine (Urd) at any pH or concentration of the eluent, the optimum separation of seven nucleobases and nucleosides was possible, as shown in Fig. 1.

The longer retention times of guanine monophosphates (GMPs) and uridine monophosphates (UMPs) can be reduced by using a higher pH and a higher concentration of the mobile phase. The effect of the pH on the capacity factor of nucleotides is shown in Fig. 4. This figure shows that the retention times of all nucleotides decrease with increasing eluent pH up to about 11. Above pH 11 most of the nucleotides were not affected by eluent pH and constant capacity factors were obtained, whereas the retention times of 5′-GMP and 3′UMP were increased with eluent pH.

A very interesting phenomenon was observed for the separation of 2′- and 3′-isomers of AMP. At lower concentration (8 mM) of mobile phase these isomers can be separated only at pH lower than 10.5, where 2′-AMP eluted faster than 3′-AMP. At higher concentration of mobile phase, better separation of these isomers was observed above pH 10.5 with the reversed order of the elution (Fig. 5).

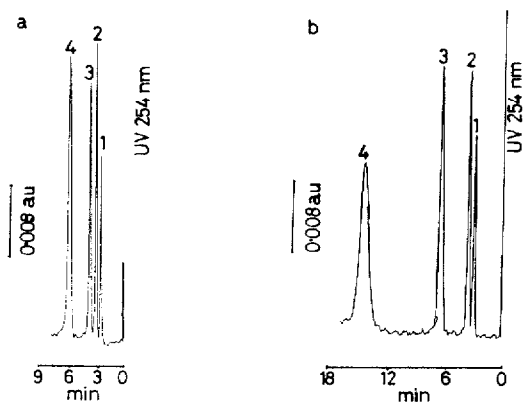The retention times of 2′- and 3′-isomers of purine nucleotides can be mini-

Fig. 3. (a) Chromatogram of nucleobases. Eluent, 8 mM carbonate buffer pH 8.6; sample volume, 2.5 μl; sample concentration, 8 mM of each base. Other conditions as in Fig. 1. Peaks: 1 = Cyt; 2 = Ura; 3 = Gua; 4 = Ade. (b) Chromatogram of nucleosides. Conditions as in (a). Peaks: 1 = Urd; 2 = Cyd; 3 = Guo; 4 = Ado.
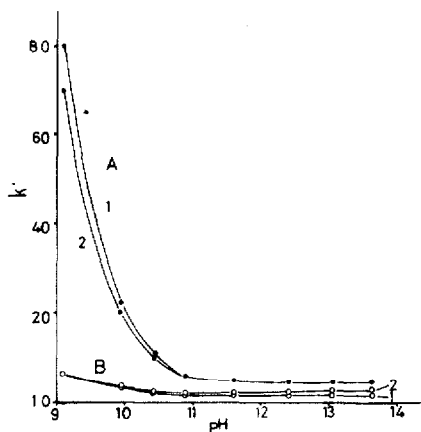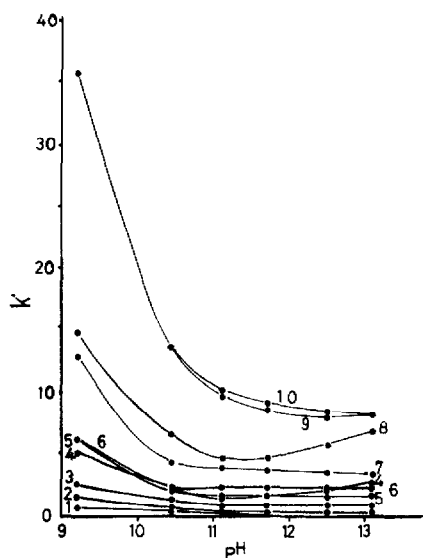
Fig. 4. Effect of eluent pH on k' of nucleotides. Curves: 1 = 5'-CMP; 2 = 2'-CMP; 3 = 5'-AMP; 4 = 5'-UMP; 5 = 3'-AMP; 6 = 2'-AMP; 7 = 3'-UMP; 8 = 5'-GMP; 9 = 2'-GMP; 10 = 3'-GMP.

Fig. 5. Effect of eluent pH and concentration on k' of 2'- and 3'-AMP isomers. Eluent: A = 8 mM carbonate buffer; B = 40 mM carbonate buffer; pH of eluent was adjusted by 0.5 M sodium hydroxide. Curves: 1 = 3'-AMP, 2 = 2'-AMP.

mized to large extent by using a higher concentration of the eluent. Retention times of purine nucleobases and nucleosides can also be reduced by using a higher eluent pH. Fig. 6 shows the complete separation of Ade, Ado, and three AMP isomers eluted within 10 min, which was attained with a higher concentration of carbonate buffer at pH 13.1.

At a lower concentration of the carbonate eluent rapid elution of all the nucleotides is not possible, even at high pH, *e.g.* 5'-GMP required 120 min for its elution with 8 mM carbonate buffer at pH 10.9. The rapid elution of all nucleotides can be achieved using carbonate eluent of higher concentration with higher pH. Of twelve nucleotides studied, ten were separated by using 40 mM carbonate buffer eluent at pH 12.35 (Fig. 7). Detection of all the nucleotides was carried out using UV as well as conductivity detection.

Phosphate buffers are more popular for the separation of nucleobases, nucleosides and nucleotides in ion-exchange chromatography[12,13,15]. Interesting behaviour of nucleotides with a phosphate buffer was observed. Fig. 8 represents the plots of the capacity factor, k', *vs.* pH of the eluent for nucleotide monophosphates. The k' values for all nucleotides decrease with increasing pH of eluent up to pH 7. But the behaviour of the nucleotides differs above pH 7. In the alkaline region, k' values for GMP and UMP increase with pH, whereas those for AMP and CMP were not affected. This can be explained on the basis of the dissociation of nucleotides. In acidic media the dissociation of all nucleotides decreases with increasing pH of the eluent. They are dissociated slightly at neutral pH and will have weak ionic interaction with the anion-exchange resin; this might result in a minimum retention time for all the
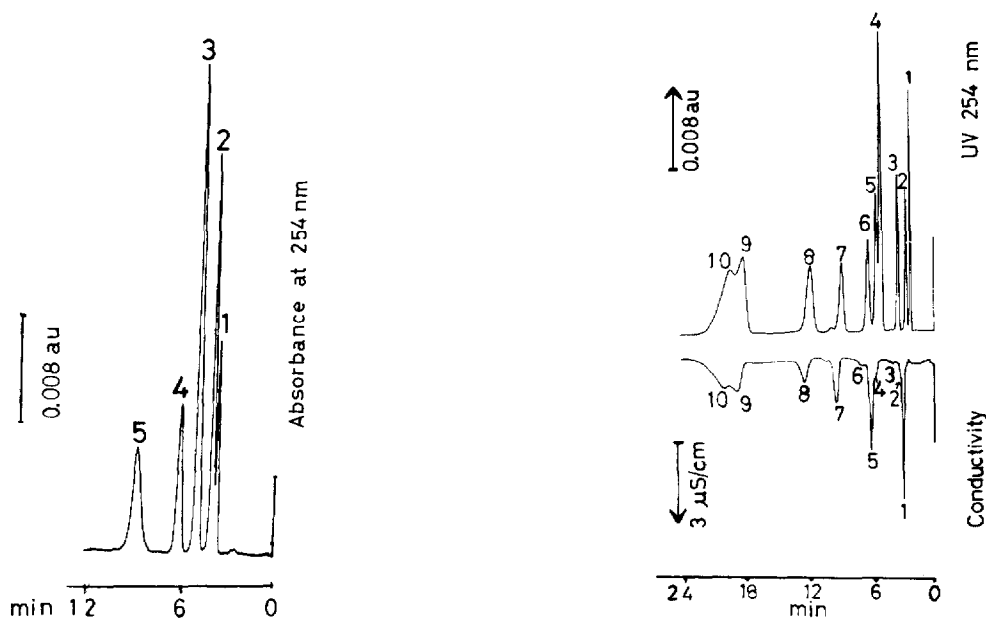
Fig. 6. Chromatogram of adenine, adenosine, and adenilic acids. Eluent, 40 mM carbonate buffer pH 13.1; sample volume, 3 μl. Other conditions as in Fig. 1. Peaks: 1 = 5'-AMP; 2 = Ade; 3 = 3'-AMP; 4 = 2'-AMP; 5 = Ado.

Fig. 7. Chromatogram of nucleotides recorded with a dual-detection system. Eluent, 40 mM carbonate buffer pH 12.35; sample volume, 8 μl. Other conditions as in Fig. 1. Peaks: 1 = 5'-CMP; 2 = 3'-CMP; 3 = 5'-AMP; 4 = 3'-AMP; 5 = 5'-UMP; 6 = 2'-AMP; 7 = 3'-UMP; 8 = 5'-GMP; 9 = 2'-GMP; 10 = 3'-GMP.

nucleotides near pH 7. The pK values for AMP and CMP are lower than 7, hence their dissociation and therefore the k' values had not been affected by varying pH in the alkaline range. The pK values for the keto groups on GMP and UMP are higher than 9, hence they might be dissociated at alkaline pH and acquire more and more negative charge with increasing pH, which would mean they would be retained longer and longer on the anion-exchange resin column.

To study the effect of concentration on the separation and retention behaviour of nucleotides, phosphate buffers with various concentrations from 5 mM to 50 mM and pH 5.5–8.6 were employed. Capacity factors of nucleotides obtained at pH 5.55 were plotted against concentration of phosphate eluent in Fig. 9. It was observed that k' of all the nucleotides decreased with increasing concentration of the eluent. Even though the retention times of 2'- and 3'-isomers of nucleotides were higher with the eluent of lower concentration, separation was better than with the eluent of higher concentration.

Figs. 8 and 9 show that the pH of the eluent has a more pronounced influence on the separation of 2'- and 3'-isomers of nucleotides than does the eluent concentration. From Fig. 8, in the isocratic elution of nucleotides, two optimum separation ranges for pH are found: pH 5–6 and pH 7.25–8. Most of nucleotides can be separated at pH 5.55, although AMP and GMP have very long retention times. After extensive study of the retention behaviour of nucleobases, nucleosides, and nucleotides, opti-
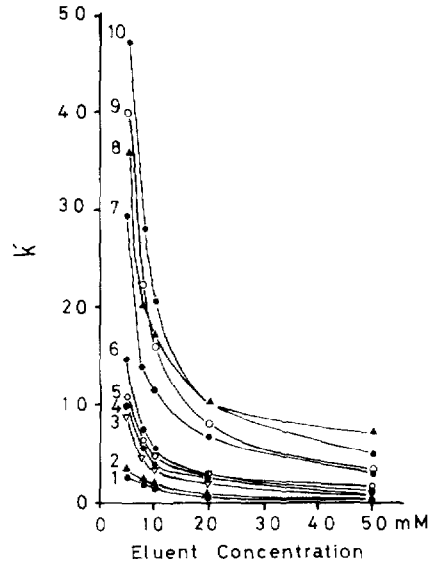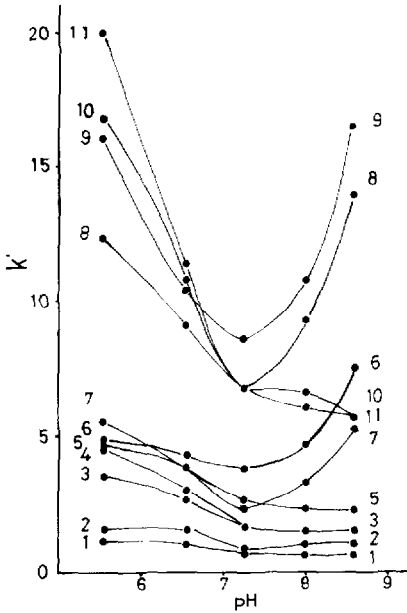
Fig. 8. Effect of eluent pH on k' values. The k' values were obtained by using 10 mM sodium phosphate buffer as eluent with various pH. Curves: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 2'-CMP; 4 = 3'-CMP; 5 = 5'-AMP; 6 = 5'-GMP; 7 = 2'(3')-UMP; 8 = 2'-GMP; 9 = 3'-GMP; 10 = 2'-AMP; 11 = 3'-AMP.

Fig. 9. Effect of eluent concentration of k' values of nucleotides. The k' values were obtained by using pH 5.55 phosphate buffer eluent with various concentrations. Curves: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 2'-CMP; 4 = 3'-CMP; 5 = 5'-AMP; 6 = 2'(3')-UMP; 7 = 2'-GMP; 8 = 2'-AMP; 9 = 3'-GMP; 10 = 3'-AMP.
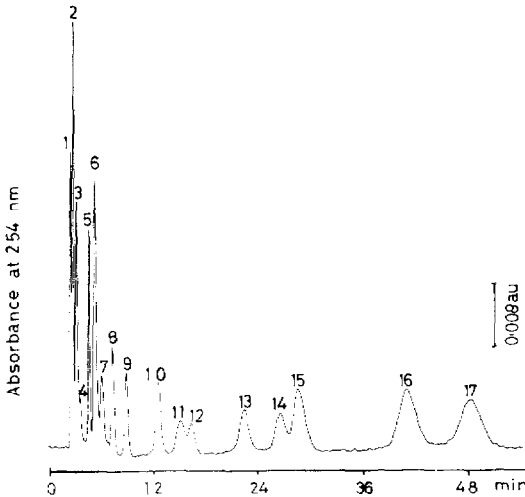

Fig. 10. Chromatogram of nucleobases, nucleosides, and nucleotides by isocratic elution with phosphate buffer eluent. Eluent, 5 mM phosphate buffer pH 7.75. Other conditions as in Fig. 1. Peaks: 1 = Cyt; 2 = Urd; 3 = Cyd; 4 = Gua; 5 = 5'-CMP; 6 = Ade; 7 = Guo; 8 = 5'-UMP; 9 = 2'-CMP; 10 = 5'-AMP; 11 = Ado; 12 = 3'-UMP; 13 = 5'-GMP; 14 = 2'-AMP; 15 = 3'-AMP; 16 = 2'-GMP; 17 = 3'-GMP.
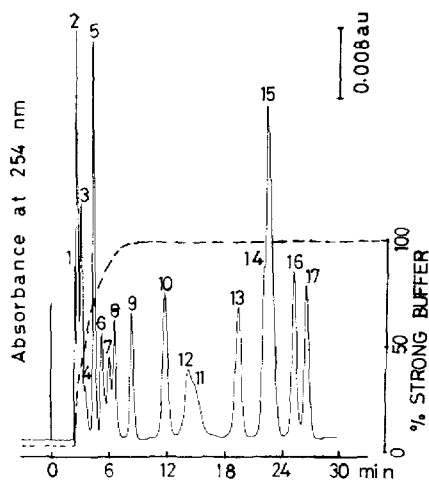
Fig. 11. Chromatogram of nucleobases, nucleosides, and nucleotides by gradient elution with phosphate buffer eluent. eluent I, 5 mM phosphate buffer pH 7.75; eluent II, 50 mM phosphate buffer pH 7.75. After 3 min of sample injection, $\sqrt[4]{X}$ mode gradient was started from 0% II to 100% II in 6 min. Other conditions and peaks as in Fig. 10.

mum isocratic separation of seventeen compounds was obtained at pH 7.75 using 5 mM phosphate buffer (Fig. 10). This condition is very critical because a slight increase or decrease in the pH of the eluent will affect the separation of 5'-UMP and Guo and cause the peaks to merge.

The separation time of 51 min with the isocratic elution can be reduced to 27 min by using gradient elution. Gradient elution was started after 3 min from the sample injection and the eluent concentration was increased from 5 mM to 50 mM in 6 min using the $\sqrt[4]{X}$ mode of a Toyo Soda Model GE-2 gradient device (Fig. 11).

CONCLUSION

A mixture of nucleobases, nucleosides and nucleotides was well separated on an ion chromatography with a dual-detection system consisting of a conductometric and a UV detector. Simultaneous determination of inorganic anions and nucleotides could be carried out by using carbonate buffer eluents and the conductometric detector. The UV detector does not respond to some inorganic ions but provides a more sensitive detection method for compounds related to nucleic acids, so the dual-detection system is useful for the analysis of a complex mixture by ion chromatography.

The pK values of nucleobases and nucleosides are higher than 9 and hence they acquire negative charge if the pH of the eluent is above their pK values, then they can be separated well with carbonate eluents. They have almost no charge at neutral pH, therefore they are poorly resolved by phosphate buffer at pH 4–8. Complete separation of nucleobases as well as nucleosides is achieved using carbonate buffer eluent.

Phosphate buffer eluents were applied to obtain the minimum separation time

by the aid of a gradient elution technique; however, the conductometric detector cannot be used with phosphate eluents, even when the isocratic elution is employed, owing to their high background conductance.

The surface agglomerated and bonded anion-exchange resin has worked well for the wide range eluent pH from 1 to 14, with excellent reproducibility. The resin was highly stable in the extreme pH conditions. The low ion-exchange capacity of the resin allows the use of eluents with a low salt concentration, which helps to keep down analytical cost, and more importantly facilitates the maintenance of the instrument by reducing the difficulties often caused by the use of a high salt concentration in the eluent.

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