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SEPARATION OF MAJOR RNA-DERIVED NUCLEOTIDES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The effects of pH, ionic strength and amount of methanol in the eluent on the retention of 5′-, 3′- and 2′-ribonucleoside monophosphates on a reversed-phase high-performance liquid chromatographic system are described. The data were used to develop suitable separation protocols for synthetic nucleotide mixtures and applied to the separation of RNA nucleotides derived by alkaline hydrolysis.

INTRODUCTION

RNA is a suitable biochemical fraction for the study of nucleotide biosynthesis from labelled precursors for several reasons: (1) radioactivity is easily incorporated from labelled acid-soluble nucleotides; (2) it is the most abundant polynucleotide in the cell and nucleotides can be selectively separated from the remaining macromolecular cell components by alkaline or enzymic hydrolysis; (3) the nucleotide composition of RNA hydrolysates is relatively simple compared with the composition of acid-soluble extracts, which contain in addition a great diversity of interfering low-molecular-weight substances.

The availability of suitable methods for resolving mixtures of RNA-derived nucleotides is a crucial requisite in such studies. Several high-performance liquid chromatographic (HPLC) procedures for the separation of RNA components on anion-exchange supports have been published [1–5], but information on the chromatographic behaviour of nucleoside monophosphates in reversed-phase systems is rather fragmentary and usually restricted to 5′-phosphates [6–10].

This paper describes the influence of pH, ionic strength and organic modifier methanol on the separation of 5′-, 3′- and 2′-purine and pyrimidine ribonucleo-

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side monophosphates on an octadecylsilica reversed-phase support. It is also shown how reversed-phase HPLC can provide suitable procedures for the separation of the major RNA-derived nucleotides.

EXPERIMENTAL

Instrumentation

A Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), consisting of a Model 440 UV detector (fixed wavelength 254 nm), a U6K injector, two pumps, M 6000A and M-45, the gradient programmer M-660 and a μBondapak C_{18} column (300×4 mm I.D., 10 μm particle size) protected by a Guard Pack module, was used. Occasionally, a 2138 Uvicord S UV detector (LKB, Bromma, Sweden) at 254 nm was used. Chromatographic profiles were registered with an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.) or a

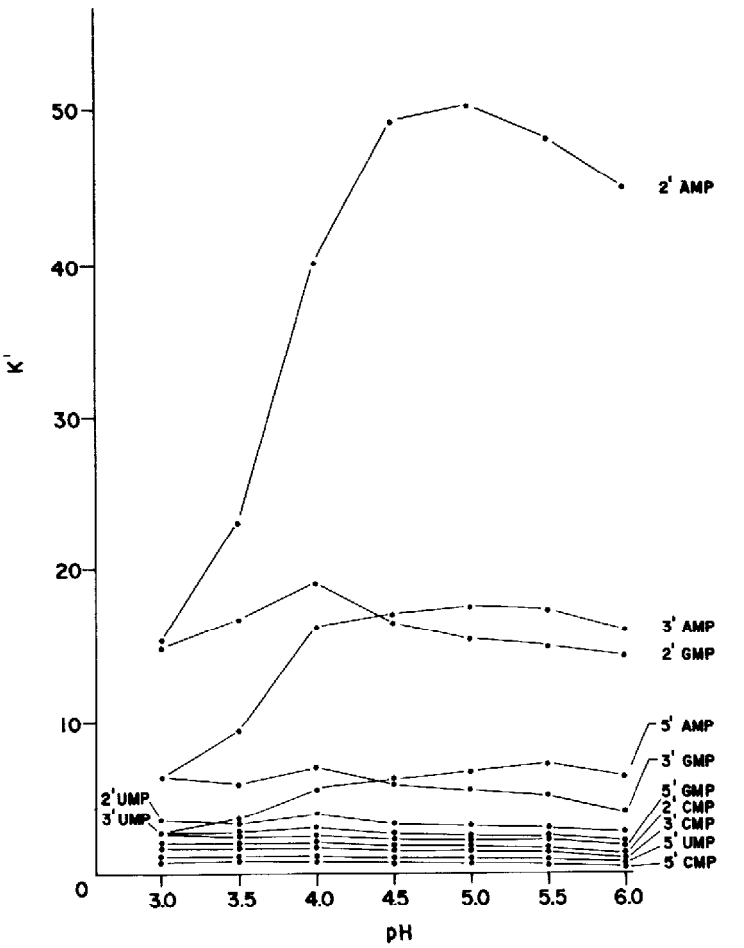


Fig. 1. Effect of mobile phase pH on the retention of 5′-, 3′- and 2′-purine and -pyrimidine ribonucleoside monophosphates. Mobile phase, 0.1 *M* potassium dihydrogen phosphate.

Hewlett-Packard Model 3395 A integrator (Avondale, PA, U.S.A.).

Chemicals

Purine and pyrimidine reference compounds were from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate was from Carlo Erba (Milan, Italy), HPLC-grade methanol was from Romil (Shepshed, U.K.). Radiochemicals were obtained from the Radiochemical Centre (Amersham, U.K.). All other chemicals were reagent grade.

Preparation of eluents, standards and samples

High-quality water was obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Eluents were prepared daily from a stock solution of 1.0 *M* potassium dihydrogen phosphate. The pH was adjusted, and the solution was degassed under reduced pressure through HATF filters (Millipore). Nucleotide standards were prepared as 1 m*M* stock solutions in water (the exact concentration was then calculated from the extinction coefficient) and frozen at −40°C until used. They were appropriately diluted with water, or mixed to the desired concentrations, usually 20–50 μM. Samples derived from RNA hydrolysis were adjusted as

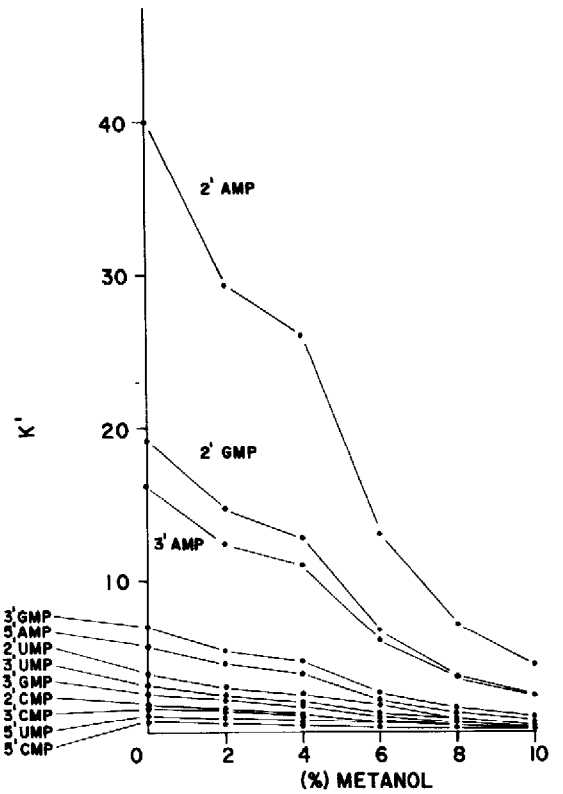


Fig. 2. Effect of the amount of methanol on the retention of 5′-, 3′- and 2′-purine and -pyrimidine ribonucleoside monophosphates. Mobile phase, 0.1 *M* potassium dihydrogen phosphate and 0–10% methanol.

closely as possible to the eluent pH and filtered through HV_4 filters (Millipore) before injection.

Chromatographic conditions

Before use, the column was equilibrated for 1 h at a flow-rate of 1.5 ml/min or overnight at 0.1 ml/min with the selected mobile phase. All separations were carried out at room temperature, flow-rate 1.5 ml/min and chart-speed 0.5 cm/min. For gradient elution, two eluents were prepared: buffer A, 0.1 *M* potassium dihydrogen phosphate and buffer B, 0.1 *M* potassium dihydrogen phosphate and 20% methanol, both adjusted to the same desired pH. At the end of a chromatogram, the gradient programme was reversed for 5 min and the column reequilibrated to initial conditions after 15 min pumping of eluent A. Additional details are given in the figure legends.

Biological samples

Dried *Artemia* embryos were obtained from S. Francisco Bay Brand (Newark, CA, U.S.A.). The treatment of embryos and the growth conditions of nauplii were as described previously [11] except that the salt concentration of the medium was five-fold diluted. *Artemia* nauplii (ca. 300 mg) were incubated in artificial seawater free of phosphates [12], in the presence of 0.3 m*M* [U-^{14}C]glycine (110

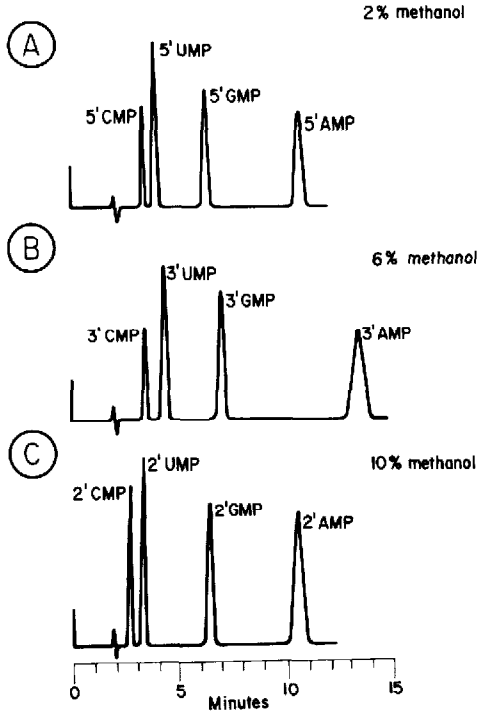


Fig. 3. Chromatographic resolution of simple mixtures of RNA nucleotide standards under isocratic elution. (A) 5′-Monophosphates; (B) 3′-monophosphates; and (C) 2′-monophosphates. Mobile phase; 0.1 *M* potassium dihydrogen phosphate (pH 4.0) and methanol as indicated.

Ci/mol) or 15 μM [8-^{14}C]guanine (54 Ci/mol) for 5 h at 30°C. The nucleic acid fraction and alkaline-derived RNA nucleotides (0.3 M potassium hydroxide, 37°C, 22 h) were obtained as described previously [13].

RESULTS AND DISCUSSION

Influence of pH, ionic strength and organic modifier

The chromatographic separation of 5′-, 3′- and 2′-ribonucleoside monophosphates was studied in the pH range 3.0–6.0. At all pH values the elution sequence,

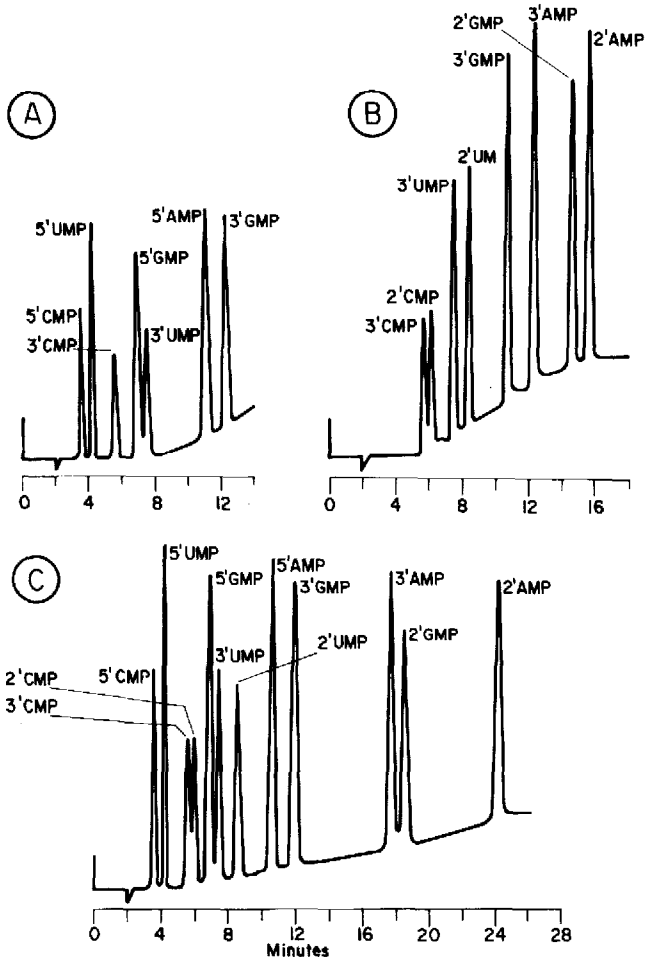
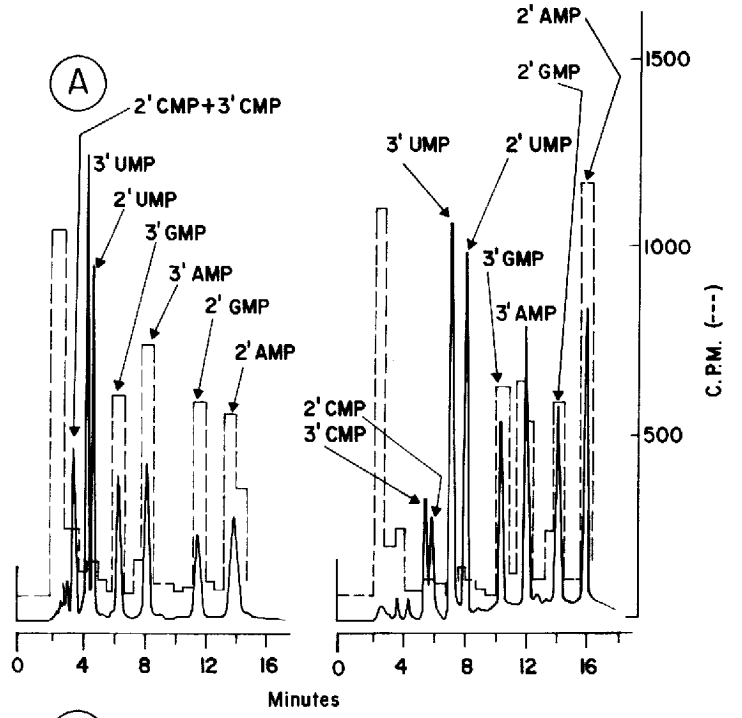


Fig. 4. Chromatographic resolution of complex mixtures of RNA nucleotide standards by gradient elution: (A) Mixtures of 5′- and 3′-monophosphates; initial conditions 0.1 M potassium dihydrogen phosphate (pH 4.0), final conditions 0.1 M potassium dihydrogen phosphate (pH 4.0) and 20% methanol; gradient from 0 to 100% eluent B, curve 7 for 15 min. (B) Mixtures of 3′- and 2′-monophosphates; initial conditions 0.1 M potassium dihydrogen phosphate (pH 3.5), final conditions 0.1 M potassium dihydrogen phosphate (pH 3.5) and 10% methanol; gradient from 0 to 50% eluent B, curve 6 (linear) for 10 min, then isocratic elution until the end of the chromatogram. (C) Mixture of 5′-, 3′- and 2′-monophosphates; initial conditions 0.1 M potassium dihydrogen phosphate (pH 4.0), final conditions 0.1 M potassium dihydrogen phosphate (pH 4.0) and 10% methanol; gradient from 0 to 50% eluent B, curve 6 (linear) for 20 min, then isocratic elution until the end of the chromatogram.


A
2' CMP+3' CMP
3' UMP
2' UMP
3' GMP
3' AMP
2' GMP
2' AMP
2' CMP
3' CMP
3' UMP
2' UMP
3' GMP
3' AMP
2' GMP
2' AMP
1500
1000
500
C.P.M. (---)
0
4
8
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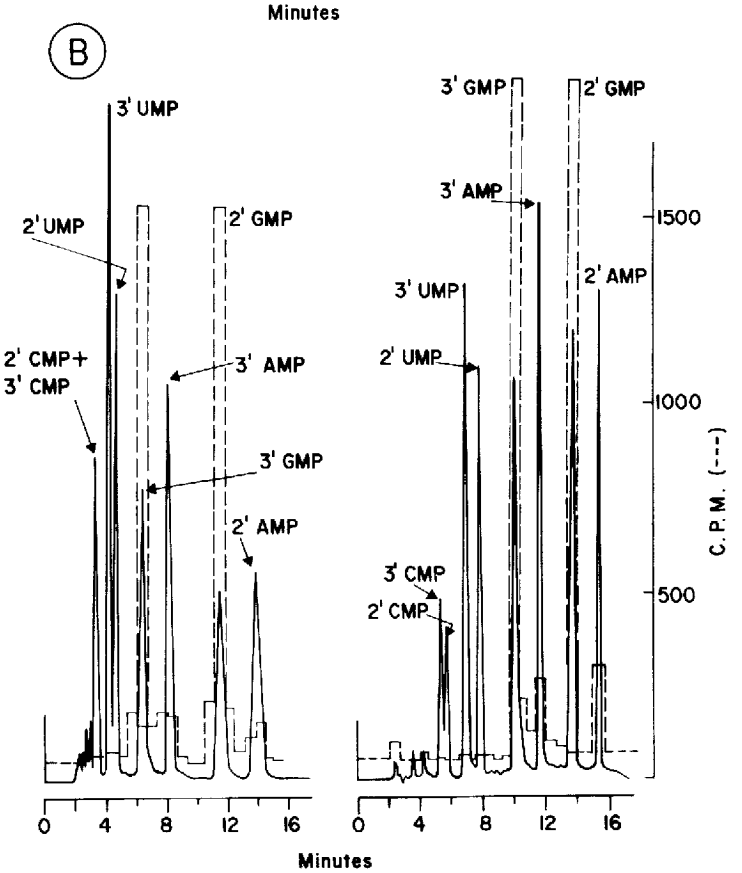
16
Minutes


B
3' UMP
2' UMP
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3' CMP
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3' UMP
2' UMP
2' AMP
3' CMP
2' CMP
1500
1000
500
C.P.M. (---)
0
4
8
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16
Minutes

for a given purine or pyrimidine, is 5′, 3′ and 2′. Fig. 1 summarizes the effect of mobile phase pH (0.1 *M* potassium dihydrogen phosphate) on the capacity factor (k') of cytidine, uridine, guanosine and adenosine monophosphates. The retention of cytidine and uridine monophosphates is scarcely dependent on mobile phase pH, the retention of guanosine nucleotides being moderately affected by pH. However, the retention of adenosine monophosphates is pH dependent, showing a dramatic increase especially when pH changes from 3.0 to 4.0, reaching maxima at pH 5.0 for 3′- and 2′-AMP and at pH 5.5 for 5′-AMP.

Changes of ionic strength between 0.02 and 0.2 *M* potassium dihydrogen phosphate have negligible effects on the elution of all the nucleotides tested. Excellent resolution of 3′- and 2′-nucleotide mixtures can be achieved isocratically at pH 3.5 and 4.0, permitting baseline resolution of all isomers with the sole exception of a partial overlapping between 3′- and 2′-CMP.

Nevertheless, under these conditions, 3′- and 2′-purine nucleotides exhibit excessively long retention times for rapid analysis. Consequently, the effect of the organic modifier, methanol, in the mobile phase was examined in the concentration range 0–10% at pH 4.0. The results (Fig. 2) show that the retention for all nucleotides decreases with increasing methanol concentration and, as expected, the most strongly retained isomers (3′ and 2′) are affected by the presence of methanol.

Although the chromatographic behaviour of free bases on reversed-phase systems can be reasonably explained in terms of pK_a [8], this is not the case for the nucleotides. Neither the observed elution order for the isomers, 5′, 3′ and 2′, nor the retention–pH dependence described here are easy to explain. The reported difficulties encountered by Zakaria and Brown [8] in explaining the chromatographic behaviour of adenosine and its 5′-phosphates, and our additional data on the retention of 3′- and 2′-nucleoside monophosphates, show how far we are from understanding the mechanisms of retention on reversed-phase systems. However, the reversed-phase separations of these compounds, although poorly understood from a mechanistic view point are of immediate use for analytical purposes.

Separation of simple mixtures of 5′-, 3′- or 2′-nucleotides

Mixtures of 5′-, 3′- or 2′-isomers can be baseline-resolved under isocratic conditions in the pH range 3.5–6.0. At pH 3.0, there is co-elution of 5′-GMP and 5′-AMP and of 3′-GMP with 3′-AMP. Although 5′-isomers can be eluted in a reasonable time without methanol in the mobile phase, the addition of the organic modifier is convenient if the separation of the 3′- or 2′-nucleotides is desired, as shown in Fig. 3.

Fig. 5. Chromatographic and radioactivity profiles from alkali-derived RNA nucleotides after incubation of *Artemia* nauplii with labelled purine nucleotide precursors. (A) Incorporation of radioactivity from [U-^{14}C]glycine. (B) Incorporation of radioactivity from [8-^{14}C]guanine. For counting radioactivity, fractions were collected every 40 s. Separation was accomplished under both isocratic (left) and gradient (right) elution: isocratic conditions: 0.1 *M* potassium dihydrogen phosphate (pH 3.5) and 5% methanol; gradient conditions: same as indicated in Fig. 4B.

Separation of complex mixtures

Mixtures of 3′- and 2′-nucleotides can be conveniently resolved at pH 3.5. However, mixtures of 5′- and 3′- or of 5′- and 2′-nucleotides are best resolved at pH 4.0. Fig. 4A and B shows the separation of mixtures of 3′- and 2′- and of 5′- and 3′-nucleotides by gradient elution with increasing methanol concentration. The use of gradients prevents excessive overlap between 2′- and 3′-CMP or between 5′-GMP and 3′-UMP, as observed in isocratic elution with 5% methanol, but the remaining peaks are still baseline-resolved. Finally, Fig. 4C illustrates the separation of all isomers at pH 4.0 with a linear gradient of methanol concentration from 0 to 10%.

However, many separations can be achieved isocratically for certain purposes, and both the short analysis time and the excellent resolution we describe overcome some of the disadvantages of the anion-exchange separations, e.g. overlapping of 2′- and 3′-isomers [2,3] and of different purine [5] or pyrimidine bases [4] containing nucleotides.

Applications

The methods described here are currently used in our laboratory in the study of purine nucleotide metabolism during the larval development of the crustacean *Artemia*. Fig. 5 shows the incorporation into RNA of radioactivity from the de novo ^{14}C-labelled precursor glycine (Fig. 5A), and from the salvage-labelled precursor [8-^{14}C]guanine (Fig. 5B). RNA nucleotides are separated in 16 min by either isocratic or gradient elution. This allows the detection of both adenine- and guanine-labelled nucleotides, which in turn permits easy determination of the percentage distribution of radioactivity and specific activity.

From the present study it is evident that, depending on the experimental needs of the investigator, many different types of separation can be achieved by adequate manipulation of the pH and organic modifier content of the mobile phase. Moreover, it should be noted that although these chromatographic procedures have been designed for use in metabolic studies, they can also provide useful tools in studies of RNA composition and in enzymology of nucleases acting on RNA, as well as for determinations of purity and specificity of such enzyme preparations. Finally, these procedures could also be of clinical interest in view of the significance of cellular levels of different ribonucleoside monophosphates in human leukemias [14].

ACKNOWLEDGEMENT

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