# CORRESPONDENCE

## Quantitation of Ribonucleotides from Base-Hydrolyzed RNA Using Capillary Zone Electrophoresis

*Sir:* Ribonucleic acid (RNA) is a biopolymer consisting of four different ribonucleosides connected by phosphate units through 3′–5′ linkages on the ribose rings. Specificity of RNA is derived by the sequence of the four bases, of which two are purines (adenine [A] and guanine [G]) and two are pyrimidines (cytosine [C] and uracil [U]). Unlike in deoxyribonucleic acid (DNA), a double-stranded biopolymer, pairing between purine and pyrimidine bases need not occur in RNA. Consequently, there is much interest in determining the ratio of all four "major" bases in different kinds of RNA, as well as the amount of modified bases (e.g. ribothymidine and pseudouridine) which may constitute up to 15% of tRNA. Additional interest is centered on the determination of the quantity of radiolabel incorporated into RNA and free nucleoside monophosphates (nucleotides) in metabolic studies.

Toward these ends, several methods of molecular discrimination have been previously employed to separate nucleotides acquired from complete hydrolysis of RNA. These include paper electrophoresis (*1*), ion-exchange chromatography (*2*), high-performance liquid chromatography (HPLC) (*3*), and isotachophoresis (*4*). Recently, capillary zone electrophoresis (CZE) has been shown to be applicable to the problem of nucleotide and oligonucleotide separation (*5–7*). We report the ability of CZE with UV absorbance detection to distinguish isomers of the four main ribonucleotides, as well as the possible use of this system in quantitation. CZE compares favorably to other separation techniques especially in the areas of limited sample quantity, resolution, and time of analysis. With on-line UV detection (254 nm), CZE provides a 5-min separation of the main ribonucleotides with a detection limit correlating to less than 0.1 ng of RNA.

### EXPERIMENTAL SECTION

Pure nucleoside monophosphate isomers as well as mixtures of the isomers were obtained from Sigma Chemical Corp., St. Louis, MO.

Bulk RNA (obtained from Sigma and from the laboratory of Professor Dean Appling, University of Texas, Austin) was subjected to 0.3 M KOH at 37 °C for 18 h. The digested sample was directly introduced into a fused silica capillary (Polymicro Technologies, Phoenix, AZ) by placing one end of the capillary into the analyte solution and raising the end 7 cm above the outlet. By continuously injecting a sample until the plug front reached the detector, we determined that this method of introduction delivers approximately 2 nL in 5 s for a 50 μm i.d. capillary. A high-voltage power supply (Model R50B, Hipotronics, Inc., Brewster, NY) capable of delivering −30 to +30 kV was used for all experiments, and a Plexiglas box outfitted with a twin interlock system prevented access to the capillary and electrodes while the system was in operation.

CZE separation was performed under two different sets of conditions. In one, the inlet was held positive with respect to a grounded outlet, and 50 mM formate, at pH between 3.7 and 4.0, was employed as the running buffer. In this case, electroosmotic flow was from anode to cathode. Alternatively, the inlet was held negative with respect to the grounded outlet, and a running buffer that contained 12.5 mM formate at pH 3.8 with 0.1 mM cetyltrimethylammonium bromide (CTAB) was used. CTAB, a cationic surfactant, changes the charge associated with the inner wall of a capillary from net negative to net positive. Consequently, the direction of electroosmosis is from cathode to anode (*8*, *9*). Prior to first use, capillaries were conditioned with the CTAB buffer for 3–4 h in order to achieve a reproducible electroosmotic flow rate. To maintain this conditioning, capillaries were stored with CTAB buffer inside when not in use. Between individual runs, the columns were rinsed with buffer solution only.

In all separations, on-column detection was accomplished with a UV absorbance spectrometer (Model UVIDEC-100 V, Japan Spectroscopic Co., Tokyo, Japan) set at 254 nm and a home-built cell holder with 0.1-mm slits. The polyimide coating on the capillaries was removed by flame and acetone wash at the site of detection.

### RESULTS

**Resolution of Ribonucleotide Isomers.** Figure 1 depicts the structure of the ribonucleotide products of base-hydrolyzed RNA. Cleavage occurs such that any single nucleoside has a phosphate group at either the 2′ or the 3′ position, and yields of the two isomers are approximately equal. The separation of a standard mix of ribonucleotide isomers at pH 3.7 is shown in Figure 2. This pH aided separation in two ways. First, at a moderately low pH the difference in the effective charges of different nucleotides is augmented, and consequently, so is the difference in electrophoretic mobilities (μe) (*10*). Second, low pH causes the electroosmotic flow rate (μeo) to decrease more in magnitude than the oppositely directed electrophoretic mobilities of nucleotides, with the result being enhanced resolution (*11*). Resolution was nearly baseline for 2′- and 3′-CMP as well as 2′- and 3′-AMP and was adequate to distinguish the isomers of both GMP and UMP. The identification of peaks was made by spiking the sample with either the 2′ or 3′ isomer of each of the different nucleotides. As shown in Figure 3, when a mixture of all 12 2′, 3′, and 5′ isomers was electrophoresed, all species were distinguishable.

**Determination of Nucleotide Composition in RNA Digests.** The conditions used to resolve the isomers of nucleotides had substantial drawbacks for quantitative analysis. Low pH caused the electroosmotic flow rate to be both slow and irreproducible. Because resolution of 2′ and 3′ isomers is irrelevant for assays of nucleotide composition in RNA, we adopted another strategy that sacrifices some resolution while gaining reproducibility and speed of analysis.

The cationic detergent CTAB was added to formate buffer and the polarity of the electrodes was reversed so that the electroosmotic flow was in the same direction as the electrophoretic mobilities of the nucleotides. Figure 4 shows the resulting electropherograms from an equimolar mixture of the four pairs of nucleotides (part a), the base-hydrolyzed digests of rabbit intestinal mucosa RNA (part b), calf liver RNA (part c) and baker's yeast RNA (part d). In each electropherogram the four nucleotide isomer pairs produced five peaks in which the only resolvable isomers were 2′- and 3′-CMP. Nevertheless, this information was sufficient to estimate the ratio of the four different bases based on relative peak heights. Moreover, each electropherogram was obtained in 5 min or less.

We found that a period of several hours was required to condition a capillary wall to the buffer containing CTAB under these conditions. During this period, the electroosmotic flow rate decreased. Once a capillary was conditioned, the elec-

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**Table I. Comparison of Determined Nucleotide Composition of Different RNAs with Previously Published Values**

| source of RNA | | % CMP | % GMP | % AMP | % UMP |
|---|---|---|---|---|---|
| calf liver | experimental | 28.3 ± 0.8 | 35.8 ± 0.4 | 17.9 ± 0.5 | 18.0 ± 0.5 |
| | published[a] | 29.1 | 35.0 | 19.5 | 16.4 |
| rabbit liver | experimental | 29.7 ± 1.4 | 33.3 ± 1.1 | 17.3 ± 0.5 | 19.7 ± 0.5 |
| | published[b] | 28.2 | 32.6 | 19.3 | 19.9 |
| baker's yeast | experimental | 18.4 ± 0.9 | 34.1 ± 0.4 | 25.5 ± 0.6 | 22.0 ± 0.6 |
| | published[a] | 17.8 | 29.4 | 24.7 | 28.1 |

[a] See ref 1. [b] West, E. S.; Todd, W. R.; Mason, H. S.; Van Bruggen, J. T. *Textbook of Biochemistry*, 4th ed.; Macmillan: New York, 1967; p 394.

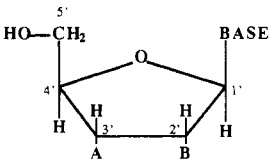


**Figure 1.** Diagram of a 2′- or 3′-ribonucleoside monophosphate. When A = phosphate, B = OH, and vice versa.

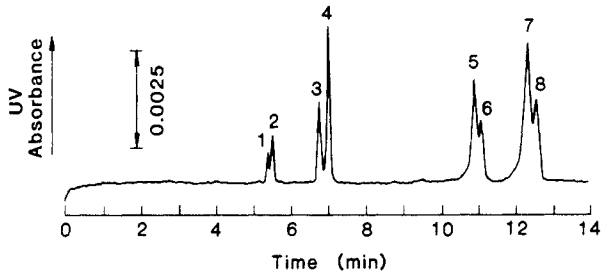


**Figure 2.** Electropherogram of mixture of 2′ and 3′ isomers of adenylate, guanylate, uridylate, and cytidylate, each at approximately 0.3 mM: 1, 2′-CMP; 2, 3′-CMP; 3, 2′-AMP; 4, 3′-AMP; 5, 3′-GMP; 6, 2′-GMP; 7, 3′-UMP; 8, 2′-UMP. The sample was run in 50 mM sodium acetate, pH 3.7, with an applied voltage of +25 kV. A 75-μm fused silica capillary was employed, with length 40 cm from anode (injection site) to detector, 60 cm from anode to cathode. Injection time was 10 s.

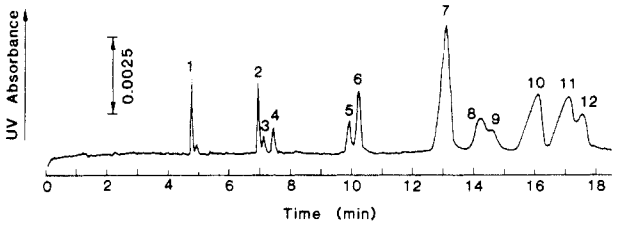


**Figure 3.** Electropherogram of mixture of 2′, 3′, and 5′ isomers of adenylate, guanylate, uridylate, and cytidylate, each at approximately 0.3 mM: 1, 5′-AMP; 2, 5′-CMP; 3, 2′-CMP; 4, 3′-CMP; 5, 2′-AMP; 6, 3′-AMP; 7, 5′-GMP; 8, 3′-GMP; 9, 2′-GMP; 10, 5′-UMP; 11, 3′-UMP; 12, 2′-UMP. All conditions are identical with those described in Figure 2 except that buffer here is at pH 4.0. Injection time was 10 s.

tropherograms were highly reproducible, unlike those obtained with the buffer system lacking detergent. In cases in which a capillary was stripped of its CTAB coating by rinsing with 0.1 M NaOH and was subsequently recoated with detergent, we found that the relative, but not the absolute, migration rates of nucleotides were conserved.

Normalization of the absorbance response of the four bases was accomplished by measuring peak heights for an equimolar standard of the four pairs of nucleotide isomers. We are thus able to state approximate mole percents of each base in RNA samples from calf liver, rabbit liver, and baker's yeast (Table I). Values are based on a mean of three runs for the standard, calf liver, and rabbit liver and a mean of two runs for baker's yeast. Uncertainty is expressed as standard deviation and represents propagation of the error in the mean of the

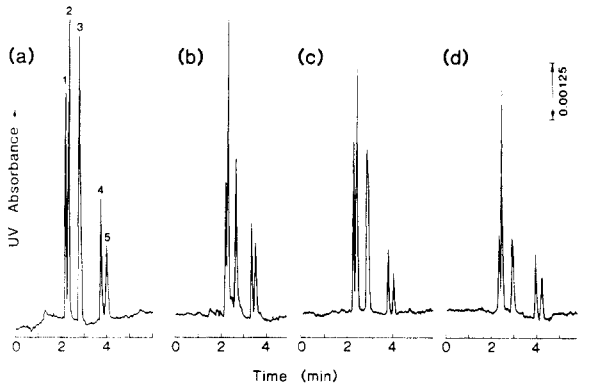


**Figure 4.** Electropherograms of (a) equimolar mixture of 2′ and 3′ isomers of AMP, GMP, UMP, and CMP; (b) base-hydrolyzed rabbit intestinal mucosa RNA; (c) base-hydrolyzed calf liver RNA; (d) base-hydrolyzed yeast RNA. For each electropherogram, peaks are as follows: 1, 2′- and 3′-UMP; 2, 2′- and 3′-GMP; 3, 2′- and 3′-AMP; 4, 3′-CMP; 5, 2′-CMP. Buffer was 12 mM sodium formate, 0.1 mM CTAB, pH 3.8. Sample was injected for 5 s at the cathode, which was at −26 kV with respect to the anode. The capillary had a 50 μm i.d. and was 29 cm from cathode to detector, 42 cm from cathode to anode. Concentration of each nucleoside monophosphate in part a was 0.5 mM.

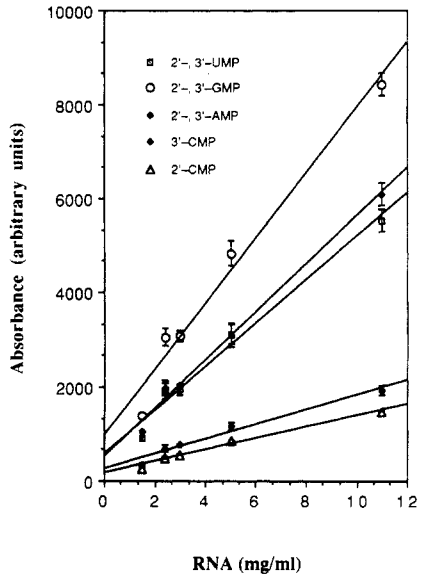


**Figure 5.** Absorbance peak heights of ribonucleoside monophosphates vs amount of yeast RNA digested. Approximately 2 nL was injected per run. Points for 2.4 mg/mL, 5 mg/mL, and 11 mg/mL represent a mean of three measurements, and points for 1.5 and 3 mg/mL represent a mean of two measurements. Error bars show standard deviation.

standard with the error in the mean of each sample group. The sources of the previously published values did not provide estimates of uncertainty.

**Estimation of RNA Amount by Measurement of Nucleotide Content.** The relationship between absorbance peak

heights and concentration of nucleotides was examined over a range of 30–500 μM and found to be linear for all bases. This suggests that the present method may be utilized for estimation of absolute RNA amounts in a tissue extract. To demonstrate this, we recorded the peak heights of nucleotides versus mass of RNA prior to base hydrolysis. Figure 5 shows the resulting plot for yeast RNA. By calculation of the response of the instrument to the quantity of a nucleotide standard injected, the amount of RNA in a sample can be determined. Furthermore, if the ratio of the four bases in a specific RNA source is known, detection of any peak is sufficient to determine the amount of RNA. Therefore, for situations in which speed is of prime importance, measurement of the uracil peak will permit such an estimation in less than 2.5 min. This method is clearly applicable to samples containing a single RNA species as well as those containing mixtures of different RNAs, as would result from bulk RNA extraction from tissue.

## DISCUSSION

The clearest advantage CZE maintains over other means of nucleotide separation is the required sample quantity. In our experiments, injection of less than 20 pg of each ribonucleotide provided electropherograms with a signal-to-noise ratio of 3:1. In comparison to HPLC and isotachophoresis the amount of sample necessary per run is less by approximately 100-fold, and in comparison to paper electrophoresis, by many orders of magnitude. The method of microcolumn liquid chromatography, however, allows a comparable detection limit of ribonucleotides, as demonstrated by Banks and Novotny (*12*).

Nucleotide resolution in CZE is as good or better than that for each of the previously demonstrated methods. In the experiments done without CTAB, all product species of RNA base-hydrosylate were resolved. HPLC and ion-exchange chromatography also effect such a separation, but isotachophoresis has been shown to distinguish only the 2′ from the 3′ isomer for CMP, and paper electrophoresis apparently has been unable to separate any of the isomers. Even for our more rapid and reproducible experiments utilizing CTAB, resolution rivals that seen in many HPLC and ion-exchange separations and is superior to that in isotachophoresis and paper electrophoresis.

Short separation time is a third attribute of CZE. Complete separation of ribonucleotides is possible in 5 min, provided a CTAB-conditioned capillary is available, and no capillary regeneration time is needed between separations. Isotachophoretic separation demands more than 20 min. HPLC requires approximately 20 min for a separation and another 20 min between runs to equilibrate the column. Paper electrophoresis and ion-exchange chromatography traditionally have taken several hours or more.

The use of electropherogram peak heights in quantitation requires either that the migration rate of the standard is exactly equal to the rates for the samples or, if velocities are different, that diffusion of bands is negligible. In this study, migration rates did show minor variations, but diffusion of nucleotides is low for the time scale of our separations. The effective diffusion coefficient for 5′-AMP, for example, has been estimated at $1.6 \times 10^{-5}$ cm$^2$/s (*11*).

In summary, the combined advantages of CZE analysis of ribonucleotides from base-hydrolyzed RNA, namely the amount of sample required, the resolution, and the time of analysis, indicate this method to be highly efficacious. In this study, no exceptional effort was made in optimizing the procedure. Also, enhanced accuracy and precision in quantitation are possible through analysis of corrected peak areas. With the prospects of further improvement and also of automation, we believe that CZE may become the method of choice for quantitating base composition of RNA and for measuring RNA amount in tissue. The feasibility of coupling CZE to a radioisotope detection system (*13*), either on-line or off (*14*), suggests the potential to monitor incorporation of radiolabels in purine and pyrimidine metabolism.

## ACKNOWLEDGMENT

We wish to thank Dean Appling and the members of his lab (University of Texas, Austin) for their advice and intestines.

**Registry No.** 5′-AMP, 61-19-8; 5′-CMP, 63-37-6; 2′-CMP, 85-94-9; 3′-CMP, 84-52-6; 2′-AMP, 130-49-4; 3′-AMP, 84-21-9; 5′-GMP, 85-32-5; 3′-GMP, 117-68-0; 2′-GMP, 130-50-7; 5′-UMP, 58-97-9; 3′-UMP, 84-53-7; 2′-UMP, 131-83-9; CTAB, 57-09-0.

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# Labeling Reaction for Time-Resolved Luminescence Detection in Liquid Chromatography

*Sir:* In high-performance liquid chromatography (HPLC), chemical derivatization (or labeling) of analytes to obtain products with better detection characteristics is a very well-known procedure to improve detection limits. Due to

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