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Note

Simultaneous analysis of nucleosides and nucleotides by high-performance liquid chromatography

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We have been developing a method[1] which permits the rapid chemical synthesis of oligoribonucleotides. It is essential to have available methods which quickly verify the nucleoside composition of the synthesized nucleotide chain. Traditionally, this result has been obtained through enzymatic degradation of the deblocked oligonucleotide chain followed by time consuming paper chromatography.

Recently there have been several reports in the literature on separations applicable to oligoribonucleotide analysis[2–7]. However, these procedures generally lack the ability to separate and identify simultaneously nucleosides and nucleotides. We wish to describe in this report the simultaneous separation and quantitation of ribonucleosides and ribonucleotides, thus allowing direct and rapid determination of enzymatic digests.

The use of a silica based weak anion-exchange column combined with a ternary solvent system has permitted the development of a rapid method for the simultaneous separation of nucleosides and mononucleotides. The instrument used was a Spectra-Physics microprocessor controlled high-performance liquid chromatography (HPLC) system, Model SP8000, equipped with an auto-injector, a helium degass system, constant temperature oven, a ternary solvent system, a dual channel printer/plotter and a SP8210 UV detector (254 nm). The system is controlled through an SP4000 Chromatography Data System. Separations were carried out on a Brownlee Labs AX-10A silica based weak anion-exchange column (LiChrosorb AN, particle size 10 μm) fitted with a guard column (6 $\times$ 0.2 cm).

Nucleosides and nucleoside 3′-phosphates were obtained from Sigma (St. Louis, MO, U.S.A.). Extinction coefficients used are listed in Table I. Pancreatic ribonuclease was also obtained from Sigma while spleen phosphodiesterase was obtained from ICN (Irvine, CA, U.S.A.). All solvents and buffers were filtered through Millipore 0.45-μm filters before use and were then continually degassed by means of helium sparging.

During operation, the column and guard column were maintained at 30°C. The flow-rate was held constant at 2 ml/min. Sufficient sample solution was injected to fill the 10-μl sample loop.

Separation of the four ribonucleosides was done isocratically by using acetonitrile–0.01 *M* ammonium dihydrogen phosphate, pH 5.0 (4:1). Resolution of

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TABLE I

EXTINCTION COEFFICIENTS FOR NUCLEOSIDES AND NUCLEOTIDES

Compound	$\varepsilon_{\lambda max}$*	ε_{254}**
A	14,900	13,700
Ap	15,400	14,300
C	9100	6700
Cp	9200	6700
G	13,600	13,600
Gp	13,400	13,300
U	7100	6200
Up	10,000	8900

* From ref. 8.
** Calculated using a Cary 17 spectrophotometer.

all components (Fig. 1) is possible in just over 5 min. Retention times are indicated and are distinct and reproducible.

Nucleotides were separable on the same column by the use of a pH gradient (programmed elution) in aqueous ammonium dihydrogen phosphate buffer (pH 5.0 to pH 3.1). Solvent conditions for separating nucleosides and nucleotides are listed in Table II, a sample chromatogram is shown in Fig. 2 and retention times are compiled in Table III. Reproducibility of these results is very good providing buffer solutions are carefully prepared.

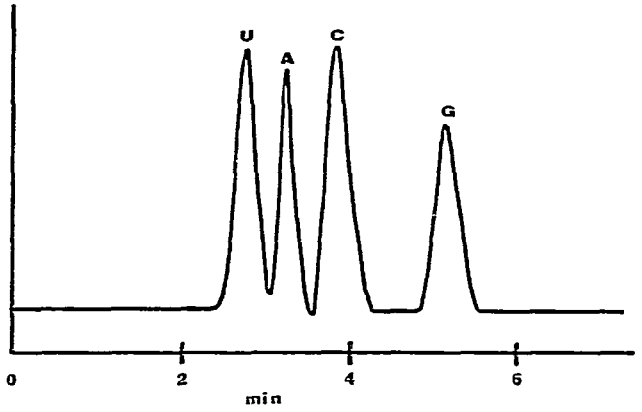

Fig. 1. HPLC separation of adenosine (A), cytidine (C), guanosine (G) and uridine (U) on a Brownlee Labs AX-10A ion exchange column using isocratic conditions. Solvent: acetonitrile–0.01 *M* $NH_4H_2PO_4$, pH 5.0 (4:1). Retention times (sec) and relative standard deviations (%) for the four nucleosides are: U (163, 1.6); A (197, 2.2); C (238, 2.2) and G (296, 1.8).

From the data it is apparent that adenosine and guanosine are not completely resolved. This is a minor problem in enzyme degradations using phosphodiesterases since only one nucleoside is produced. For total nucleoside analysis, the isocratic conditions are used. For enzyme digests, nucleotide amounts in the order of 5 $O.D._{260}$

TABLE II

PROGRAMMED SOLVENT SYSTEM FOR SIMULTANEOUS SEPARATION OF NUCLEOSIDES AND NUCLEOTIDES

Time (min)	*Solvent composition (%)* *		
	0.0025 M $NH_4H_2PO_4$, pH 5.0	*H_2O*	*0.5 M $NH_4H_2PO_4$, pH 3.1*
0	100	0	0
1	100	0	0
2	0	100	0
5	0	50	50
12	0	0	100
18	0	0	100
20	100	0	0

* Changes linearly between values.

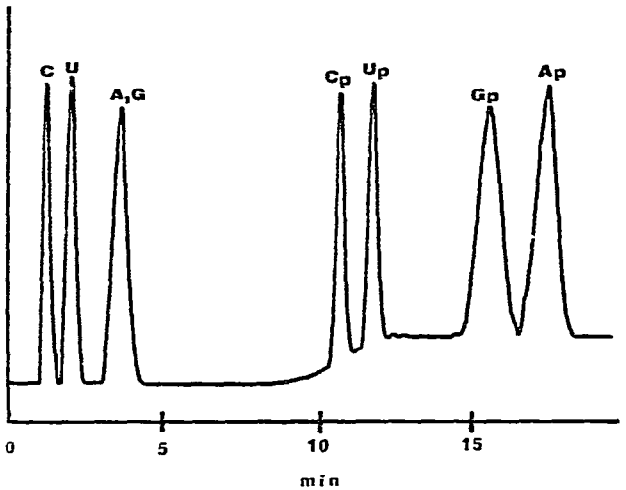

Fig. 2. HPLC separation of the ribonucleosides A, C, G and U and their 3'-monophosphates on the AX-10A ion exchange column using the solvent conditions of Table II. A and G have similar retention times using this program (Table III).

TABLE III

RETENTION TIMES OF NUCLEOSIDES AND NUCLEOTIDES DURING PROGRAMMED SEPARATION

Compound	*Retention time (sec)*	*Relative standard deviation (%)*
C	83	1
U	122	0.7
A	207	2
G	216	1
Cp	632	0.2
Up	695	0.1
Gp	925	0.2
Ap	1016	0.2

units are used. The results from degradations of CpA and ApA are shown in Fig. 3a and b. Ratios of units are calculated automatically by the microprocessor using the ε_{254} values in Table I.

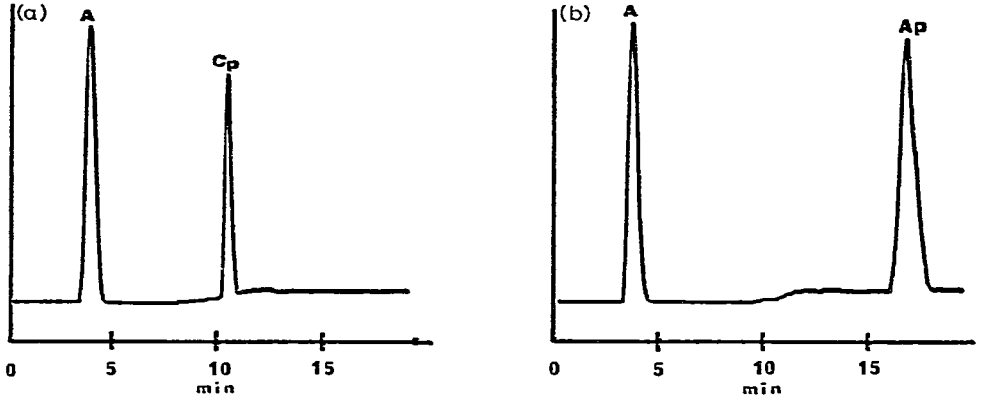

Fig. 3. HPLC analysis of enzyme degradations of dinucleotides: (a), pancreatic ribonuclease A degradation of CpA; (b), spleen phosphodiesterase degradation of ApA.

The method described in this report thus permits rapid analysis of ribonucleoside and ribonucleotide mixtures and is particularly useful for analysis of enzyme degradations of ribonucleotides.

Peak areas were found to be accurate to within $\pm 2\%$ for all compounds except adenosine 3'-monophosphate (Ap) and guanosine 3'-monophosphate (Gp) which were accurate within $\pm 5\%$.

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