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Note

Anomalous separation of 2′- and 3′-ribonucleotides using ion suppression reversed-phase high-performance liquid chromatography

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Earlier separations of 2′- and 3′-ribonucleotides by Horvath *et al.*[1] used anion-exchange chromatography, with small-bore (1 mm) and long (200 cm) columns and a gradient system operating at elevated temperatures, to achieve separations in *ca.* 60 min. Using conventional ion-exchange methods, operating at room temperature with a 25-cm anion-exchange column and gradient elution, there was only separation of 2′- and 3′-adenosine monophosphate (AMP) while the isomers of the other nucleotides were not well separated[2]. Liquid–liquid partition chromatography, using reversed-phase columns with bonded non-polar stationary phases[3], can separate DNA and RNA components[4]. Reversed-phase high-performance liquid chromatography (HPLC), when combined with ion suppression or ion-pair chromatography[5], is able to separate closely related compounds or samples containing ionic and non-ionic species.

In our studies with ion suppression using high ionic strength eluents and standard length RP-8 columns (250 × 4.6 mm), separations of the 2′- and 3′-isomers of the four major ribonucleotides, cytidine monophosphate (CMP), uridine monophosphate (UMP), guanosine monophosphate (GMP) and AMP, are accomplished in *ca.* 30 min with essentially baseline separation. The separations are achieved when operating at room temperature, isocratically, under convenient, readily reproducible conditions.

MATERIALS AND METHODS

The 2′- and 3′-ribonucleotides (sodium salts) were obtained from Sigma. Ammonium dihydrogen phosphate (monobasic), certified A.C.S., was from Fisher Scientific. All solutions were prepared in distilled water.

The 2′- and 3′-isomers of the ribonucleotides were detected using a Dupont Model 837 variable-wavelength detector, monitoring absorbance at a wavelength of 265 nm. The column used was a reversed-phase LiChrosorb RP-8 from Unimetrics. A Rheodyne sample injector with a fixed 20-μl sample loop was used to apply the sample to the column. The eluent was 0.6 M $NH_4H_2PO_4$ (pH 3.5), run at a flow-rate of 1.0 ml/min at room temperature. The chromatograms were recorded on a Heath recorder at a chart speed of 0.5 cm/min at an output voltage of 10 mV.

To protect the column, at the end of each day, the column is washed with

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distilled water for 30 min to remove the high salt solution. This prevents damage to the silica backbone of the packing material by the high concentration of phosphate ions, analogous to the effect of eluent above pH 7.5[6].

RESULTS AND DISCUSSION

Figure 1 shows the chromatographic separation of a mixture of the 2′- and 3′-isomers of the four ribonucleotides using *ca.* 60 μg of each isomer. The 3′-isomer eluted before the 2′-isomer for each of the four nucleotides, which is opposite to the sequence reported by ion exchange[1,2]. The 2′- and 3′-isomers of the pyrimidine ribonucleotides eluted faster than the corresponding isomers of the purine ribonucleotides. This is illustrated in Table I. The 2′-GMP peak is low (Fig. 1), possibly due to sample degradation. Some ultraviolet-absorbing material associated with the 2′-GMP sample having a retention time of 21 min may represent a breakdown product. The retention times obtained in Fig. 1 can be determined with a precision greater than ±0.1 min.

This system produces adequate separation of DNA 5′-deoxyribonucleotides, and the anomalous separation of the 2′- and 3′-isomers of ribonucleotides was un-

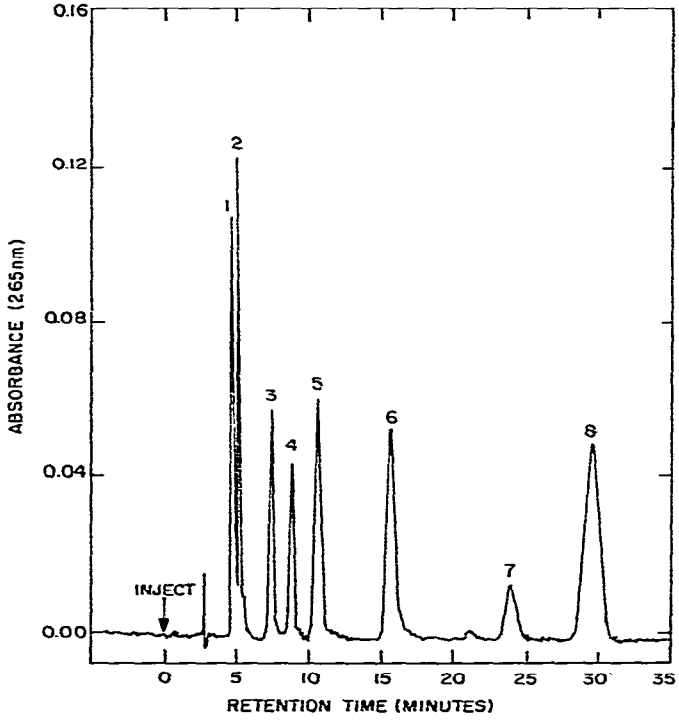

Fig. 1. Chromatograms of a mixture of the 2′- and 3′-isomers of the four ribonucleotides CMP, UMP, GMP and AMP, on a reversed-phase RP-8 column (250 × 4.6 mm). A 20-μl sample of a mixture of 1 m*M* concentrations of each of the 2′- and 3′-ribonucleotides was injected. The separations were achieved by eluting with 0.6 *M* $NH_4H_2PO_4$ (pH 3.5) at a flow-rate of 1.0 ml/min at room temperature, with detection at 265 nm. Peaks: 1 = 3′-CMP; 2 = 2′-CMP; 3 = 3′-UMP; 4 = 2′-UMP; 5 = 3′-GMP; 6 = 3′-AMP; 7 = 2′-GMP; 8 = 2′-AMP.

expected. By varying the salt concentration of the eluent from 0.05 *M* to 0.8 *M* at pH 3.5, an optimum separation of the 2′- and 3′-ribonucleotide isomers was obtained at 0.6 *M*. The higher the ionic strength, the greater is the retention time for the solutes[7,8]. The pH was optimized at 3.5 by studying the chromatographic separation obtained at a salt concentration of 0.6 *M* over a pH range between 2 and 5.

The retention times of the pyrimidine ribonucleotides[7] were independent of pH over the range between 3.5 and 6.0. In the case of the purine ribonucleotides, the retention time of GMP did not vary with pH, but for AMP there was a significant increase in retention time in going from pH 3.5 to 6.0. Increasing the salt concentrations over a range from 0.1 *M* to 0.5 *M* increased the retention times of AMP, thus improving the resolution of its 2′- and 3′-isomers. For the pyrimidine ribonucleotides, essentially no variation in retention time with salt concentration was observed. We have observed analogous results with deoxyribonucleotides[8].

The reproducibility of the system, when operating over a prolonged period, is greater than $\pm$ 1.5 min, and a progressive increase in retention times is an indication of the condition of the column. When retention times increase by more than 1.5 min, the column should be reactivated to ensure optimum efficiency.

Our studies of *in vitro* radiation damage to nucleic acids require an efficient, reproducible, high-resolution technique to separate the many, often similar products. Ion-pair chromatography represents a powerful new approach to reversed-phase HPLC, for the separation of closely related compound's not presently separable by conventional chromatography. The isocratic, reversed-phase ion-pair HPLC system reported here, operating at room temperature, under mild conditions and at high salt concentrations is suitable for the direct application of physiological samples and cell extracts.

CONCLUSIONS

The separation of the 2′- and 3′-isomers of the 4 ribonucleotides: cytidine monophosphate, uridine monophosphate, guanosine monophosphate and adenosine monophosphate has been demonstrated using isocratic, reversed-phase HPLC.

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