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MICROBORE PACKED-COLUMN ANION-EXCHANGE LIQUID CHROMATOGRAPHY OF NUCLEOBASES, NUCLEOSIDES AND NUCLEOTIDES

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SUMMARY

Low-capacity anion-exchange resin was packed into a 45 cm × 0.19 mm I.D. fused-silica tubing and applied to micro-column liquid chromatography of nucleobases, nucleosides and isomers of nucleotides.

The effects of the chromatographic conditions on the elution behaviour of these compounds were studied. The efficiency of the microbore packed column was comparable with that of the conventional size column.

INTRODUCTION

Liquid chromatographic (LC) analysis of biologically important compounds related to nucleic acids has been carried out for many years. A number of papers have appeared on the separation of nucleobases, nucleosides and nucleotides by ion-exchange chromatography[1–7], and reversed-phase liquid chromatography has recently been applied successfully[8–14]. Generally, nucleobases and nucleosides are relatively weak bases, which at slightly acidic or neutral conditions carry no ionic charge on their molecules; however, nucleotides contain phosphate groups which are dissociated under acidic and neutral conditions. Therefore, in cation-exchange chromatography and reversed-phase chromatography, nucleotides are eluted fastest, followed by nucleosides and nucleobases. In contrast in anion-exchange chromatography nucleotides in the anionic form interact with the fixed cation groups on the ion-exchanger. The retention of nucleotides is enhanced by the hydrophobic interactions between the resin matrix. Thus, nucleotides are eluted slower than the corresponding nucleosides or nucleobases. Anion-exchange chromatography can be used to separate nucleotide isomers.

Low capacity ion-exchange materials with a silica gel core have been developed for high-performance liquid chromatography (HPLC) and applied to chromatography of compounds related to nucleic acid[15,16]. Recently, chemically stable low-capacity ion-exchange resins have been developed for ion chromatography[17–20]. These resins were designed mainly for the analysis of inorganic ions, such as alkali–metal

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ions, halogen ions, nitrate ion, nitrite ion, and sulphate ion. In previous reports, however, it has been shown that these columns are useful for the analysis of carboxylic acids[21,22] and compounds related to nucleic acids[23]. They can be operated using eluents with a very low ionic strength which show a low background in UV detection and, therefore, can achieve a better sensitivity than when highly concentrated eluents are necessary. The low salt concentration of the eluents is also favourable for the maintenance of the instruments.

Miniaturization of the LC column is being studied by many workers. Recently, Kucera summarized the work on microbore columns in his book, and introduced results of the separation of some deoxyribonucleosides and their corresponding mononucleotides on a reversed-phase material packed in a 1.0 mm I.D. stainless-steel tube[24]. Microbore columns have proved to have advantages over the conventional 4.0–4.6 mm I.D. columns. The low consumption of packing material, eluent and sample is good not only economically but also for the possible development of ancillary and related techniques.

Flexible fused-silica tubing is very suitable for use as microbore columns because of its smooth and chemically inert inner surface, flexibility and mechanical stability. The nature of the tubing make it easy to reduce the column diameter to 0.1–0.3 mm with high column efficiency[25].

In previous papers, anion-exchange chromatography using fused-silica tubing microbore columns packed with newly developed low-capacity anion-exchange resins was reported. A surface-agglomerated and -bonded anion-exchange resin column was used in the construction of a micro-column ion chromatograph by using a cation-exchange hollow-fibre suppressor combined with a micro-flow conductivity cell[26]. A UV detector was applied to a microbore packed column with the same resin[27]. A totally porous low-capacity anion-exchange resin column was also used for microbore column chromatography of isomeric aromatic acid derivatives[28] and isomeric phenolic compounds[29].

This paper describes the application of a microbore column of cross-sectional area *ca.* 1/500 at that of the conventional size column, packed with an anion-exchange resin, to HPLC of a mixture of nucleobases, nucleosides and nucleotides, including isomers.

MATERIALS AND METHODS

The microbore column chromatograph used in this work was as previously reported[26–28]. A Milton Roy Minipump, a Jasco Uvidec 100 II UV detector, and a Jasco microloop sample injector, Model 422, with a fixed sample volume of 0.05 μl were employed.

Flexible fused-silica tubing was obtained from Scientific Glass Engineering, North Melbourne, Australia. A totally porous low-capacity anion-exchange resin, TSK gel IC-anion PW (30 μequiv/ml, particle diameter 9 $\pm$ 1 μm) was recently developed by Toyo Soda (Tokyo, Japan) for ion chromatography. The resin was slurry-packed with carbonate buffer under a pressure of 250 kg/cm^2 by means of a Jasco Model A 700 pump in the constant pressure mode. The column was 450 $\times$ 0.19 mm I.D. Eluents were prepared from ammonium dihydrogen phosphate and diammonium hydrogen phosphate. The concentration of phosphate was varied from

5 m*M* to 200 m*M*, and the pH was varied from 4.4 to 9.85. The eluent from a conventional size pump was split before the micro injector. The smaller portion of the split eluent was led to the column via the micro injector and the larger portion was returned to the eluent reservoir.

Nucleobases, nucleosides, and nucleotides were obtained from Kohjin (Tokyo, Japan) or Sigma (St. Louis, MO, U.S.A.) and were dissolved to 8 m*M* as the stock solution in water except for Ade, Gua, Ado and Guo: these compounds were dissolved by the addition of ammonium solution. All other chemicals were purchased from Nakarai Chemicals (Kyoto, Japan).

RESULTS AND DISCUSSION

A chromatogram of four nucleobases is shown in Fig. 1. The eluent was 50 m*M* ammonium phosphate buffer (pH 7.75). The flow-rate of the pump was adjusted to maintain the column pressure at 45 kg/cm^2 and the splitting ratio was 120:1. Under these conditions, a flow-rate of 1.8 μl/min was maintained at ambient temperature. Nucleosides were separated by using ammonium phosphate (pH 4.90) as eluent. Nucleobases and nucleosides are practically neutral in net charge at these conditions and their separation is based mainly on their affinity for the ion-exchange resin matrix through hydrophobic interactions rather than ion-exchange interaction. Therefore it was observed that nucleosides were eluted faster than the corresponding

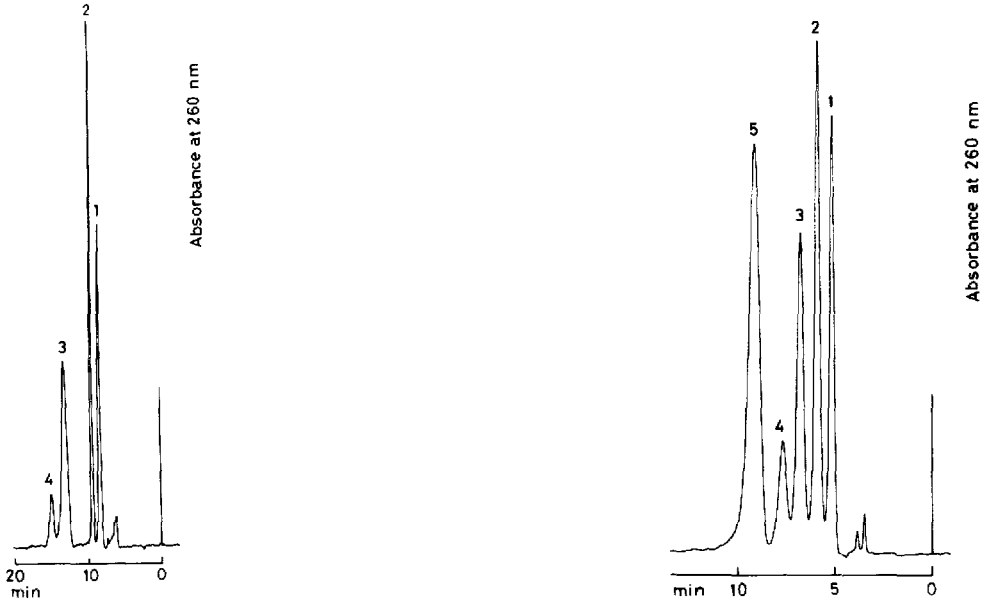


Fig. 1. Chromatogram of nucleobases. Column, TSK gel IC-anion PW, 450 × 0.19 mm I.D.; eluent, 50 m*M* phosphate buffer (pH 7.75); column pressure, 45 kg/cm^2; eluent flow-rate, 1.8 μl/min; splitting ratio, 120:1; column temperature, ambient; detection, 260 nm; sample volume, 0.05 μl. Peaks: 1 = Cyt; 2 = Ura; 3 = Ade; 4 = Gua. Sample amount, 100 pmol of each compound except Gua (7.3 pmol).

Fig. 2. Chromatogram of nucleosides. Eluent, 50 m*M* phosphate buffer (pH 4.90); other conditions as in Fig. 1. Peaks: 1 = Cyd; 2 = Urd; 3 = Thd; 4 = Guo; 5 = Ado. Sample amount, 50 pmol of each compound except Guo (7.4 pmol).

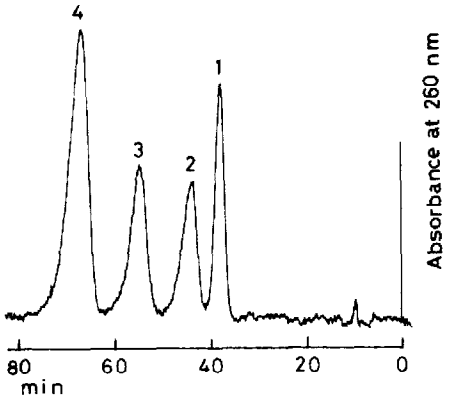
nucleobases owing to the hydrophilicity of the sugar groups in nucleosides which reduced the hydrophobic interactions. Five nucleosides eluted within 11 min in the following order: Cyd > Urd > Thd > Guo > Ado (Fig. 2). The amounts of these samples ranged from 2.1 to 13.4 ng.

Nucleotides have phosphate group which dissociates under acidic conditions to carry a negative charge. Therefore, as would be expected with anion-exchange chromatography, all nucleotide isomers were retained through the ion-exchange effect acting in concert with the hydrophobic interactions, resulting in larger k' values than those of the corresponding nucleobases or nucleosides.

Nucleotide isomers were separated on the column. A chromatogram of four AMP isomers is shown in Fig. 3; 3',5'-cAMP eluted between 5'-AMP and 2'-AMP. A mixture of 5'-, 3'- and 2'-CMP isomers and 2',3'-cCMP was chromatographed under the same conditions, and the isomers were separated within 27 min in the order cCMP > 5'-CMP > 2'-CMP > 3'-CMP. The other nucleotide isomers were also separated.

Calibration curves for Cyt, Cyd and 5'-CMP were determined by plotting their peak heights against the sample amounts loaded on the column (Fig. 4). The linear range was more than two orders for each compounds, and the detection limit was down to 2 pmol for Cyt and Cyd when the signal-to-noise ratio was 5, whereas that of 5'-CMP was larger than other two peaks owing to peak broadening under the elution conditions used. When the sample size was more than 1 nmol the peak shape was skewed and broadened, and the calibration curve plotted by the peak height became non-linear. However, the linear range, could be extended by plotting the peak area instead of the peak height.

The effect of the phosphate concentration of the eluent on the retention of nucleic acid related compounds was examined. The k' values of nucleobases eluted by pH 7.75 phosphate and the k' values of nucleosides eluted by pH 4.90 phosphate are plotted against the eluent concentration in Fig. 5. Only a moderate effect of



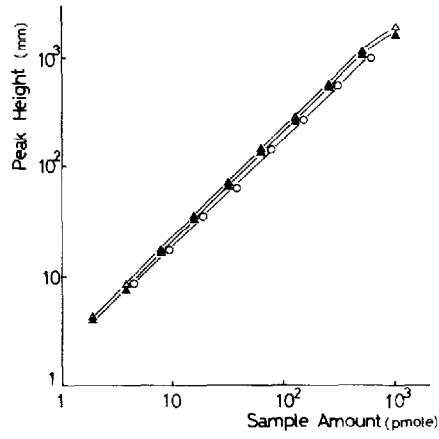


Fig. 3. Chromatogram of four AMP isomers. Eluent, 20 mM phosphate buffer (pH 7.25); other conditions as in Fig. 1. Peaks: 1 = 5'-AMP; 2 = 3',5'-cAMP; 3 = 2'-AMP; 4 = 3'-AMP. Sample amount, 50 pmol of each compound.

Fig. 4. Calibration curve. Conditions as in Fig. 1. Curves: Cyt (△), Cyd (▲), 5'-CMP (○).

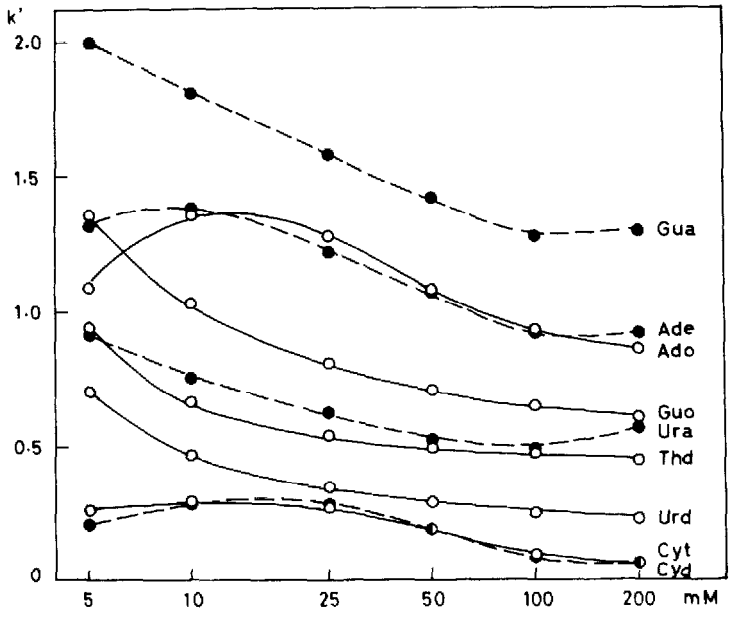


Fig. 5. Effect of phosphate concentration of the eluent on the k' values of nucleobases and nucleosides. Eluent: nucleobases, phosphate buffer (pH 7.75); nucleosides, phosphate buffer (pH 4.90). Other conditions as in Fig. 1.

phosphate concentration on the k' values was observed for these compounds. The k' values of Gua, Ura and their corresponding nucleosides decreased monotonically with increasing phosphate concentration, whereas at lower phosphate concentrations, the behaviour of Ade, Cyt and their corresponding nucleosides was anomalous. The k' values of these compounds increased with the concentration, and maximum values were obtained at *ca.* 10 m*M* for Ade and Ado, and *ca.* 20 m*M* for Cyt and Cyd. Above this range, the k' values decreased with increasing phosphate concentration of the eluent. The effect of the eluent concentration on the elution behaviour of nucleobases and nucleosides was relatively small; however, the effect can not be explained at present.

At pH 4.4, nucleotides are negatively charged and interact strongly with the anion-exchange resin. The k' values of nucleotides were much larger than those of the corresponding nucleobases and nucleosides. The retention of nucleotides largely depended on the phosphate concentration of the eluent: when it was increased from 25 m*M* to 200 m*M*, the k' of 5'-AMP, for instance, showed a remarkable reduction from 11 to 1.4. Phosphate ions in the eluent compete with the dissociated nucleotides for the ion-exchange sites on the anion-exchange resin. The increase in the concentration of the phosphate ion weakens the interaction between the nucleotide molecules and the resin to reduce the k' values of nucleotides.

In all cases, nucleobases, nucleosides and nucleotides with purine bases were eluted slower than those with pyrimidine bases.

In ion-exchange chromatography, the pH of the eluent has an important influence on the behaviour of the solute ions. The effect of the pH on the retention of nucleotides was investigated, and the relationship between the k' value of nucleotides

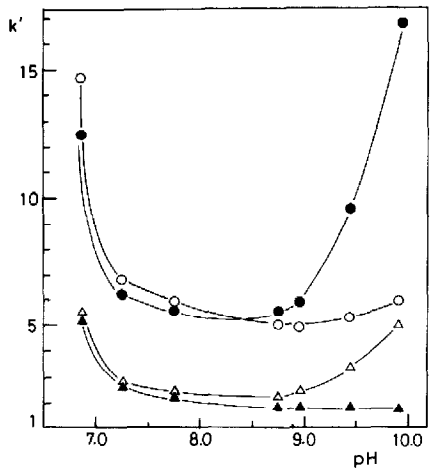


Fig. 6. Effect of eluent pH on the k' values of nucleotides. Eluent, 50 mM phosphate buffer. Curves: 5'-AMP (○); 5'-GMP (●); 5'-UMP (△); 5'-CMP (▲). Other conditions as in Fig. 1.

and the eluent pH is shown in Fig. 6. In the low pH range, the k' values of all nucleosides decreased with increasing pH, and above pH 8 the values for 5'-AMP and 5'-CMP were slightly affected by the eluent pH. In contrast, above pH 9 the k' values of 5'-GMP and 5'-UMP increased remarkably with increasing pH. The right-hand side of Fig. 6 can be explained by the dissociation of hydroxy groups on the purine and the pyrimidine rings in 5'-GMP and 5'-UMP, respectively. The dissociation constants (pK values) of these hydroxy groups are 9.3 and 9.4, respectively, therefore in the higher pH range in Fig. 6, the fraction of the molecules that carry the negative charge increased with eluent pH and these nucleotides were adsorbed more and more strongly on the anion-exchange resin. In contrast to these nucleotides, 5'-AMP and 5'-CMP have no dissociable groups in the alkaline pH range used in

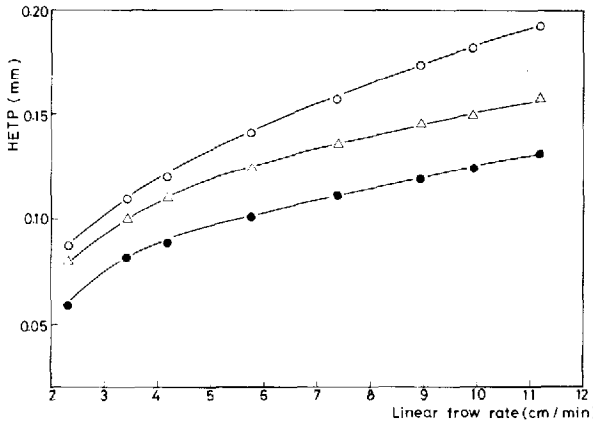


Fig. 7. Effect of eluent flow-rate on HETP. Eluent: Ura and Urd, 17 mM phosphate buffer (pH 6.95); 5'-UMP, 24 mM phosphate buffer (pH 8.95). Column temperature, 43.5°C; other conditions as in Fig. 1. Curves: 5'-UMP (○); Urd (△); Ura (●).

this study, and so the eluent pH had little effect on the k' values of these two nucleotides.

The efficiency of the column is a function of various parameters, *e.g.* the eluent flow-rate, the temperature, the viscosity of the eluent and the nature and size of the solute molecules. The effect of eluent flow-rate was examined. In general, a minimal value of the height equivalent to a theoretical plate (HETP) is observed at very low flow-rates. HETP values of 5'-UMP, Urd and Ura increased with increasing flow-rate of the eluent, and the minimal values were obtained at the lowest flow rate applied in this study (Fig. 7).

The column efficiency of the present microbore packed column was compared with that of a conventional size column. Data from the manufacturer were used to calculate the HETP for a conventional size column packed with the same packing material. This value was *ca.* 0.10 mm for the chloride ion peak eluted at a flow-rate of 7.9 cm/min, and for the present microbore column, an HETP value of 0.11 mm for Ura at the same flow-rate was obtained from the curve in Fig. 7. The present results show that the microbore packed column has an efficiency comparable with that of the conventional size column.

The effect of column temperature on the retention behaviour of nucleobases, nucleosides and nucleotides was studied. Logarithms of the k' values obtained at temperatures from 16°C to 40°C were plotted against the reciprocal of the absolute

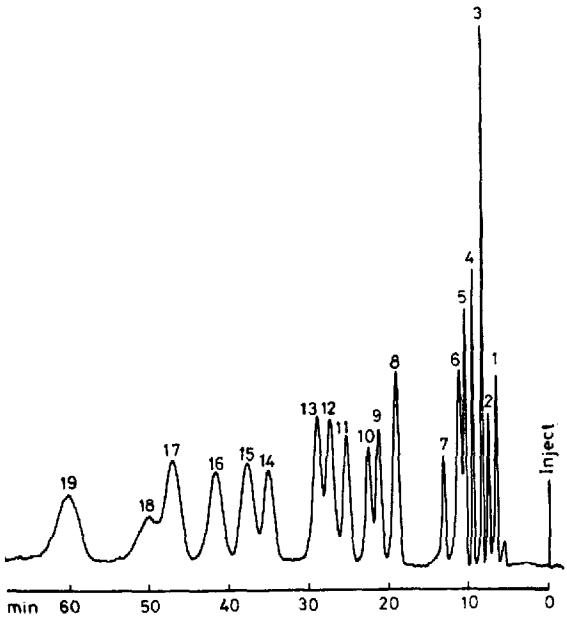


Fig. 8. Chromatogram of nucleobases, nucleosides and nucleotides. The column was pre-equilibrated with 17 mM phosphate buffer (pH 7.15). Eluent, 30 mM phosphate buffer (pH 7.15); flow-rate, 1.43 μl/min; column pressure, 40 kg/cm^2; other conditions as in Fig. 1. Peaks: 1 = Cyd; 2 = Urd; 3 = Cyt; 4 = Ura; 5 = Guo; 6 = Ado; 7 = Gua; 8 = Ade; 9 = 5'-CMP; 10 = 5'-UMP; 11 = 2'-CMP; 12 = 3'-CMP + 2'-UMP; 13 = 3'-UMP; 14 = 5'-GMP; 15 = 5'-AMP; 16 = 2'-GMP; 17 = 2'-AMP; 18 = 3'-GMP; 19 = 3'-AMP. Sample amount: peaks 1–8, 21.5 pmol of each compound; peaks 9–19, 26 pmol of each compound.

temperature, and a linear relationship was obtained for four nucleosides and four 5'-nucleotides. Nucleobases also yield straight lines above 20°C; however, at lower temperatures, the curves deviated upward from linearity.

The separation conditions for a mixture of four nucleobases, four nucleosides and twelve isomeric nucleotides were investigated, and the optimum resolution for the mixture was obtained (Fig. 8). Before the sample injection, the column was equilibrated with 17 m*M* ammonium phosphate (pH 7.15) and then the flow-line was washed and replaced by the stronger eluent from the reservoir to the splitter, which was placed just before the sample injector. To obtain a good separation of nucleobases and nucleosides, pre-equilibration of the column by the eluent at a concentration between 10 m*M* to 20 m*M* is recommended. A good resolution of nucleotides was achieved with an eluent concentration between 30 m*M* to 40 m*M* at a pH below 8.0. Under these conditions, however, the 3'-CMP peak merged with the 2'-UMP peak, and the latter was not completely resolved from the 3'-UMP peak. By eluting with 50 m*M* phosphate buffer (pH 4.90), excellent resolution of six nucleotides between 5'-CMP and 3'-UMP was achieved, and these peaks eluted in the same order as in Fig. 8, except that 2'-UMP eluted faster than 3'-CMP. A stepwise pH gradient elution was carried out to accelerate the elution of the peaks after 3'-CMP; however, sufficient resolution of the peaks from 3'-UMP to 2'-GMP was not achieved.

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