CHROM. 4822

ANALYSIS OF RIBONUCLEOTIDES AND DEOXYRIBONUCLEOTIDES USING HIGH-PRESSURE LIQUID COLUMN CHROMATOGRAPHY

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(Received April 16th, 1970; revised manuscript received May 12th, 1970)

SUMMARY

Conditions for the separation of some mononucleotides, dinucleotides, nucleoside diphosphates and deoxymononucleotides on a pellicular anion-exchange column are described. These chromatographic systems allow quantification and identification of nucleotide materials in nanomole quantities and can be achieved within 100 min. The applicability of the present systems to the analysis of base composition of polynucleotides has been demonstrated by analyzing some oligonucleotides of known structure.

INTRODUCTION

In the sequence analysis of RNA molecules, characterization and quantification of base components is an important operation. Paper and thin-layer chromatography and electrophoresis have been used commonly as well as various column chromatographic methods[1,2]. Recently, SHMUKLER[3] reported the separation of ^{32}P-labeled AMP, ADP and ATP on a commercial nucleic acid analyzer. Analysis of some ribonucleosides and bases using a similar column has been reported also by HORVATH AND LIPSKY[4].

This communication describes the separation of some mono- and dinucleotides, nucleoside diphosphates and deoxymononucleotides on a pellicular anion-exchange column. In essence the present method involves a high-pressure liquid chromatography ion-exchange system. A mixture of mononucleotides can be cleanly separated within 1 h and is quantifiable for as little as 0.3 nmole of each mononucleotide. In addition, some dinucleotides, nucleoside diphosphates and deoxymononucleotides can be separated on the column.

MATERIALS AND METHODS

Mononucleotide samples, Cp (2′ + 3′), Up (2′ + 3′), Ap (3′) and Gp (2′ + 3′)

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obtained from P-L Biochemicals were used as standards. They were prepared as aqueous solutions whose concentrations were determined by measurement of the O.D.$_{254m\mu}$ of an aliquot in 0.2 *M* KH_2PO_4, pH 8.5. Nucleoside diphosphates (Li salts) were obtained from Mann Research Laboratories.

Four dinucleotides, ApCp, GpCp, ApUp, and GpUp were prepared by digestion of a sample of *Escherichia coli* sRNA (General Biochemicals) with pancreatic RNase. The digest was chromatographed on EDTA-washed Whatman No. 3 MM paper, in *n*-propanol–ammonia–water (55:10:35). The band containing dinucleotides was eluted with 0.05 *M* NH_4OH, and re-spotted on washed Whatman No. 3 MM paper for electrophoresis (0.05 *M* NH_4COOH, pH 3.0; 2000 V for 3 h). The dinucleotides were eluted with 0.05 *M* NH_4OH, evaporated to dryness, and redissolved in distilled water. A sample of each dinucleotide was hydrolyzed in 0.3 *N* KOH at 37° for 18 h. Each dinucleotide was identified by measurement of its mononucleotide components obtained from the KOH hydrolysate.

Apparatus

A Picker Nuclear LCS-1000 analyzer equipped with an anion-exchange column was used for this study.

Preparation of buffers

In all buffers prepared for the analyzer, the precise adjustment of pH was found to be of the utmost importance. Concentrated and dilute buffers described here contained Fisher "ACS Certified" KH_2PO_4. The salt used for buffers in linear gradient systems was not purified further, but that for exponential gradient systems (see nucleoside diphosphates, dinucleotides below) was purified as follows: Distilled water was added to 800 g KH_2PO_4 to a volume of 1200 ml. The mixture was boiled gently until all salt had dissolved, then cooled slowly and allowed to stand overnight at room temperature. The crystals which had formed were discarded; the supernatant was filtered through cotton to remove gross impurities. The concentration of KH_2PO_4 in the filtered supernatant was determined. Appropriate dilutions were then made to make the desired buffer concentrations; the pH was adjusted with concentrated H_3PO_4, using a Leeds and Northrup pH meter.

Operational conditions

While not in use, the column was maintained at 40°. Prior to each chromatographic run, the column was conditioned at high flow rates for 10 min with dilute degassed buffer at 40°, then at 70° (or the temperature to be used during the analysis) for about 30 min at which time the baseline usually had leveled off. Samples were injected with a Hamilton syringe. Elution was effected with a linear or exponential gradient at a flow rate of 0.2 ml/min. Chart paper speed was 1 in./5 min.

Sample preparations and elution conditions

System I. This buffer system was used for the separation of mononucleotides. Samples containing between 0.25 and 2.7 nmole of each mononucleotide in a solution containing 0.49 μmole $KClO_4$, 0.26 μmole $HClO_4$ and 0.80 μmole KH_2PO_4, pH 8.5, were injected onto the column. The column was maintained at 70°. Initially, the mixing chamber contained 40 ml of dilute buffer (0.005 *M* KH_2PO_4, pH 2.4). Gradient-

forming concentrated buffer (1.0 *M* KH_2PO_4, pH 3.6) was introduced into the mixing chamber 10 min after the beginning of the run, at a rate of 0.1 ml/min.

System II. This system was used for the separation of ribo- and deoxyribomononucleotides. Samples containing between 0.3 and 2.0 nmole of each mononucleotide in a solution containing 0.12 μmole $KClO_4$, 0.12 μmole $HClO_4$ and 1 μmole KH_2PO_4 were injected onto the column. The column was maintained at 70°. The dilute buffer in System II was 0.001 *M* KH_2PO_4, pH 3.5, filled to a level of 50 ml in the mixing chamber. Without any gradient delay, concentrated buffer, KH_2PO_4, 1.0 *M*, pH 4.3 flowed into the mixing chamber at a rate of 0.1 ml/min.

System III. For the separation of nucleoside diphosphates and dinucleotides, samples were made up as for System II for mononucleotides; the volume injected contained about 0.5 μg of each diphosphate. Dilute buffer was 0.01 *M* KH_2PO_4, pH 2.4, and filled the mixing chamber to 40 ml. Concentrated buffer (1.0 *M* KH_2PO_4, pH 3.6) was added, without any gradient delay, at a rate of 0.2 ml/min, *i.e.*, at a rate equal to the flow of buffer through the column. This established a system with an exponential gradient.

RESULTS AND DISCUSSION

Separation of mononucleotides

R values of the mononucleotides separated by System I are shown in Table I. Fig. 1 illustrates a typical run. The system separates 2′ and 3′ isomers with the ex-

TABLE I

R VALUES FOR SOME NUCLEOTIDES IN THREE BUFFER SYSTEMS

Nucleotide	*R values (cm)*		
	System I	*System II*	*System III*
Cp (2′)	1.3	9.9	
Cp (3′)	1.3	11.5	
Up (2′)	13.0	14.6	
Up (3′)	15.3	16.4	
Ap (2′)	4.3	19.8	
Ap (3′)	8.6	24.5	
Gp (2′)	23.2	25.8	
Gp (3′)	28.2	31.2	
CDP			14.4
UDP			24.8
ADP			27.3
GDP			44.6
ApCp			18.2
GpCp			32.3
ApUp			33.7
GpUp			51.6
dCMP		10.4	
dAMP		18.2	
dGMP		26.3	

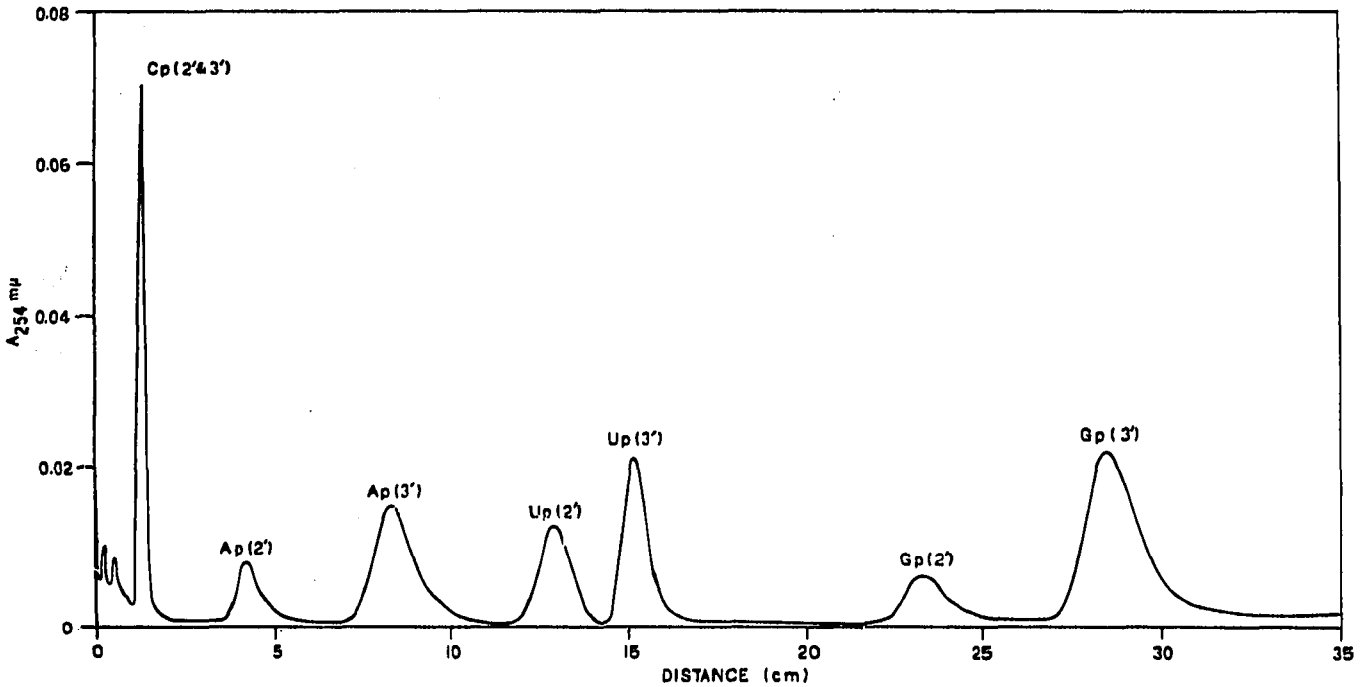

Fig. 1. Chromatogram of mononucleotides. Chromatographic conditions are described in the text.

ception of those of Cp which are eluted as a single peak. The extreme sharpness of Cp (2′ + 3′) as well as its proximity to the "noise" peak (marking beginning of run) would seem to increase the error in the quantitation of Cp. In spite of this apparent disadvantage, standard curves for this system show limited variation in the points depicting Cp values (Fig. 2). The linearity of all four curves indicates that the measure-

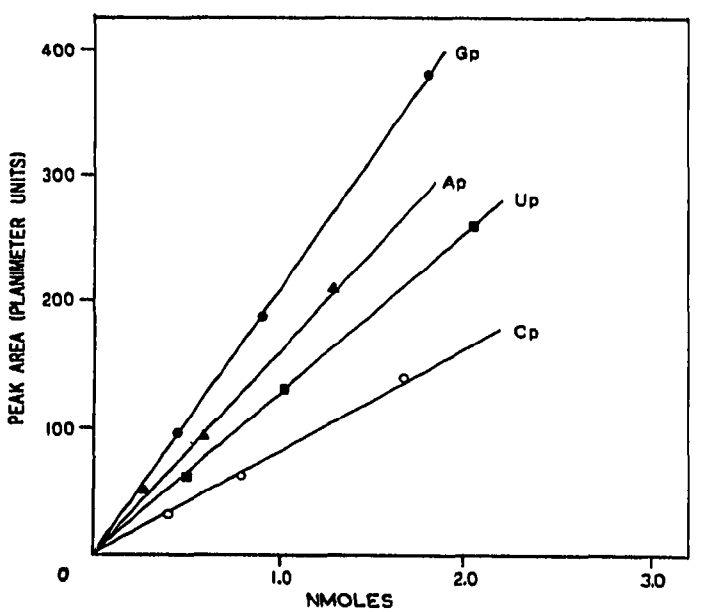

Fig. 2. Standard curves for mononucleotides showing the linear relationship between quantities of each mononucleotide and peak area of chromatogram. Chromatographic system I was used.

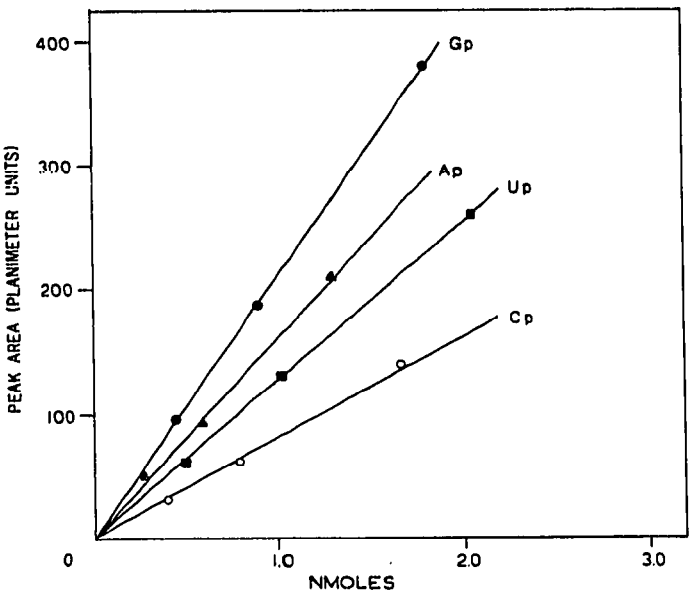

Fig. 3. Standard curves for mononucleotides. Chromatographic system II was used.

ment of peak areas from System I can be used for quantitation of the mononucleotides.

The additional possibility in System I of contamination of Cp peaks with nucleosides (which would be eluted from the column near the beginning of the run) suggested that an improved separation system should be developed in which Cp peaks were retained on the column for a longer time. It was found that System II had such an effect.

R values of some mononucleotides separated by System II are shown in Table I. Standard curves for these mononucleotides using System II are shown in Fig. 3. The linearity of these curves indicates that quantitative measurement of each nucleotide may be obtained through measurement of peak areas.

TABLE II

CHARACTERIZATION OF PRODUCTS FROM ALKALINE HYDROLYSIS OF DINUCLEOTIDES

Dinucleotide	*Mono-nucleotide products*	*R*	*Area*	*nmoles*	*Ratio*
ApCp	Cp (3′)	12.2	510	6.63	
	Ap (2′)	20.3	537	7.44	1.22
	Ap (3′)	25.0	722		
ApUp	Up (3′)	17.1	366	2.93	
	Ap (2′)	20.3	256	3.48	1.19
	Ap (3′)	25.2	333		
GpUp	Up (3′)	16.7	38	0.31	
	Gp (2′)	26.0	27	0.29	1.07
	Gp (3′)	31.6	34		
GpCp	Cp (3′)	11.2	40	0.52	
	Gp (2′)	26.5	56	0.56	1.08
	Gp (3′)	32.1	61		

It was of particular interest to test the applicability of the present systems to the analysis of base composition of polynucleotides. Table II shows the data of such analysis on dinucleotides of known structure.

Hence, the two systems used for the separation of mononucleotides each have their own merits. System I is valuable because it separates the four mononucleotides completely from each other although the 2′ and 3′ isomers of Cp cannot be measured individually. However, the system remains useful for measurement of total Cp, Ap, Up and Gp, respectively, as illustrated by the analysis of dinucleotides (Table II).

System II for mononucleotides gives excellent separation of 2′ and 3′ isomers of all four mononucleotides with the exception of Ap (3′) and Gp (2′), which overlap. This system is therefore ideal for analysis of KOH hydrolysates of oligonucleotides derived from T_1 digests of RNA, since these oligonucleotides should not contain Gp (2′). Several such analyses have been done and have been reported previously[5].

Separation of nucleoside diphosphates

Table I also shows the *R* values obtained for some nucleoside diphosphates. The increase in salt concentration of the buffer to a much higher level during the exponential gradient run led to the problem of a "significant" rise in the baseline after a period of about 50 min. Since the run described above took 105 min, purification of the buffers was necessary (see MATERIALS AND METHODS) in order to eliminate this baseline rise during as much of the run as possible. With the use of "purified" KH_2PO_4, the baseline increased less than 0.002 O.D. units during the first 100 min of a run.

Separation of dinucleotides

In addition to being a method for base composition analysis, it would be useful if the present method can also be suited as a finger printing method for studies of the primary structure of RNA's. Thus, we examined the elution pattern of the dinucleotides produced from a pancreatic ribonuclease digest of RNA. The *R* values of the four dinucleotides are shown in Table I.

Separation of deoxynucleotides

The results shown above represent only a beginning in the development of buffer systems for the purpose of separating nucleic acid components. Although the present data deal with ribonucleotides, preliminary data show that a mixture of dCMP, dAMP, and dGMP may also be separated on this column using System II. *R* values for such compounds are shown in Table I.

ACKNOWLEDGEMENTS

The authors are indebted to Professor V. M. INGRAM for his interest and support and the use of his laboratory during the course of this investigation. We also wish to thank Picker Nuclear for the loan of an LCS-1000 analyzer.

This investigation was supported by a grant No. AM 08390 from the U. S. Public Health Service to Professor INGRAM.

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