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# SIMULTANEOUS SEPARATION OF RIBONUCLEOTIDES, NUCLEOSIDES AND NITROGEN BASES BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON COLUMNS WITH RADIAL COMPRESSION

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## SUMMARY

A method is described for the simultaneous analysis of twelve major nucleotides, certain biochemically essential nucleosides and nitrogen bases. The separation was achieved using an ion-pair reversed-phase high-performance liquid chromatographic (HPLC) system with a radially compressed NovaPak $C_{18}$ cartridge and tetrabutylammonium phosphate (TBA) as a mobile phase modifier under acetonitrile gradient conditions. The effects of TBA and acetonitrile on the retention of major nucleotides were studied. The retention of nucleotides increased significantly with an increase in the TBA concentration from 0 to 2 m*M* and remained constant up to 5 m*M* TBA. The logarithm of the capacity factor decreased in proportion to the concentration of acetonitrile in the mobile phase. The separation of nitrogen bases, nucleosides and some of their deoxy forms was performed on an isocratic ion-pair reversed-phase HPLC system with pentanesulphonic and heptanesulphonic acid using a $\mu$Bondapak $C_{18}$ column. The described chromatographic procedures were applied to the separation of various biological extracts.

## INTRODUCTION

A shift in nucleic acid metabolism observed in pathology, specifically cancerogenesis, involves substantial alterations in the activity of catabolic, anabolic and interconversion enzymes[1,2]. The importance of purine metabolism was confirmed by the discovery of deficiencies of certain major enzymes in patients with immunodeficiency[3,4]. The enzyme–substrate interactions which play an essential role in the nucleic acid metabolism of cells and tissues largely depend on the composition and content of free nucleotides, nucleosides and bases[5].

At present, high-performance liquid chromatography (HPLC) is considered to be the most promising method for the determination of purine and pyrimidine metabolites[6]. The biochemical significance of information on the intracellular nucleotide composition necessitates the development of a chromatographic method to make it possible to analyse the full range of nucleic acid derivatives. Ion-pair reversed-phase

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HPLC permits a partial modification of the stationary phase towards the anion- or cation-exchange retention mechanism, and affords the possibility of designing tissue-specific chromatographic procedures for the simultaneous analysis of the whole purine/pyrimidine derivative pool, from bases and nucleosides to their triphosphates[7].

## EXPERIMENTAL

### *Instrumentation*

The HPLC system (Waters Assoc., Milford, MA, U.S.A.) consisted of M510 and M45 solvent delivery systems, Model 441 (254 nm) and Lambda-Max 480 (280 nm) absorbance detectors, a U6K universal injector and a Model 660 solvent programmer.

Separation was carried out at room temperature with a 5-$\mu$m NovaPak $C_{18}$ cartridge (10 cm × 8 mm I.D.) on a Z-Module radial compression separation system and a 10 $\mu$m $\mu$Bondapak $C_{18}$ stainless-steel column (30 cm × 3.8 mm I.D.). The eluate was monitored at 254 and 280 nm. The detector responses were recorded and calculated with a Model 730 Data Module dual-pen integrator. Peaks were identified by observing the retention times and peak height ratios at 254 and 280 nm.

### *Chemicals*

Nucleic bases, nucleosides and their mono-, di- and triphosphates were purchased from Sigma (St. Louis, MO, U.S.A.). PIC reagent A (tetrabutylammonium phosphate), PIC reagent B5 (pentanesulphonic acid) and PIC reagent B7 (heptanesulphonic acid) were obtained from Waters Assoc. Acetonitrile was provided by Merck (Darmstadt, F.R.G.).

### *Chromatography*

Water was passed through a Norganic cartridge (Millipore). Ammonium phosphate buffer with acetonitrile and an ion-pairing reagent as a mobile phase modifier (PIC A or PIC B5 and PIC B7) was filtered through a 0.45-$\mu$m filter (Millipore) and degassed for 20 min before use. The flow-rate was 2 ml/min at a pressure of 1000 p.s.i. for the NovaPak $C_{18}$ cartridge and 2500 p.s.i. for the $\mu$Bondapak $C_{18}$ column.

### *Preparation of tissue extracts*

Mouse thymus and liver were immersed in liquid nitrogen, weighed and powdered with a pestle in a porcelain mortar. Tissue homogenate was prepared in a Potter homogenizer (1 g of tissue per 10 ml of perchloric acid). The homogenate obtained was centrifuged for 10 min at 6000 rpm on a K-23 centrifuge (G.D.R.). The supernatant was neutralized to pH 7.0 with concentrated potassium hydroxide solution and kept for 24 h at 4°C, after which the sediment was separated by brief centrifugation, and the supernatant was stored at −45°C.

Blood was drawn from the ulnar vein of healthy donors and myocardial infarction patients into preliminarily cooled tubes with sodium citrate, and treated with 1 *N* perchloric acid (5 ml of perchloric acid per ml of blood).

Lymphocytes were isolated from human and mouse blood and mouse thymus by centrifugation in Ficoll ($\rho$ = 1.090) for 45 min at 400 *g* and 4°C. The cell sus-

pension was twice rinsed in medium 199 and the cell pellet was treated with 1 *N* perchloric acid (1 ml per $10^8$ cells).

## RESULTS AND DISCUSSION

The simultaneous separation of nucleotides, nucleosides and nitrogen bases was achieved by an addition of tetrabutylammonium phosphate (TBA) to the mobile phase at a small ionic strength (10 m*M* ammonium dihydrogen phosphate). As a result, a portion of the $C_{18}$ stationary phase was modified towards anion-exchange properties. Figs. 1 and 2 show the dependences of nucleotide retention on the TBA and acetonitrile concentrations in the mobile phase. An increase in the TBA concentration to 0.002 *M* led to a substantial increase in the retention of purine nucleotides (Fig. 1). The same results were obtained for pyrimidine nucleotides. Similarly to anion-exchange chromatographic systems, the capacity factor of the sample nucleotides was proportional to the charge, *i.e.*, the number of phosphate moieties. An increase in the organic component content of the mobile phase brought about a linear reduction in ln *k'*, irrespective of the TBA concentration (Fig. 2). The retention of nucleosides and bases during an increase in TBA concentration in the mobile phase was reduced insignificantly.

These results afford the basis for the development of procedures of two types: the separation of nucleotides under isocratic and organic solvent gradient conditions; and the simultaneous separation of bases, nucleosides and nucleotides under the conditions of an organic solvent gradient.

Even at small ionic strengths, isocratic conditions do not allow a simultaneous separation of mono-, di- and triphosphates to be achieved with satisfactory selectiv-

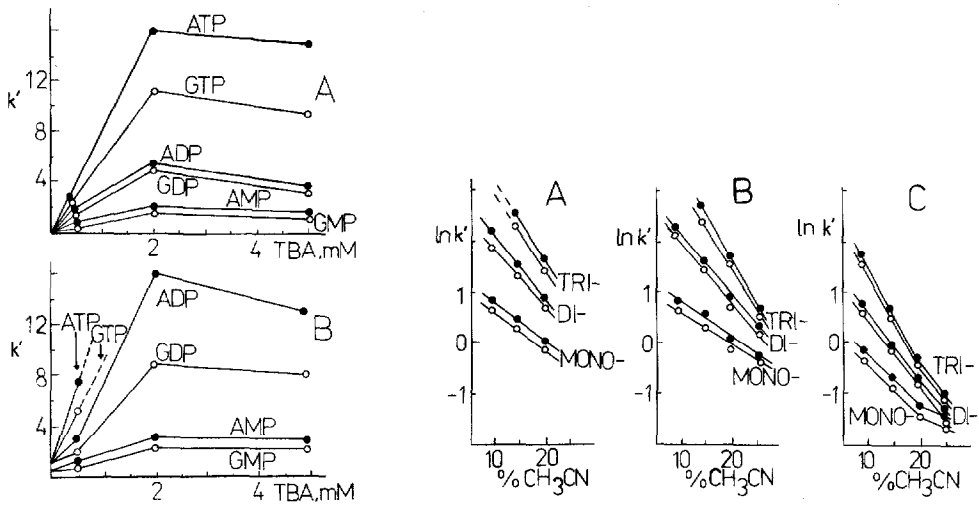



Fig. 1. Dependence of the retention coefficient (*k'*) of adenine and guanine nucleotides on the TBA concentration in the mobile phase. (A) 15% acetonitrile in the mobile phase; (B) 10% acetonitrile in the mobile phase.

Fig. 2. Dependence of *k'* of cytidine (○) and uridine (●) mono-, di- and triphosphates on the acetonitrile concentrations in the mobile phase. (A) 5 m*M* TBA, (B) 2 m*M* TBA and (C) 0.5 m*M* TBA in the mobile phase.

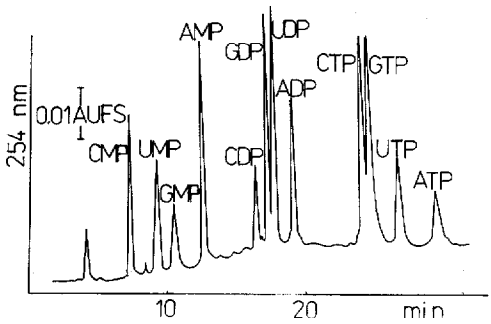



Fig. 3. Separation of twelve major nucleotides by ion-pair reversed-phase HPLC. Column: NovaPak $C_{18}$ in a Z-Module system with radial compression at room temperature. Mobile phase: (A) 10 m*M* ammonium dihydrogen phosphate–2 m*M* PIC A (pH 5); (B) 85%A + 15% acetonitrile (pH 7.5); gradient, 0–100% B; curve, 6,10 min. Flow-rate, 2 ml/min.

ity. We attempted to find the optimal conditions for the separation of twelve major nucleotides, but a low selectivity of monophosphates combined with substantial ATP retention ($k' > 20$) prevented us from obtaining the desired result. Isocratic conditions, however, are utilized in ion-pair reversed-phase HPLC to solve specific problems such as the separation of bases, nucleosides and their monophosphates[8,9], adenine nucleotides[10] and oligonucleotides[11].

Therefore, the optimal resolution of the whole nucleotide spectrum required an ion-pair reversed-phase HPLC system with an acetonitrile gradient from 0 to 10% (Fig. 3). However, the conditions used are perfectly suitable for the separation of purine and pyrimidine nucleosides and bases at the initial stage of elution which is devoid of nucleotides. This allowed us to carry out a simultaneous separation of neutral bases, nucleosides and charged nucleotides in a single run (Fig. 4). Optimization of the separation was achieved by varying such parameters as pH, ionic strength and time of column equilibration with the initial buffer. An increase in ionic strength leads to a decrease in the retention of all nucleotides (according to the anion-exchange separation mechanism). Under the conditions used, the optimal ionic strength was within the range 10–15 m*M* ammonium dihydrogen phosphate. Further, the selectivity of the separation of nucleosides and bases is affected by variations in pH within the range of their basic ionization constants, $k_{ab}$ (pH 3–5[12]). On the other

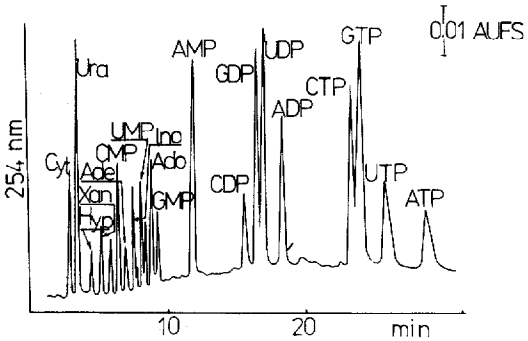



Fig. 4. Separation of major nucleotides, nucleosides and bases by IP RP HPLC. Conditions as in Fig. 3.

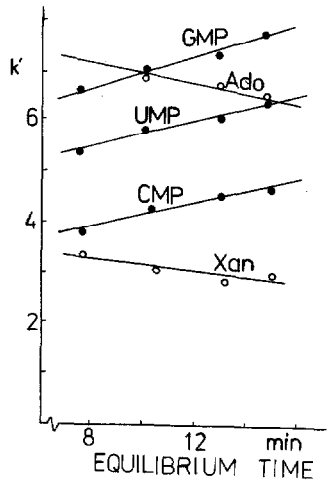
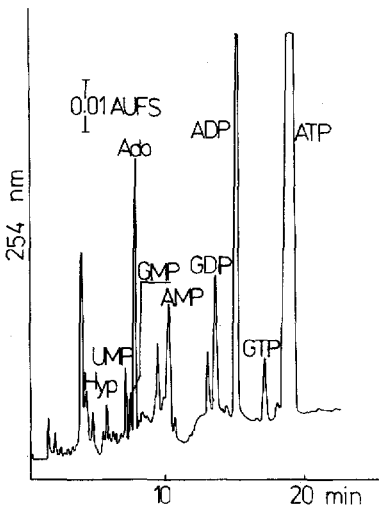



Fig. 5. Dependence of $k'$ on the time of column equilibration with the initial buffer.

hand, an increase in pH to 7.5 leads to the conversion of nucleotides into anion species and, therefore, to their maximal retention in the anion-exchange chromatographic system. Both of these factors determined the gradient range variations, pH 5–7.5. Optimization and fine regulation of the selectivity of this chromatographic separation can be achieved by altering the time of column equilibration with the initial buffer (Fig. 5). The latter affects, and in a contrary way, only the retention of bases and nucleosides on the one hand, and monophosphates on the other hand. This method allows one to obtain the optimal separation of biological samples with different spectra of nucleic acid precursors. Figs. 6 and 7 exemplify the separation of



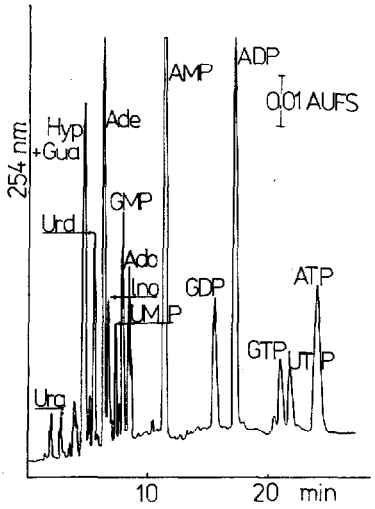




Fig. 6. Chromatogram of the acid-soluble pool of human blood. Conditions as in Fig. 3.

Fig. 7. Chromatogram of the acid-soluble pool of C3HA mouse thymus. Conditions as in Fig. 3.

the acid-soluble pool of human blood and liver of C3HA mice. It should be pointed out that the separation was carried out without the preliminary fractionation of biological samples, and the data on the total composition of purine and pyrimidine bases was obtained in a single run.

The analysis of the nucleotide composition of the acid-soluble pool of various tissues and organs[1] showed the presence of a specific nucleic acid precursor distribution. The specific composition of nucleic acid components is characteristic of cells from the liver, blood and immunogenic organs (spleen, thymus, bone marrow, lymph nodes) and biological fluids, urine, plasma, spinal fluid, etc. This specificity necessitates the development of appropriate methodology for the separation and analysis of nucleic acid components in each individual case. Specifically, the analysis of the composition of nucleosides and bases in the acid-soluble pool of cells from lymphoid organs and tissues (thymus, lymph nodes, spleen, bone marrow) showed insufficiency of the available reversed-phase separation procedures under both isocratic and gradient conditions[3]. No optimization of the separation conditions in ion-pair reversed-phase HPLC with TBA afforded the separation of Gua and Hyp, which eluted as one peak. The weak bases Cyt and Cyd, having a low hydrophobicity, are not identified on the background of the front of weakly retained polar and charged compounds[7]. As is known, a predominance of protonated molecules in the solute at a given pH leads to an increased retention of purine and pyrimidine compounds in a cation-exchange chromatographic system[8]. Hence a modification of the stationary phase towards the cation-exchange retention mechanism in ion-pair reversed-phase HPLC, *e.g.*, with pentanesulphonic acid (PIC B5) and heptanesulphonic acid (PIC B7), results in a substantial increase in the elution time of cytosine and guanine compounds (Fig. 8). The separation of the main pool of purine and pyrimidine nu-

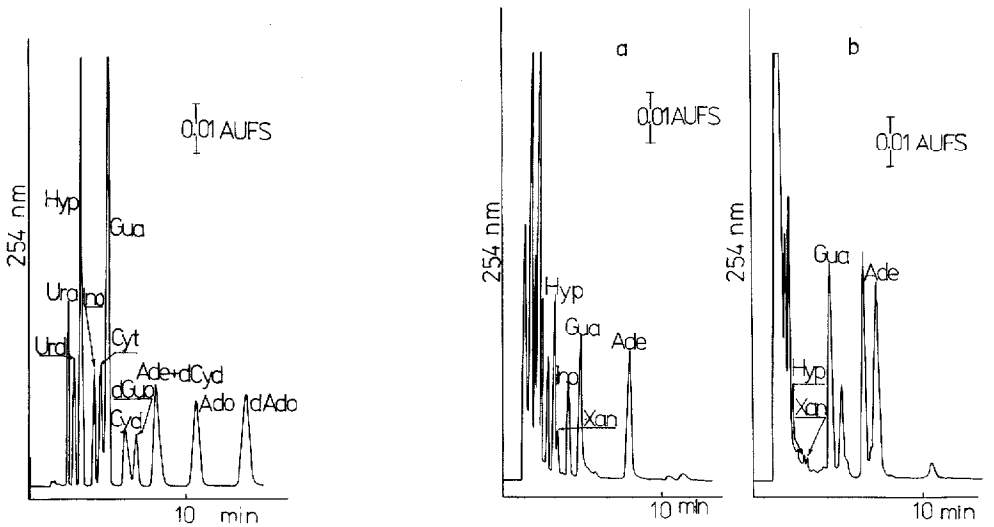



Fig. 8. Separation of nitrogen bases and nucleosides by ion-pair reversed-phase HPLC. Column: μBondapak $C_{18}$ at room temperature. Mobile phase: 10 m*M* ammonium dihydrogen phosphate– 2 m*M* PIC B5–2.5 m*M* PIC B7–8% methanol (pH 3.3). Flow-rate, 2 ml/min.

Fig. 9. Chromatogram of the ASP of T-lymphocytes from (a) human blood and (b) spleen of C3HA mice. Conditions as in Fig. 8.

cleosides and bases together with some of their deoxy forms under the optimal conditions is shown in Fig. 8. Fig. 9 illustrates the application of this method to the acid-soluble pool of T-lymphocytes of man and C3HA mice.

In conclusion, the application of ion-pairing reagents in reversed-phase HPLC affords the optimal separation and analysis of the whole spectrum of nucleotides in any biological sample.

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