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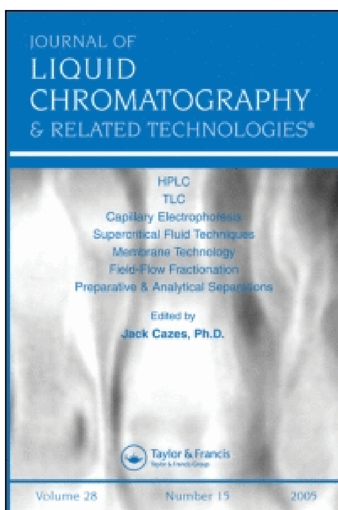

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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High Pressure Liquid Chromatography Determination of the 5'-Terminal Residue of Small RNA Molecules

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To cite this Article Vandenberghe, Antoon and De Wachter, Rupert(1982) 'High Pressure Liquid Chromatography Determination of the 5'-Terminal Residue of Small RNA Molecules', Journal of Liquid Chromatography & Related Technologies, 5: 11, 2079 — 2084
To link to this Article: DOI: 10.1080/01483918208067618
URL: http://dx.doi.org/10.1080/01483918208067618

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2079-2084 (1982)

HIGH PRESSURE LIQUID CHROMATOGRAPHY DETERMINATION OF THE 5'-TERMINAL RESIDUE OF SMALL RNA MOLECULES

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ABSTRACT

Under the appropriate conditions, high pressure liquid chromatography of alkaline hydrolysates of short RNAs allows the identification of the 5'-end group under the form of a nucleoside 5', 3'(2')-bisphosphate. The separation conditions were elaborated with an artificial mixture of nucleoside mono- and bisphosphates, tested with an alkaline hydrolysate of *Escherichia coli* 5 S rRNA, and applied to the identification of the *Artemia salina* 5 S rRNA end group.

INTRODUCTION

Modern sequencing techniques for small RNAs follow essentially two types of strategies. The first one is direct gel reading, which requires terminal labeling followed by partial base specific enzymatic (1,2) or chemical (3) cleavage and subsequent separation on polyacrylamide gel of the reaction products. The second involves random partial hydrolysis, limited in principle to one knick per molecule, followed by kinase labeling of the 5'-OH

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groups of the fragments extending to the 3'-end of the intact RNA. After separation on gel, each fragment is completely hydrolysed and the ^{32}P-labeled end identified as a nucleoside bisphosphate (4-6).

With both approaches the identification of the 5'-terminal residue of the RNA poses some problems. When the direct reading method is applied to RNA ligated with [5'-^{32}P] pC3'p (7) at the 3'-end, a heavy band of undegraded material usually obscures the reading of the 5'-penultimate band. Labeling at the 5'-end by kination with [γ-^{32}P] ATP requires prior dephosphorylation with alkaline phosphatase, with the risk that the slightest nicking by a contaminating nuclease creates extra end groups. With the random hydrolysis procedure the 5'-terminal residue is not labeled and its identification requires a separate experiment anyway.

We here present a rapid and sensitive method that overcomes the aforementioned problems. The 5'-terminal nucleotide is identified under the form of a nucleoside bisphosphate by HPLC of a complete alkaline hydrolysate of about 20 μg of unlabeled RNA.

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MATERIALS AND METHODS

Marker Nucleotides and Ribosomal RNA

The four nucleoside bisphosphates were prepared from the corresponding nucleoside 3'(2')-monophosphate mixed isomers (Sigma Chemical Company, St. Louis, Mo) by phosphorylation of the 5'-OH with $POCl_3$ (8). The reaction products were purified on Dowex 1 X 2 (Serva, Heidelberg) by ion exchange chromatography (9). The 5',3'(2')-bisphosphates were obtained as mixed isomers.

5 S ribosomal RNA from E. coli MRE 600 was prepared according to Monier (10) and Artemia salina 5 S RNA was isolated as described earlier (11).

Alkaline Hydrolysis of 5 S rRNA

20 μg of the 5 S rRNA was dissolved in 2.5 μl of 0.3 M NaOH and incubated overnight at 37° in a capillary. After hydrolysis, the sample was diluted ten-fold with water and injected directly onto the HPLC column.

HPLC Equipment and Separation Conditions

Separations were carried out on a PARTISIL SAX (Reeve Angel, Clifton, N.J.) column of 250 x 4.6 mm fitted to a Model 3500 high pressure gradient chromatograph from Spectra-Physics (Santa Clara, Calif.) equipped with a dual wavelength detector at 254 and 280 nm. Linear gradients were used by mixing 0.01 M H_3PO_4 and 1 M H_3PO_4, both adjusted to pH 2.2 with NH_3, as low and high concentration eluent. The gradient was programmed from 0 to 100% of the highest concentration eluent over a period of 100 min at ambient temperature and with a flow rate of 1.2 ml/min.

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RESULTS AND DISCUSSION

Under the described separation conditions, the nucleoside monophosphates are well separated into the amino- and keto-derivates and precede the nucleoside bisphosphates as shown in Fig. 1a. The 2'- and 3'-isomers elute together, except for A2'p and A3'p which are slightly separated. The effect is more pronounced in the case of the adenosine bisphosphates where the 2'-isomer interferes with the cytidine bisphosphate peak. This does not impede the end group analysis, since cytidine bisphosphate, contrary to adenosine bisphosphate, will be detected as a single peak and in addition gives a much higher response at 280 nm than at 254 nm. If desired, the interference can be avoided by hydrolysing the RNA with a mixture of RNases, which obviates the formation of 2'-isomers. However, use of RNases involves a more laborious sample preparation since they should be eliminated before application on the column to avoid problems with later separations

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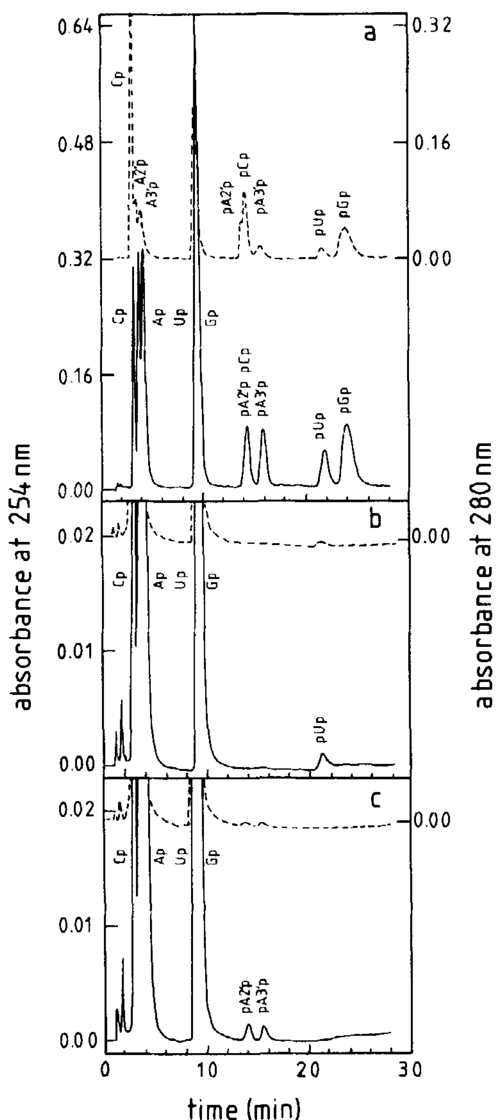

FIGURE 1. HPLC analysis of nucleoside mono- and bisphosphates. Separation conditions are as described in Materials and Methods. Full lines correspond to absorbance at 254 nm. Dashed lines indicate the absorbance at 280 nm, registrated with the same sensitivity but represented with the baseline shifted upward. (a) Separation of a text mixture containing approximately 20 μg of each nucleoside monophosphate and 5 μg of each of the nucleoside bisphosphates. All markers are composed of 2'- and 3'-mixed isomers but only in the case of adenosine are they separated. pCp gives a weak signal at 254 nm and is masked by pA2'p, but becomes visible at 280 nm. (b) Test with an alkaline hydrolysate of 5 S rRNA from E. coli showing pUp as the 5'-terminal hydrolysis product. (c) Determination of the 5'-terminus of Artemia salina 5 S rRNA showing pAp as the end group.

of nucleic acid material. A second objection is the presence of 2',3'-cyclic phosphates resulting from incomplete digestion and this will in turn complicate the pattern.

Different pH values where tested for the separation, but it was not possible to find conditions achieving the separation of the four nucleoside monophosphates as well as the four bisphosphates. The choice of pH 2.2 results in a separation of the four bisphosphates and has the advantage that the presence of pseudouridine in the alkaline hydrolysate can be detected in the same analysis (12) since it migrates between the Ap and (Gp + Up) peaks.

Using the 5 S rRNA from E. coli as a test, the 5'-terminus was identified as pUp as illustrated in Fig. 1b, in agreement with the reported sequence (13). Application of our method to the 5 S rRNA of Artemia salina showed pAp to be the terminal hydrolysis product (Fig. 1c). Since approximately 20 μg of 5 S rRNA, or 0.5 $A_{260\ nm}$ units, are used per experiment, the pNp peak contains about 1/120th of this amount, or 0.004 $A_{260\ nm}$ units. This is sufficient to give a distinct signal.

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ACKNOWLEDGEMENT

We thank Raymond De Baere for preparing the 5 S ribosomal RNAs from Escherichia coli and Artemia salina. Our research is supported in part by an FKFO grant.

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