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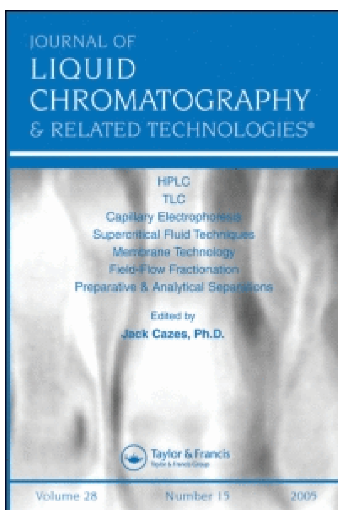

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THE SEPARATION OF THE MAJOR RIBONUCLEOTIDES, NUCLEOSIDES, AND BASES IN REVERSE-PHASE ION-PAIR CHROMATOGRAPHY

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ABSTRACT

The separation of 12 major nucleotides was achieved on a reversed-phase HPLC system with the radially compressed NovaPak C_{18} column and tetrabutylammonium phosphate (TBA) ion pairing reagent by acetonitrile gradient elution. The effect of tetrabutylammonium phosphate and acetonitrile on the retention of the major nucleotides in this system was studied. It was found that in k' decreases in a proportion to the concentration of acetonitrile in the mobile phase. The maximum of nucleotide retention was found to be at TBA concentration of about 2 mM, the amount of acetonitrile in the mobile phase being constant. A simultaneous separation of main nucleotides, nucleosides, and bases was achieved under optimum conditions.

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INTRODUCTION

High-performance liquid chromatography is the most suitable method for analysis of nucleic acids components and their derivatives in biological samples. In recent yeasr, the method of reversed-phase liquid chromatography has found broad application for analysis of the composition of bases and nucleosides in acid-soluble fractions of cell cultures, biological fluids (blood,lymph, urine) and tissues (1,2,3,4) whereas the nucleotide pool is mostly analyzed by anion-exchange chromatography (5,6, 7).

Some authors tried to separate the nucleotides by reversed-phase HPLC (8,9). However, low retention of nucleotides (particularly di- and triphosphates) on the reversed phase supports enables to solve either rather narrow or special problems (10,11).

The methods of ion-pair chromatography on a reversed phase have recently started developing; for instance, they were applied to the analysis of adenylates (12), oligonucleotides (13), simultaneous determination of mononophosphates of nucleotides and some nucleosides and bases (14,15,16). Also, attempts have been made to separate a more complete set of nucleotides by ion-pair chromatography methods (17).

At the same time, the reverse-phase ion-pair chromatography is very promising for a simultaneous separation of the whole range of purine and pyrimidine derivatives, including bases, nucleosides, as well as nucleoside mono-, di-, and triphosphates. The goal of our study was analysis dependences of the retention times of the major mono-, di-, and triphosphates of nucleosides on a reversed-phase NovaPak C_{18} system on the concentration of the ion-pairing reagent and the percentage of the organic modifier (acetonitrile) in the mobile phase.

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We also try to evaluate the possibility of a simultaneous separation of nucleotides, nucleosides, and nucleic bases on this system by their gradient elution.

EXPERIMENTAL

HPLC Instrumentation

A Waters high-performance liquid chromatography system was used (Waters Associates, Milford, Mass., USA), which consisted of an M510 and M45 solvent delivery system, model 441 and Lambda-Max model 481 absorbance detectors, U6K Universal Liquid Chromatograph injector, model 660 solvent programmer and Z-Module RCSS Radial Compression separation system. Absorbance was measured at 254 nm and 280 nm and area and retention time were calculated using a model 730 Date Module.

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Chemicals

The following chemicals were used: $NH_4H_2PO_4$ (Fisher Scientific Co., Fairlown, NJ); acetonitrile (Merck, Darmstadt, FRG). Nucleic bases, nucleoside and their mono-, di- and triphosphates were purchased from Sigma Chemical Co., (St.Louis, MO, USA). TBA (tetrabutylammonium phosphat) as PIC Reagent A were obtained from Waters Assoc., USA).

Chromatography

A 0.01 M $NH_4H_2PO_4$ buffer containing TBA in 0.0005 M, 0.002 M, and 0.005 M concentrations with acetonitrile added to 10%, 15%, 20%, 25% concentrations in the final solution was used as eluent. To remove organic impurities, water was passed through a Norganic cartridge (Waters Assoc., USA). The buffer containing ammonium dihydrogen phosphate and tetrabutylammonium was filtered through a 0.45 μ HA filter and acetonitrile through a 0.45 μ FH filter (Millipore, Intertech., France). The final buffer was degassed under vacuum for 20 min and

its pH was adjusted to 7.0 by adding concentrated NH_4OH. 0.01 M $NH_4H_2PO_4$ with TBA added to 0.002 M and 0.005 M was amployed as buffer A in gradient elution; buffer B was based on buffer A with acetonitrile added to a 15% concentration. The gradient used ranged from zero to 100% B, # 6, 10 min.

The separation was carried out on a radially packed Z-Module system with a NovaPak C_{18} plastic cartridge (100 x 8 mm) (Waters). The column was operated at 1000 psi, flow rate 2 ml/min and at room temperature.

RESULTS

1. The Effect of TBA Concentration on the Retention of Nucleotides

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Figure 1 A,B shows the dependence of the capacity factor of the major purine nucleotides on the concentration of the ion-pairing reagent for two different contents of acetonitrile in the eluent. A strong increase in the retention of nucleotides can be noted for TBA concentration growing from zero to 0.002 M, the increase being much greater for 10% of acetonitrile in the eluent than for 15%. Similar results were obtained for pyrimidine nucleotides (Figure 2 A,B). It is noteworthy that like in anion-exchange chromatography, nucleotides can be definitely divided into three groups according to their retention times and in correlation with the charge of the phosphate groups.

The increase of TBA concentration above 2 mM does not affect the retention of nucleotides and in the case of di- and triphosphates k' values are slightly decreased.

2. The Effect of Acetonitrile on the Retention of Nucleotides

Figure 3 A,B,C presents the dependence of ln k' of the major pyrimidine nucleotides on the percentage of

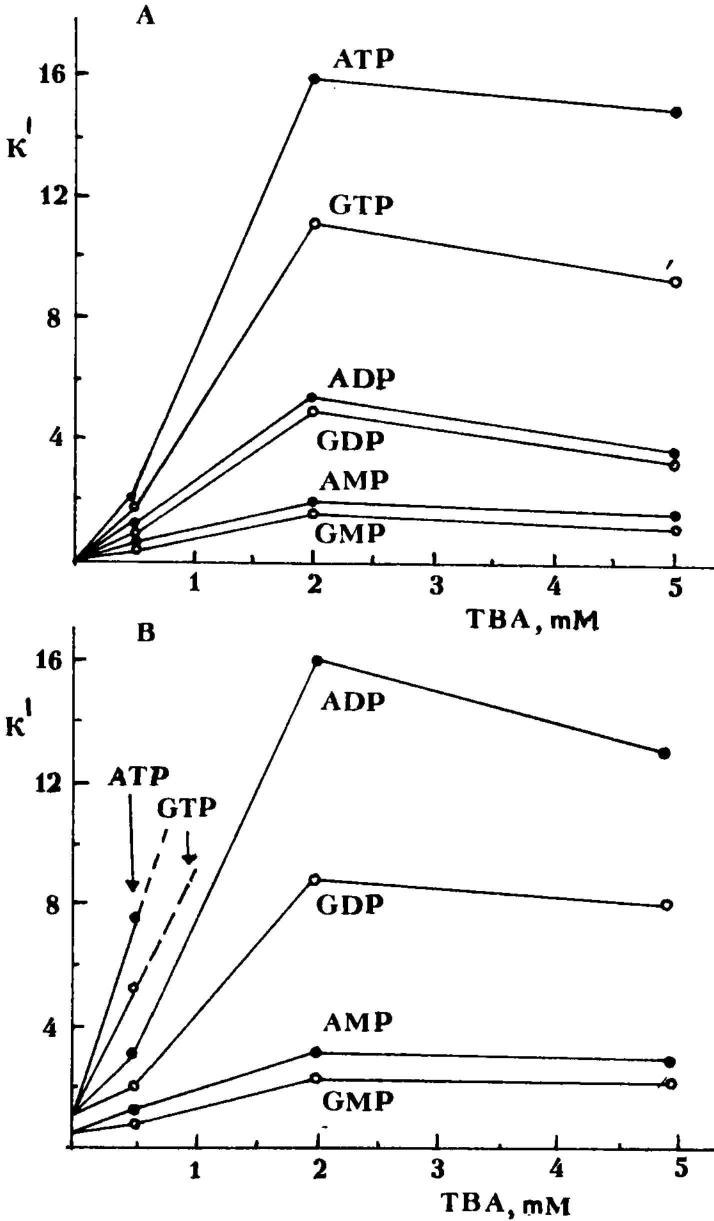

Figure 1. Plot of k' vs. concentration of pairing ion (TBA) in mobile phase for the adenine and quanine nucleotides.
A - 15% CH_3CN in mobile phase
B - 10% CH_3CN in mobile phase.

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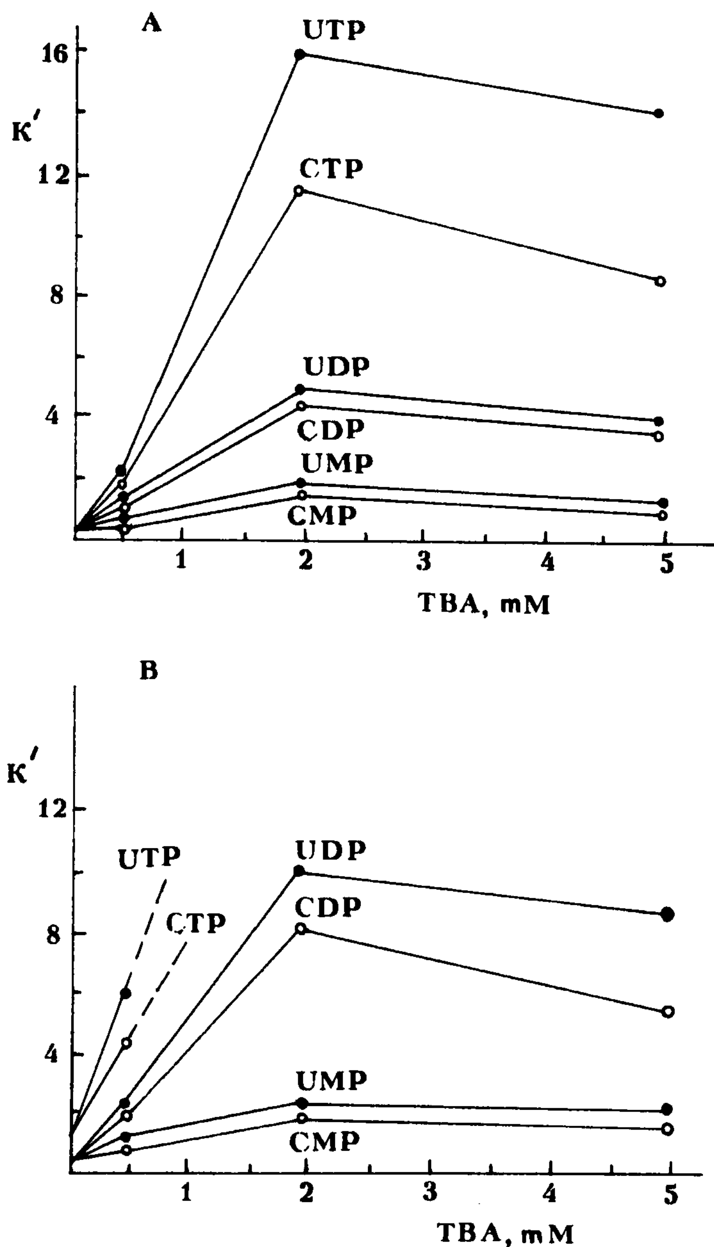

Figure 2. Plot of k' vs. concentration of pairing ion (TBA) in mobile phase for the cytosine and uracil nucleotides.
A - 15% CH_3CN in mobile phase
B - 10% CH_3CN in mobile phase.

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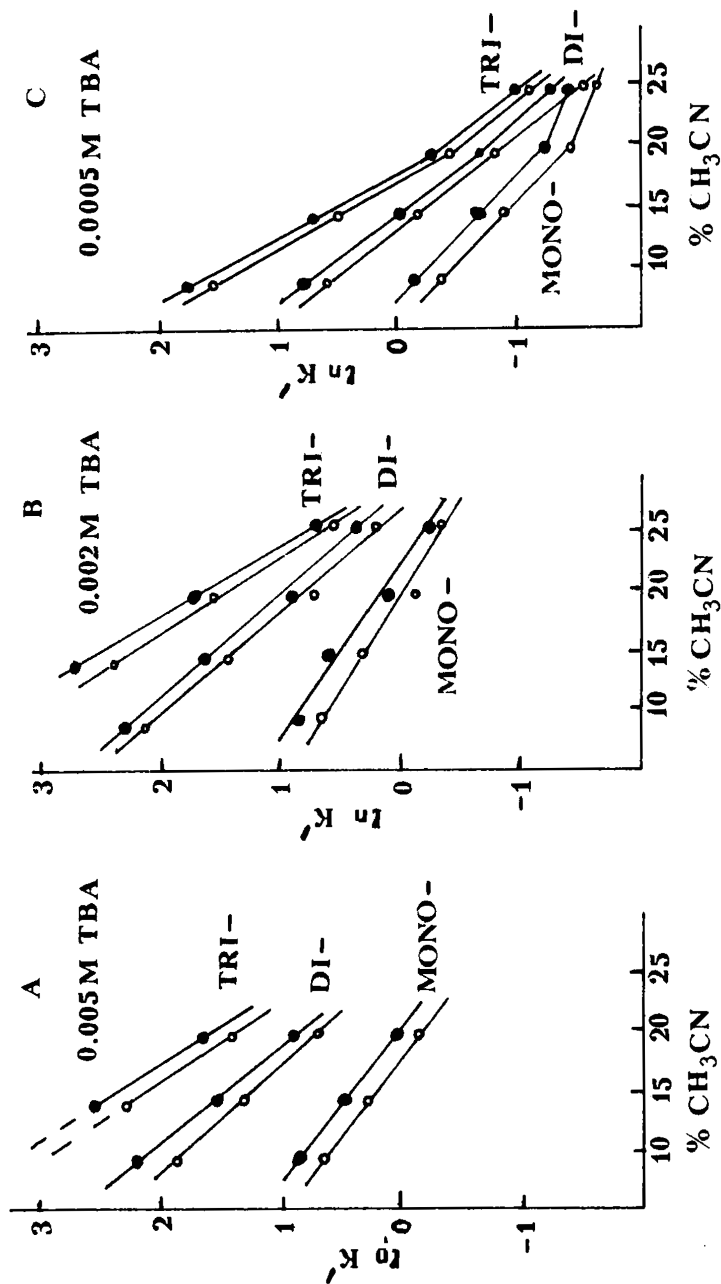

Figure 3. Plot of ln k' vs. % of acetonitrile for the mono-, di- and tri-phosphate nucleosides of cytidine (-o-) and uridine (-o-) at several TBA concentrations.

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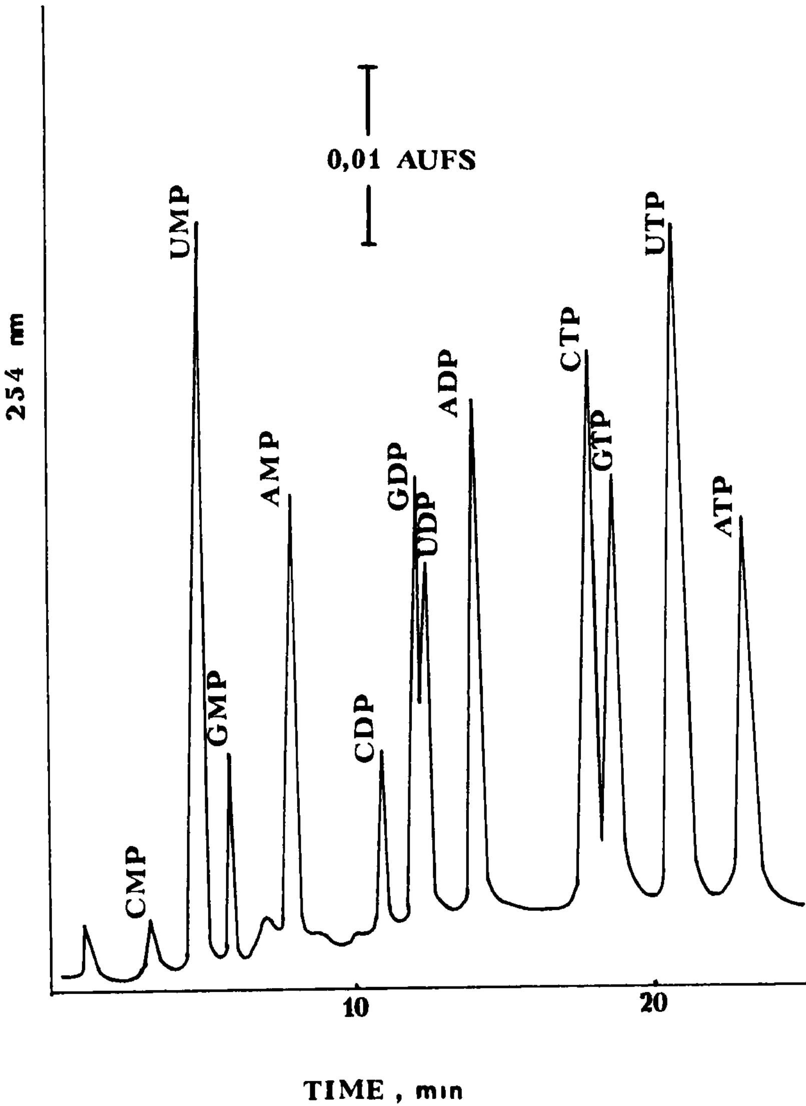

Figure 4. Separation of 12 nucleotide standards with 0 to 15% acetonitrile gradient (# 6, 10 min) at 0,005 M TBA concentration in mobile phase, flow rate 2.0 ml/min.

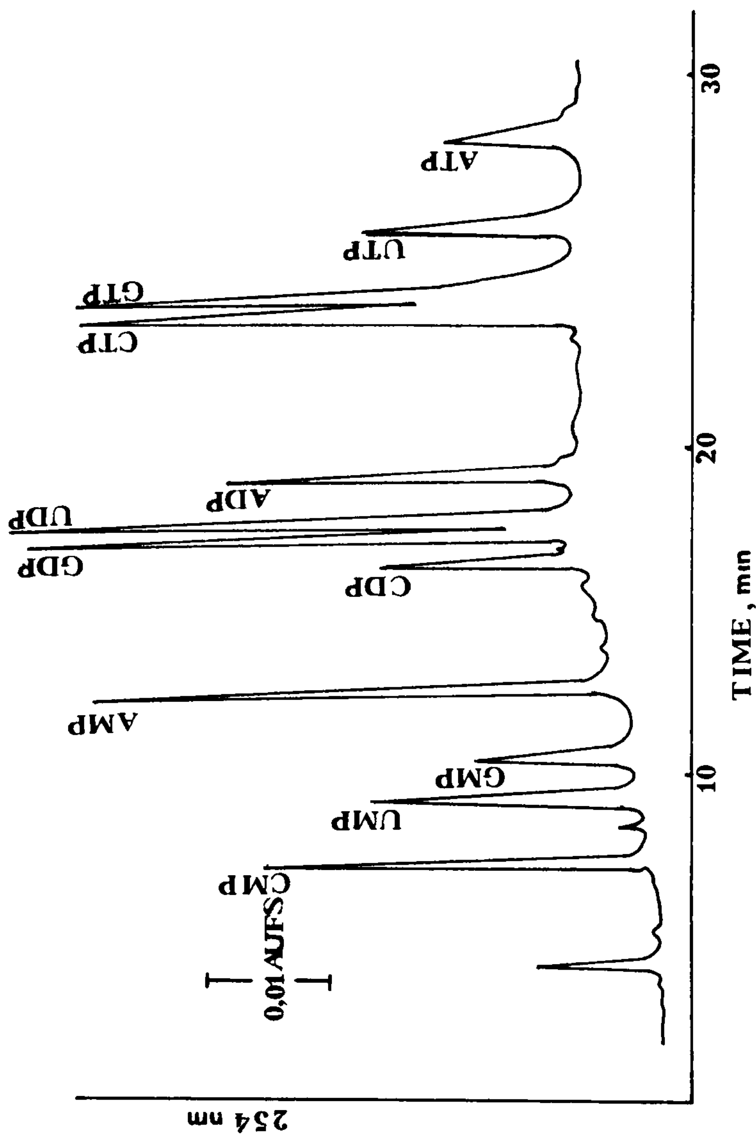

Figure 5. Separation of 12 nucleotide standards with 0 to 15% acetonitrile gradient (# 6, 10 min) at 0,002 M TBA concenration in mobile phase, flow rate 2.0 ml/min.

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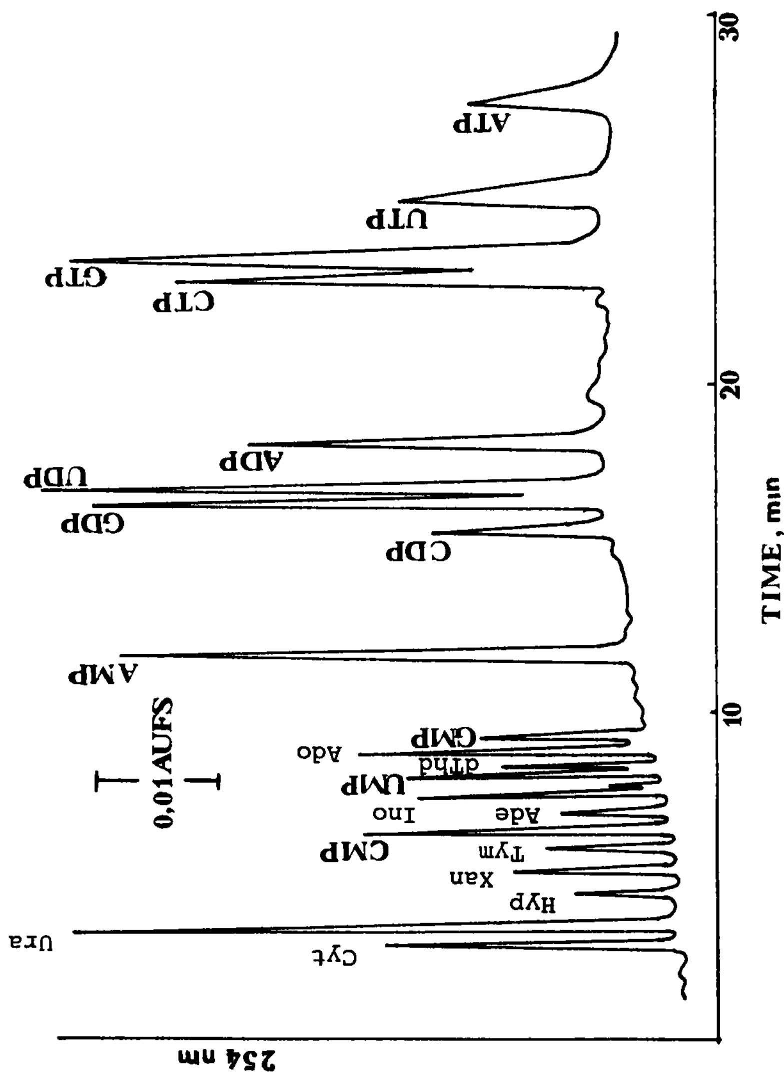

Figure 6. Separation of the major nucleic bases ribonucleosides and nucleotides with 0 to 15% acetonitrile gradient (# 6, 10 min) at 0,002 TBA concentration. Flow rate 0.2 ml/min.

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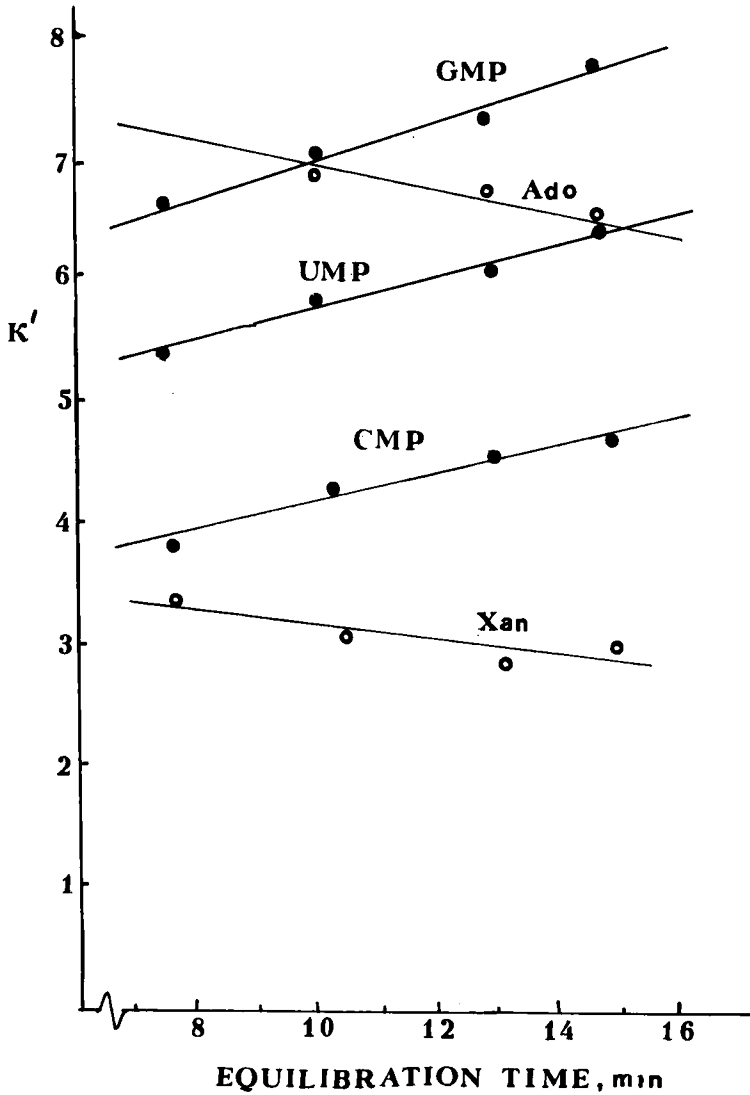

Figure 7. Plot of k' vs. equilibration time by the starting buffer for the Ado, Xan, CMP, UMP, 6MP.
Conditions as for Figure 5.

acetonitrile in the eluent at different TBA concentrations. In all instances ln k' linearly upon the increase of acetonitrile concentrations. In the case of low (0.0005 M TBA) concentrations of the ion-pairing agent, a slight deviation from linearity towards larger retention times appears. Similar results were obtained for purine nucleotides.

3. The Separation of the Major Nucleotides, Nucleosides and Bases in Acetonitrile Gradient

Figures 4,5 show the results of separation of the major nucleotides for two concentrations of the ion-pair reagent: 0.002 M and 0.005 M TBA. Good separation of all 12 nucleotides can be seen. Nucleosides and bases were separated under the same chromatographic conditions. Figure 6 depicts the results of chromatographic separation of the mixture of bases, nucleosides and their mono-, di-, and triphosphates in acetonitrile gradient. All the results were obtained after 10 min of equilibration of the column with the initial buffer. Figure 7 presents the dependence of the capacity factor, k', for xanthine, adenosine and three monophosphates on the equilibration time of the column with the starting buffer. An increase of the equilibration time reduces k' of bases and nucleosides and, on the contrary, raises k' of monophosphates.

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DISCUSSION

The adding of a counterion in the eluting buffer results in its binding with the stationary phase of the C_{18} column due to hydrophobic interaction of the aliphatic groups of the column's packing material and tetrabutylammonium cation. The relationship governing the interaction (14) is described by the equation:

$$C_s = \alpha \cdot C_m^{\beta} \qquad (1)$$

where C_s is the concentration of TBA on the stationary phase;

C_m is the concentration of TBA in the mobile phase;

α, β - are the constants of interaction of TBA with the stationary and mobile phases.

Due to above interaction positively charged groups arise on the stationary phase, which determine the possibility of the retention of nucleotides as a function of the negative charge of their phosphate groups. An increase of TBA concentration in the mobile phase gives rise to a corresponding enhancement of the amount of the counterions on the stationary phase, according to Equation (1), thus intensifying the retention of negatively charged nucleotides (Figures 1,2). The existence of a retention maximum at 0.002 M TBA concentration can testify to the saturation of interaction achieved between the ion-pairing reagent and the stationary phase for a certain compoisiton of the aqueous-organic phase of the eluent (18,19) and also to the prevalence (on further growth of TBA concentration) of the formation of TBA complexes with the charged sample and their dissolution in the aqueous-organic phase (15). The existence of the retention maximum gives grounds for a finding of the most suitable concentrations of the ion-pairing reagent and the organic solvent in the "NovaPak C_{18}- acetonitrile-containing buffer" system giving rise to maximum retention and selectivity in relation to nucleotides.

An increase of the acetonitrile concentration leads to a linear reduction of ln k' of nucleotides at all TBA concentrations (Figure 3). This seems to be due to the enhancement of hydrophobic interactions of the aliphatic moieties of the ion-pairing reagent with the aqueous-organic phase and to a corresponding decrease of TBA concentration on the stationary phase. The equation governing this interaction (14) is the following:

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$$k' = A \cdot C_s e^{B \cdot C_A}$$
$$\text{or} \quad k' = A \cdot \alpha \cdot C_m \cdot e^{B \cdot C_A} \qquad (2)$$

where C_A is the concentration of acetonitrile in the mobile phase;

A,B are the constants of interaction of the ion-pairing reagent with the mobile and stationary phases.

The use of isocratic conditions for separation of nucleotides, at constant percentage of acetonitrile, either does not enable the separation of monophosphates or cannot allow the elution of triphosphates. The exponential dependence of k' on the concentration of the organic modifier enables to perform simultaneous separation of all nucleotides by linear gradient elution with final buffer containing 15% of acetonitrile (Fig. 4). The initial region of elution of monophosphates is extended due to the gradient whereas di- and triphosphates are eluted in isocratic mode (15% acetonitrile in buffer). Like in anion-exchange separation the chromatographic pattern is characterized by formation of three groups of components related to the charge of the molecule or to the number of phosphate groups. Monophosphates are eluted in order similar to that in reverse-phase chromatography: CMP → UMP → GMP → AMP; however in case of di- and triphosphates uracil and guanine nucleotides are eluted in different order. In all cases adenine nucleotides come out the last in the group and somewhat separately, what is very convenient for the analysis of the majority of the biological samples with predominant content of adenylates. The conditions found in (17) allow to separate for quantitative estimation more than 10-12 biologically important nucleotides and coenzymes like

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ATP, ITP, IDP, IMP, NAD, c-AMP. However very close elution of some mono- and diphosphates in the beginning of the run makes impossible the application of these conditions for separation of biologically important nucleosides and bases together with the components under study. The problem of separation of nucleosides and their monophosphates is solved (14), but di- and triphosphates are not eluted in this case.

The separation of nucleosides and bases in the chromatographic conditions found demonstrates the significant selectivity of the system for these compounds, and thus the markedly lower registration threshold for them than for later eluting nucleotides. This fact indicates the possibility to use this chromatographic system for analysis of biological samples (as like as acid-soluble fractions, cell suspensions), which are characterized by an order of magnitude lower content of nucleosides and bases in comparison with that of nucleotides.

For a given biological experiment it is necessary to have an appropriate set of standards related to the studied substrates of biochemical reactions and to the specific composition of the biological sample. In this connection it may be necessary to change slightly the separation conditions (concentration of ion-pair reagent and final percentage of organic solvent). In order to find the optimum separation conditions of the biological samples one should have the information about the effect of pH and ionic strength of the starting eluent on the retention and resolution of the components in a given chromatographic system "NovoPak C_{18}-PICA-buffer with acetonitrile".

The application of ion-pair reagent together with organic modifier makes this chromatographic system similar to ion-exchange one with the same long equilibration time after returning to the gradient start. The

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equilibration appears to comprise setting an equilibrium between TBA concentration in the mobile and stationary phases at a certain aqueous-organic eluent composition. The results presented in Fig. 7 demonstrate the opposite dependences of k'-values of bases, nucleotides and monophosphates on the increase of equilibration time. Thus, the small variations in equilibration time can be used for fine regulation of k'-values of monophosphates, nucleosides and bases in order to improve resolution in the region of their combined elution.

Thus, the study of retention of nucleotides on reverse-phase column in the presence of ion-pair reagent allows to find the conditions for simultaneous separation of the major nucleotides in acetonitrile gradient. The chromatographic conditions found enable to perform separation of nucleotides and biologically important nucleosides and bases during one run, that is important for many fields of biochemical experiments.

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