Journal of Chromatography, 174 (1979) 409–419

CHROM. 11,794

ION-EXCHANGE SEPARATION OF NUCLEIC ACID CONSTITUENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received February 14th, 1979)

SUMMARY

The high-performance liquid chromatographic separation of a large variety of nucleic acid constituents on a silica-based, weak-anion exchange column was accomplished. Using this technique it was possible to achieve some relatively difficult separations, such as the separation of 2′-, 3′-, and 5′-AMP, and the separation of a mixture of ribo- and deoxyribo-nucleosides and -nucleotides. A number of other separations are demonstrated by isocratic or gradient elution. These include the separation of a mixture of nucleoside monophosphates, the separation of a mixture of nucleoside mono-, di-, and triphosphates, the separation of a mixture of nucleosides and bases, and the separation of a mixture of nucleotide oligomers. These chromatographic separations were accomplished using relatively simple experimental procedures at ambient temperatures and involved relatively short analysis times. Excellent separations were obtained, in most cases, by adjustment of buffer concentration and pH, or by addition of an organic modifier. In some cases, it was necessary to use gradient elution to achieve optimum resolution.

INTRODUCTION

The analysis of nucleic acid constituents by high-performance liquid chromatography (HPLC) has been reviewed by several workers[1–3]. The need for efficient chromatographic analysis of these compounds has developed, in part, in order to more fully understand the central role of nucleic acids in heredity and cell function. Intracellular patterns of nucleotides and nucleosides provide information on the metabolic state of the cell. Altered levels of these compounds in biological fluids can indicate changes in nucleic acid metabolism in tissues associated with certain disease states or drug responses. Separation of nucleosides, nucleotides, and free bases is necessary in the determination of base composition of nucleic acids, the synthesis of specific polynucleotides, and the elucidation of gene sequences, while studies of the prebiotic evolution of nucleic acids require the separation of a different subset of these

compounds. Because of the similarities in their physical and chemical properties, separation of nucleoside monophosphate isomers has been a difficult analytical problem and in the past has been achieved through use of radio-labeling techniques combined with chromatography (*i.e.*, paper chromatography or electrophoresis)[4,5]. In this paper, we describe a rapid method for the separation of the 2′-, 3′-, and 5′-isomers of AMP. In addition, we describe the rapid separation of nucleosides, nucleotides, oligonucleotides, and bases using a silica-based,bonded-phase,weak-anion exchanger. It is particularly useful in the separation of nucleoside monophosphate isomers and separation of ribo- from deoxyribo-nucleosides or -nucleotides.

EXPERIMENTAL

Anion-exchange chromatography of synthetic mixtures of nucleosides, nucleotides, purines, and pyrimidines was carried out on 4 mm × 30 cm MicroPak AX-10 column (Varian, Walnut Creek, Calif., U.S.A.). The AX-10 column is a difunctional weak anion-exchange bonded phase prepared on 10-μm Lichrosorb Si-60 silica, and has an exchange capacity of ~2 mequiv./g (5 mequiv. per column as determined by titration with orthophosphoric acid). Nucleosides, nucleotides, and bases were obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.), Sigma (St. Louis, Mo., U.S.A.), and P-L Biochemicals, (Milwaukee, Wisconsin, U.S.A.); all were chromatographically pure with the exception of 3′-AMP (ICN Pharmaceuticals,) which contained some 2′-AMP as an impurity. Standard solutions of these compounds (10^{-3} *M*) were prepared and aliquots of the standard solutions were then taken and used to make up mixtures. One drop of chloroform was added to each standard solution to inhibit bacterial growth. Glass-distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and pesticide quality acetonitrile was obtained from MC&B Manufacturing Chemists (Los Angeles, Calif., U.S.A.). Analytical reagent grade potassium dihydrogen phosphate was obtained from linckrodt (Phillipsburg, N.J., U.S.A.). Buffers were prepared in triply distilled water or in HPLC water, obtained from J. T. Baker (Phillipsburg, N.J., U.S.A.). The pH of buffers was adjusted using ortho-phosphoric acid or sodium hydroxide solution and pH was measured using a Corning Digital 110 expanded scale pH meter equipped with a combination pH electrode. In the case of acetonitrile-containing eluents, the phosphate buffer was first prepared by dissolving KH_2PO_4 in water to give the desired concentration, followed by adjustment of the pH to the appropriate value, and acetonitrile was then added to give the reported acetonitrile content, by volume.

Gradient separations and some isocratic separations were performed using a Varian Model 5020 liquid chromatograph (Varian Aerograph) equipped with three solvent proportioning valves and a Valco manual loop injector. Eluents were monitored by UV absorbance at 254–270 nm using a Varichrom variable wavelength detector.

Isocratic separations were also performed using an LC System Support Unit I (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) equipped with a manual loop injector (Glenco Scientific, Houston, Texas, U.S.A.). Eluents were monitored by UV absorbance at 254 or 259 nm using a Schoeffel Spectroflow monitor SF770 in conjunction with a Schoeffel GM770 monochrometer (Schoeffel Instrument, Westwood, N.J., U.S.A.). Data recording and integration were performed using a Hewlet-

Packard 3380A integrator-recorder (Hewlett-Packard, Palo Alto, Calif., U.S.A.). All chromatographic separations were performed at room temperature. Typically, 25-μl injections were made and solute concentrations were *ca.* 10^{-4} *M*. Flow-rates ranged from 0.8 to 2.0 ml/min.

Perchloric acid extract of Balb-C mouse liver (prepared by the method of Khym[6]) was kindly provided by Dr. F. Klein, University of California Medical School, San Francisco, Calif., U.S.A.

RESULTS AND DISCUSSION

A variety of columns and experimental conditions have been used for the separation of nucleoside monophosphate isomers by HPLC. Partial resolution of AMP isomers using anion-exchange chromatography with gradient elution has been reported[7], as well as an unsuccessful attempt to separate 2'-AMP and 3'-AMP on a silica-based strong cation exchanger[8]. More recently, the successful separation of the 2'- and 3'-isomers of AMP and GMP on a strong anion-exchange resin was reported[9].

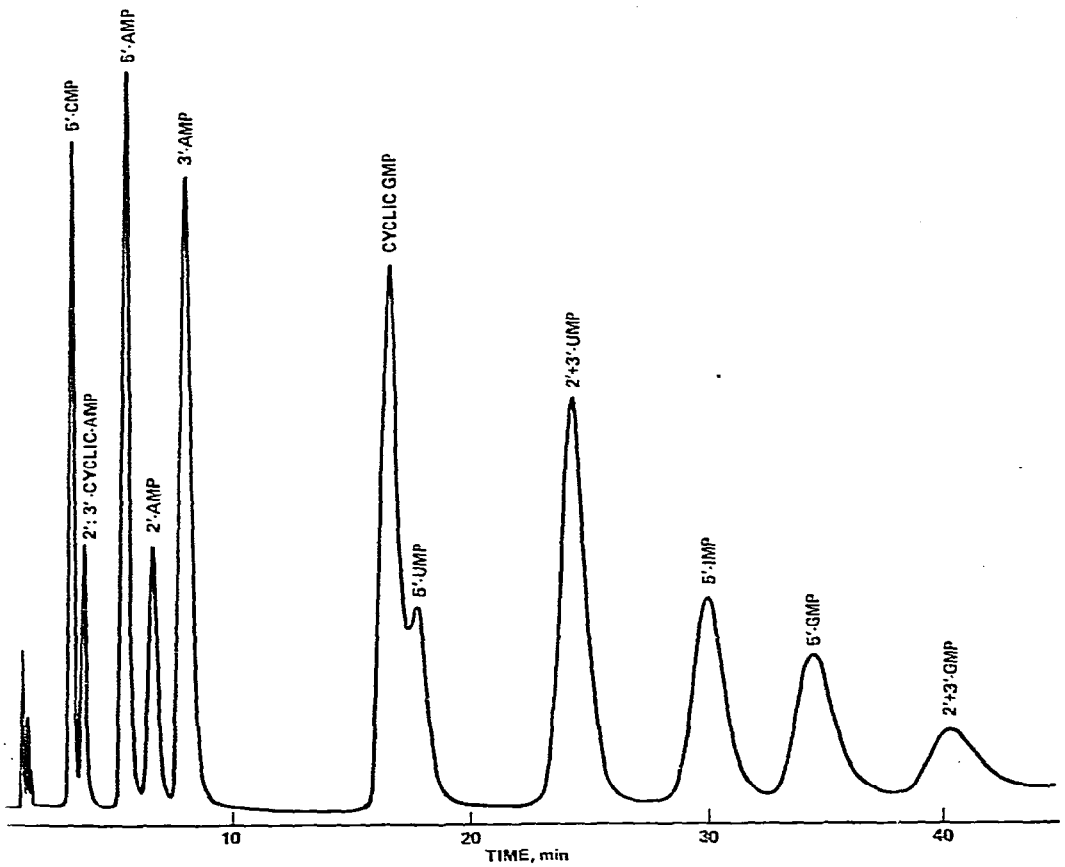


Fig. 1. HPLC separation of nucleoside monophosphates by isocratic elution with 0.01 *M* KH_2PO_4 (pH 3.0). Flow-rate, 120 ml/h; detection, 254 nm, 0.08 AUFS. The three peaks eluting at 1–2 min are nucleoside impurities contained in some of the nucleotides.

TABLE I

ISOCRATIC ELUTION TIME OF A VARIETY OF NUCLEIC ACID CONSTITUENTS AS A FUNCTION OF BUFFER pH AND ACETONITRILE CONTENT

Compound	Buffer pH or composition[a]						
	pH 2.25	pH 2.85	pH 3.52	pH 5.56	pH 7.0	Mixed buffer No. 1 (80% CH_3CN)[b]	Mixed buffer No. 2 (20% CH_3CN)[c]
2'-AMP	7.52	14.23	45.60				18.21
3'-AMP	8.56	16.42	52.66				20.36
5'-AMP	7.52	11.70	23.10			>120	15.34
Adenine		2.56	3.11	4.04	5.25	5.06	
Adenosine		2.56	3.15	3.61	4.00	4.24	
ADP	6.36	11.82	32.01				
ATP	>120	>120					
ApA[d]		3.75					
ApApA[d]		25.71					
2'-CMP	7.08	9.95	28.09				13.30
3'-CMP	6.13	9.95	27.40				11.87
5'-CMP	5.70	6.95	14.94				8.09
Cytosine		2.44	2.57	3.44	4.72	9.48	
Cytidine		2.44	2.67	3.30	3.99	10.69	
2'-UMP	34.03	50.42					
3'-UMP	37.46	50.60					
5'-UMP	28.23	38.46					
Uracil		3.56	3.76	3.75	3.84	2.49	
Uridine		3.40	3.48	3.48	3.60	3.36	
UDP	>120	>120					
UTP	>120	>120					
2'-GMP	36.48	66.00					
3'-GMP	35.07	65.30					
5'-GMP	22.57	59.94					
Guanine		3.10	4.03	4.53	4.90	5.61	
Guanosine		3.65	3.90	4.00	4.26	6.28	
deoxy-5'-AMP	6.57	16.12	33.07				14.53
deoxy-5'-CMP	5.02	6.74	14.19				7.22
deoxy-5'-UMP	28.81	40.78					
deoxy-5'-GMP		55.96					
2'-deoxy-3'-AMP	7.47	14.99	40.45				
2'-deoxy-3':5'-cyclic AMP		11.08	20.25				10.30
3':5'-cyclic AMP	6.21	9.78	17.51				9.64

[a] Unmixed buffers are prepared from 0.01 M KH_2PO_4 and adjusted to indicated pH by addition of H_3PO_4. Flow rates are 0.8 ml/min unless otherwise noted.

[b] 80% CH_3CN – 20% (0.01 M KH_2PO_4 adjusted to pH 2.85). Flow rate, 1.5 ml/min.

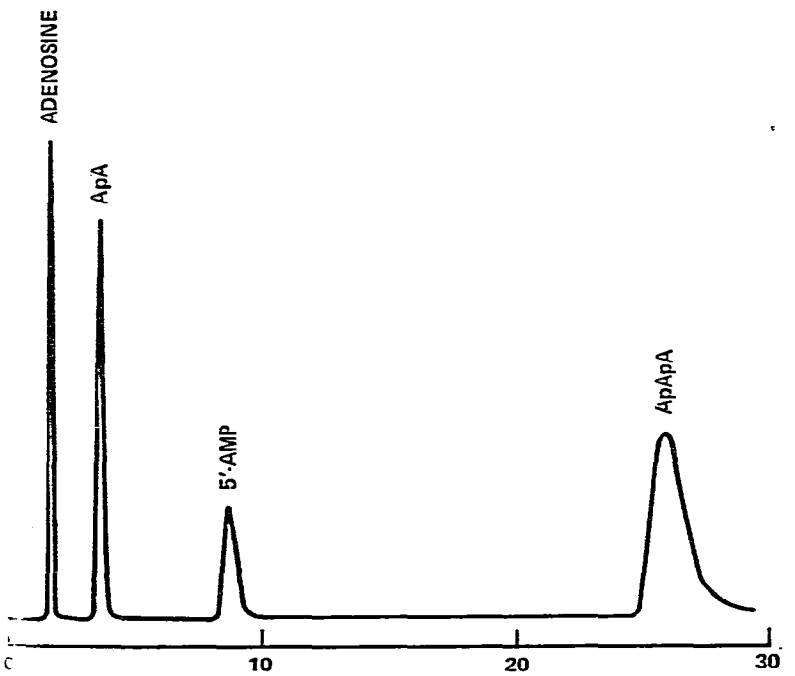
[c] 20% CH_3CN – 80% (0.01 M KH_2PO_4 adjusted to pH 2.85). Flow rate, 1.0 ml/min.

[d] Flow rate, 1.33 ml/min.

The complete resolution of 2′-, 3′-, and 5′-CMP has been achieved[4] in about 15 h using a Dowex-1 (formate) column. It has been suggested[10] that 2′-, 3′-, 5′-isomer separations can also be obtained for other nucleotides using similar conditions, but analysis times are prohibitively long. Typically, the separation of the 5′-isomer from the 2′- and 3′-isomers is readily accomplished, whereas the separation of the 2′- and 3′-isomers has proven to be difficult[11].

As shown in Fig. 1, an excellent separation of a mixture of nucleoside monophosphates was obtained under isocratic conditions using 0.01 *M* KH_2PO_4 by adjustment of buffer pH and flow-rate. With the mobile phase pH adjusted to 3.0, 2′-, 3′-, 5′-, and 2′:3′-cyclic AMP were completely separated within 10 min. Most of the other nucleoside monophosphates are also separated under these conditions, with the exception of the 2′-, 3′-combinations. Attempts were made to achieve a better separation for 2′- and 3′-UMP, 2′- and 3′-CMP, and 2′- and 3′-GMP by varying the buffer pH from 2.25 to 7. It was not possible to resolve any of these conditions (Table I). By using the same mobile phase adjusted to pH 2.85, it was possible to separate a mixture of adenosine, 5′-AMP, ApA and ApApA (Fig. 2). Such separations of low-molecular-weight polynucleotides are useful in monitoring the synthesis or degradation of nucleotide oligomers, and in the study of precursor interactions in prebiotic evolution of nucleic acids.

Rapid HPLC analysis of nucleosides has commonly been performed using reversed-phase methods[12–14]. However, it is often of interest in biological samples to analyze bases, nucleosides, and nucleotides simultaneously, which necessitates the use



g. 2. HPLC separation of adenosine, 5′-AMP, ApA and ApApA by isocratic elution with 0.01 KH_2PO_4 (pH 2.85). Flow-rate, 80 ml/h; detection, 259 nm, 0.0025 AUFS.

of ion-exchange separations. It previously had been difficult to achieve conditions for isocratic ion-exchange separation of nucleotides under which nucleosides are also resolved; using aqueous buffers, nucleosides are generally poorly retained. With a silica-based ion exchanger, addition of an organic modifier to the mobile phase decreases the absolute solubility of solutes in the mobile phase and thus increases retention times of nucleosides and bases. An organic modifier also serves to increase the relative affinity of deoxynucleosides for the stationary phase compared to ribo compounds, enhancing the resolution of the two classes. Gradient programming may then be used to elute the more highly charged nucleotides by increasing the ionic strength and pH of the mobile phase. Complete isocratic separation of a mixture of four bases and four nucleosides was accomplished using a mixture of 20% 0.01 *M* KH_2PO_4 (pH 2.85) and 80% acetonitrile (Fig. 3). By increasing both the molarity and pH of the aqueous mobile phase, it was possible to resolve a complex mixture of nucleoside mono-, di-, and triphosphates (Fig. 4).

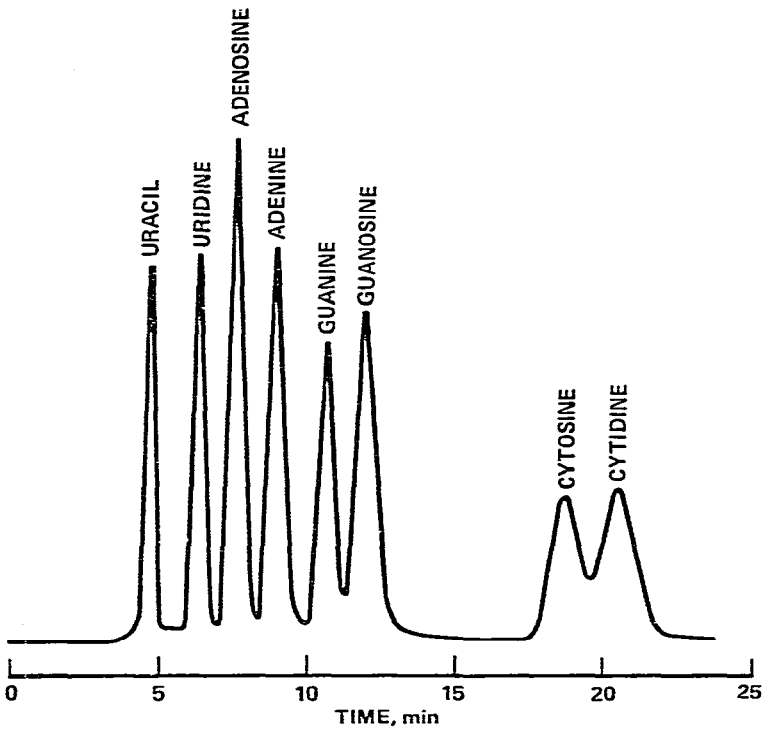


Fig. 3. HPLC separation of nucleosides and bases by isocratic elution with 80% acetonitrile–20% 0.01 *M* KH_2PO_4 (pH 2.85). Flow-rate, 48 ml/h; detection, 259 nm, 0.01 AUFS.

The separation of ribo- from deoxyribonucleotides has been successfully carried out on anion-exchange resins using gradient elution[6,15]; however, complete elution of all nucleotides required 1–3 h, and some pairs of ribo- and deoxyribonucleoside monophosphates were poorly resolved. Separation of ribo- and deoxyribonucleoside mono-, di-, and triphosphates has been attempted using a silica-based, strong anion exchanger, but it was not possible to obtain simultaneously good resolution and a satisfactory analysis time[16,17].

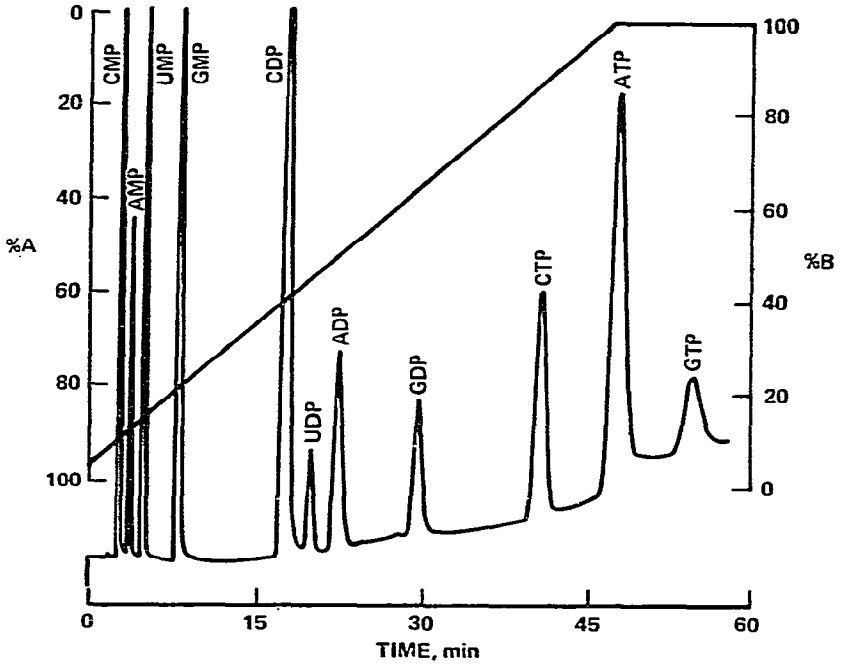
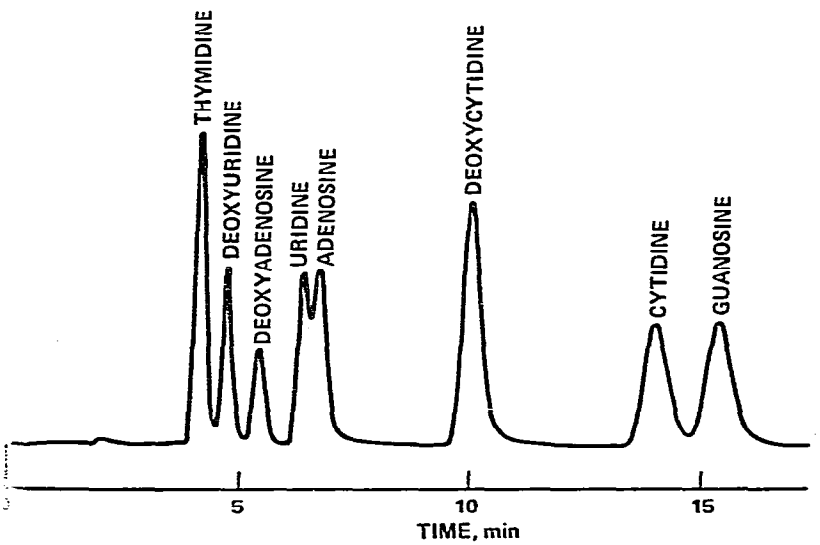


Fig. 4. HPLC separation of nucleoside 5′-mono-, di-, and triphosphates by gradient elution. A = 0.01 *M* KH_2PO_4, pH 2.85; B = 0.75 *M* KH_2PO_4, pH 4.4. Gradient program: 5–100% B; step 1, +2% B/min for 47.5 min; step 2, isocratic, 100% B for 12.5 min. Flow-rate, 120 ml/h; detection, 260 nm, 0.5 AUFS (0–15 min), 0.2 AUFS (15–60 min).



ig. 5. HPLC separation of nucleosides and deoxynucleosides by isocratic elution with 82% etonitrile–18% 0.0125 *M* KH_2PO_4. Flow-rate, 120 ml/h; detection, 270 nm, 0.2 AUFS.

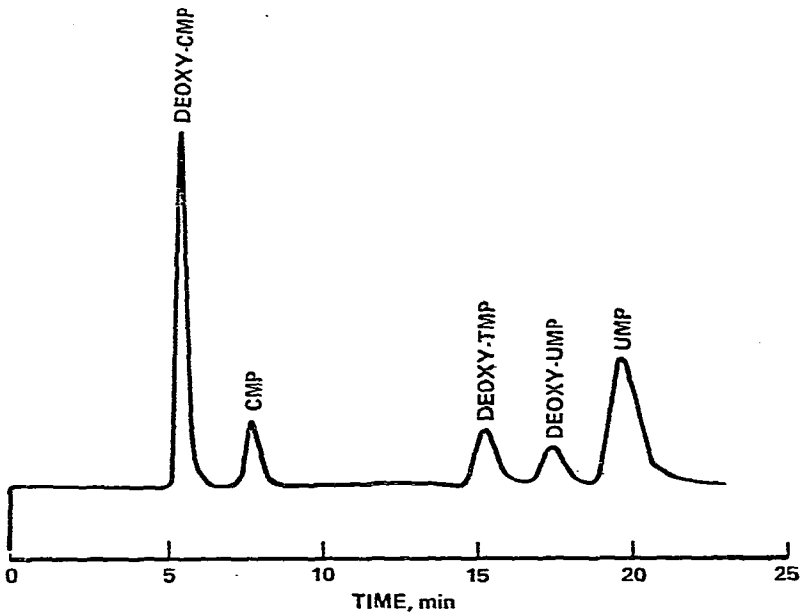


Fig. 6. HPLC separation of 5'-nucleotides and deoxynucleotides by isocratic elution with 40% acetonitrile–60% 0.01 *M* KH_2PO_4 (pH 2.85). Flow-rate, 120 ml/h; detection, 270 nm, 0.2 AUFS.

The separation of mixtures of nucleosides and deoxynucleosides or nucleotides and deoxynucleotides was readily obtained isocratically. The separation of a mixture of nucleosides and deoxynucleosides (Fig. 5) was achieved by elution with

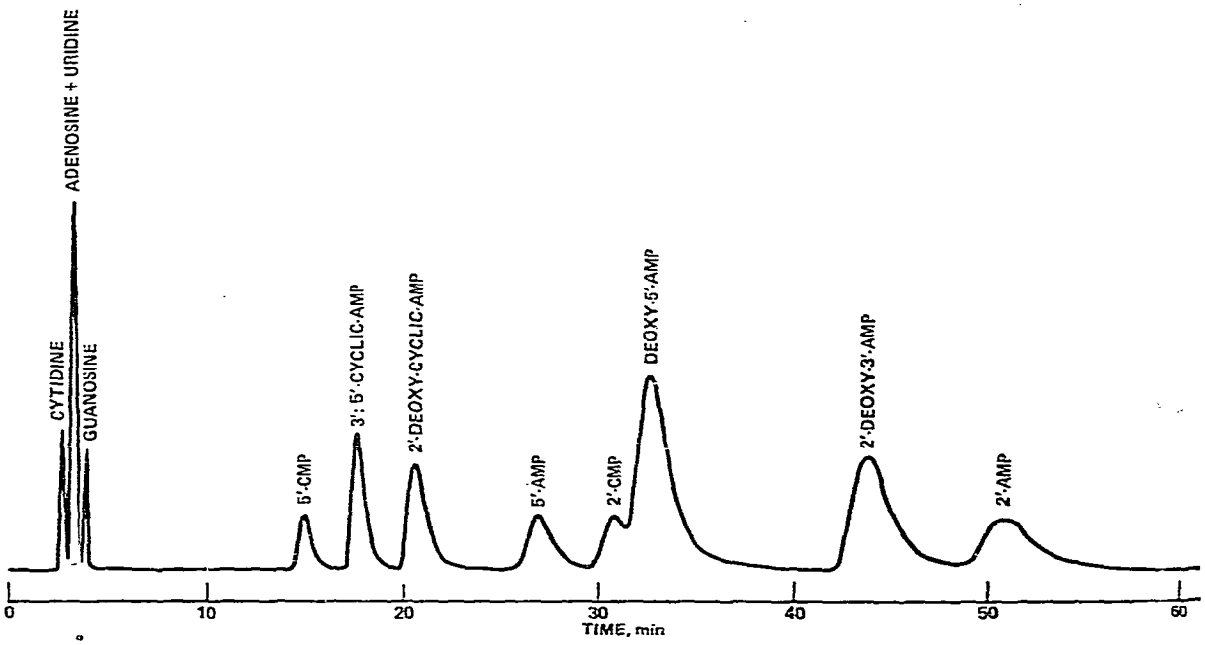


Fig. 7. HPLC separation of nucleosides, nucleotides, cyclic nucleotides, and deoxynucleotides by isocratic elution with 0.01 *M* KH_2PO_4 (pH 3.52). Flow-rate, 48 ml/h; detection, 259 nm, 0.01 AUFS.

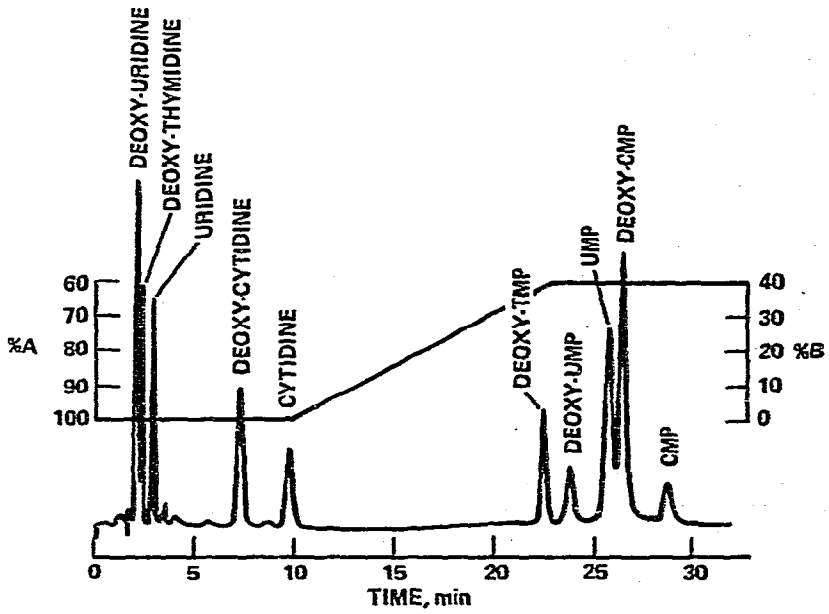
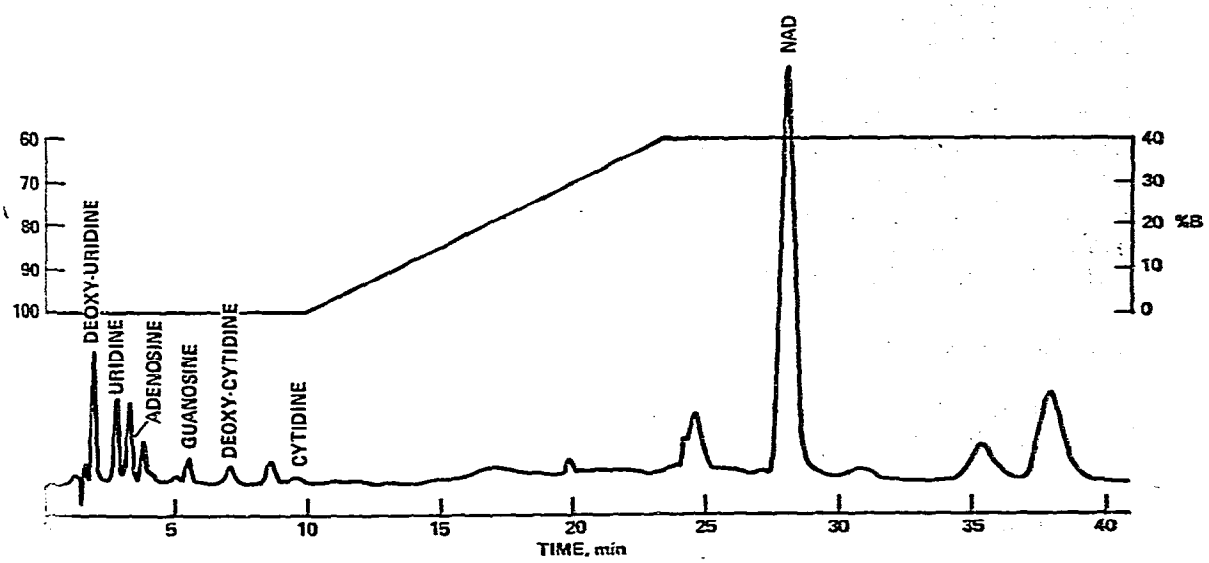


Fig. 8. HPLC separation of nucleosides, deoxynucleosides, and 5'-nucleotides and deoxynucleotides by gradient elution. A = 80% acetonitrile–20% 0.01 *M* KH_2PO_4 (pH 2.85); B = 0.01 *M* KH_2PO_4, pH 2.85. Gradient program: 0–40% B; step 1, isocratic, 0% B for 10 min; step 2, +3% B/min for 13.33 min; step 3, isocratic, 40% B for 6.67 min. Flow-rate, 120 ml/h; detection, 260 nm, 0.05 AUFS.

82% acetonitrile–18% 0.0125 *M* KH_2PO_4, whereas the separation of a mixture of nucleotides and deoxynucleotides (Fig. 6) was accomplished by elution with 40% acetonitrile–60% 0.01 *M* KH_2PO_4 (pH 2.85). (Note that deoxy-CMP is completely resolved from CMP and deoxy-TMP from UMP; in a reported analysis by gradient



g. 9. HPLC separation of perchloric acid extract of Balb-C mouse liver. 10 μl was injected, uivalent to 2 mg of liver. Conditions same as those in Fig. 8.

elution on an anion-exchange resin, these pairs were poorly resolved[15].) A mixture of nucleosides, nucleotides, and deoxynucleotides was partially resolved isocratically using an aqueous mobile phase (Fig. 7).

The effect of mobile phase pH and addition of organic modifiers on retention of a wide range of nucleic acid constituents was investigated (Table I). In some cases (*e.g.*, 2′- and 3′-CMP) changing the pH provided partial resolution of coeluting compounds, while addition of acetonitrile allowed separation of the less polar bases and nucleosides. An excellent separation of nucleosides, nucleotides, deoxynucleosides, and deoxynucleotides was achieved using a gradient elution of decreasing acetonitrile concentration (Fig. 8). This technique was used to analyze the nucleic acid constituents in an extract of Balb-C mouse liver (Fig. 9).

CONCLUSION

The separations described demonstrate that a large variety of nucleic acid components can be chromatographically separated in a minimum of time using relatively simple experimental techniques. Many of the separations can be accomplished without the use of gradient elution by optimizing the buffer concentration and pH or, in the case of nucleosides and bases, by the addition of an organic modifier to the buffer. Using these techniques, it has been possible to readily accomplish relatively difficult separations, such as the separation of 2′-, 3′-, and 5′-AMP or the separation of deoxynucleotides and nucleosides.

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CMP	Cytidine monophosphate
UMP	Uridine monophosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate
GMP	Guanosine monophosphate
ApA	Adenylyl-(3′→5′)-adenosine
ApApA	[Adenylyl-(3′→5′)]$_2$-adenosine
NAD	Nicotinamide-adenine dinucleotide

Note: All nucleotides are the 5′-isomer unless otherwise specified.

ACKNOWLEDGEMENT

This work was performed, in part, under a National Research Council Associateship granted to E. Edelson.

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