Journal of Chromatography, 454 (1988) 195–203
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 797

ANALYSIS OF DOUBLE-STRANDED POLY(A) · POLY(U) MOLECULES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. LIAUTARD, S. COLOTE, C. FERRAZ and J. SRI-WIDADA
U-249 INSERM, CRBM du CNRS et Université Montpellier I, B.P. 5051, 34033 Montpellier Cedex (France)
A. PICHOT
Laboratoire EXPANSIA, B.P. 6, 30390 Aramon (France)
and
J. P. LIAUTARD*
U-249 INSERM, CRBM du CNRS et Université Montpellier I, B.P. 5051, 34033 Montpellier Cedex (France)
(First received March 11th, 1988; revised manuscript received May 18th, 1988)

SUMMARY

The behaviour of different batches of synthetic Poly(A) · Poly(U) in reversed-phase high-performance liquid chromatography (HPLC) was studied. They consist of large molecules mainly in the form of a double strand. Differences in the elution patterns were correlated with properties detected by conventional methods such as electrophoresis, centrifugation, fusion analysis or enzymatic digestions. Under the present conditions, contamination by products and precursors used during synthesis was detectable, but was absent in most of the preparations. The differences in elution patterns between batches appear to be correlated with the size of the molecules synthesized. The chromatograms suggested that Poly(A) · Poly(U) molecules contain single-strand portions at least transiently. The presence of such portions was confirmed by enzymatic digestion with S1 nuclease. The rapidity, reproducibility and ease of reversed-phase HPLC qualify this technique as a tool for routine analysis.

INTRODUCTION

The homoribopolymers Poly(A) and Poly(U) associate to form a helix, whose structure resembles that of a native DNA[1]. In some cases, however, more complex structures have been described in mixtures of Poly(A) and Poy(U). For example, associations of two Poly(U) molecules with one Poly(A) molecule[1–3] or two Poly(A) molecules with one molecule of Poly(U) have been reported. Moreover, because they are synthesized *in vitro* using polymerizing enzymes such as polynucleotide phosphorylase (PNPase), the length of the resulting polymers is heterogeneous. Partly single-stranded polymers could therefore be present in the mixture.

Poly(A) · Poly(U) molecules are known to exert biological effects on the immune system[5]. For instance, spontaneous mammary tumours have been reduced by treatment with Poly(A) · Poly(U) molecules[6].

0021-9673/88/$03.50

Analysis of the biological properties of these molecules calls for an exact determination of the degree of purity and especially the exact structure of the different batches. Many methods can be used to analyze such molecules, *e.g.*, gel electrophoresis, centrifugation, melting analysis and enzymatic studies. However, a single method giving a general view of most of the properties, would be of great value. We have used reversed-phase high-performance liquid chromatography (HPLC) because the separation of large nucleic acids on a reversed phase is relatively complex, depending on the base composition[7], secondary structure[8] and constraints present in the molecule[9].

In the present study, we analyzed Poly(A) · Poly(U) polymers by reversed-phase HPLC and compared the results with those obtained by conventional methods. We show that most of the properties found by conventional analysis can be correlated with the particularities of the reversed-phase HPLC elution profile.

MATERIALS AND METHODS

Materials used

Poly(A), Poly(U) and Poly(A) · Poly(U) were obtained from Boehringer (F.R.G.) and from Expansia (France). Agarose type II was obtained from Sigma, acetonitrile (HPLC grade) from Touzart & Matignon and phosphate and polynucleotide kinase from Boehringer.

Methods employed

Reversed-phase HPLC. Reversed-phase HPLC was performed on a Gilson apparatus equipped with a LiChrosorb RP-18 column (25 cm × 0.4 cm; particle size, 10 μm; pore size, 75 Å; Merck) and an UV detector at 254 nm, essentially as described previously[7]. Poly(A) · Poly(U) was analyzed using a linear gradient elution, from 100% (v/v) buffer I (0.2 *M* ammonium acetate, pH 6.5) to 40% (v/v) buffer II (50% acetonitrile in water) in 40 min. A flow-rate of 1 ml/min was used throughout Poly(A) · Poly(U) was dissolved in 150 m*M* sodium chloride and kept overnight at 4°C before injection. The nucleosides were analyzed using the same buffers, but the gradient was performed in 20 min.

Other methods. Electrophoresis was performed on 1.5% agarose gel in TBE buffer (90 m*M* Tris–borate pH 8, 5 m*M* EDTA[9] with ethidium bromide at 3 V/cm for 4 h.

To analyze the base composition, the polymers were hydrolysed overnight in 0.3 *M* potassium hydroxide at 37°C. After neutralization, the phosphate groups were removed with alkaline phosphatase.

Centrifugation in a 5–20% sucrose gradient in 150 m*M* sodium chloride and 10 m*M* Tris–HCl, pH 7.4, was performed in a Kontron centrifuge using a TST 41-14 rotor at 30 000 rpm for 20.5 h. Gradients were fractionated and the optical density was determined. A mean sedimentation coefficient was deduced at the maximum absorption, according to McEwen[10].

The buoyant density in caesium chloride at 42°C was determined by centrifugation in 50% caesium chloride at 30 000 rpm for 72 h using a TST 41-14 rotor. The resulting gradient was fractionated and the refractive index and optical density at 260 nm were determined in each fraction.

Melting point and hyperchromicity [(OD_{260} max − OD_{260} min)/OD_{260} min] were determined in 10 m*M* Tris–HCl, pH 7.4 and 150 m*M* sodium chloride by increasing the temperature from 20°C to 70°C in 25 min.

RESULTS

Conventional analysis of Poly(A) · Poly(U) batches

Numerous preparations were studied. We present only three batches, A, B and C, which summarize the results obtained.

The mean molecule size was determined by two methods, *i.e.*, sucrose centrifugation and agarose electrophoresis, which gave similar results. Fig. 1 shows the gel electrophoretic pattern of the nucleic acid. The molecular weight was estimated by comparison with standards obtained by digestion of lambda-phage DNA with restriction enzymes. Preparation C consisted of heterogeneous polymers with a maximum of around 300 base pairs, preparation B had larger molecules and preparation A contained essentially large molecules. These results were confirmed by sucrose gradient centrifugation (Table I).

The base composition was determined by HPLC after alkaline hydrolysis and removal of the phosphates with alkaline phosphatase. The only bases present were uridine and adenosine, and their molar ratio was close to 1 (see Table I).

The secondary structure was analyzed by the melting point method. The results summarized in Table I confirm that Poly(A) · Poly(U) molecules are indeed double-stranded, with a melting point corresponding to published data[11]. The shape of the melting curve shows the presence of small molecules in preparation C, and to a lesser extent in preparation B. The melting curve of batch A confirms that the molecules are large, with few small molecules if any. The double-stranded nature of the

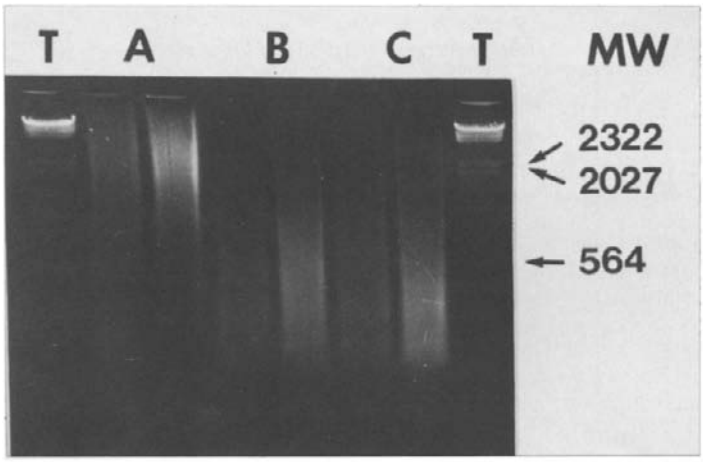

Fig. 1. Size determination of the three representative batches of Poly(A) · Poly(U) by agarose gel electrophoresis. The three batches (A, B, C), dissolved in 150 mM sodium chloride and 30% sucrose, were applied to a 1.5% agarose gel in TBE buffer containing 0.5 μg/ml ethidium bromide (see Materials and Methods). Electrophoresis was performed at 3 V/cm for 4 h. Lambda-phage DNA digested by Hind III was added for size markers (T). MW = Molecular weight.

TABLE I

ANALYSIS OF THE Poly(A) · Poly(U) PREPARATIONS BY CONVENTIONAL METHODS

The three batches were analyzed by sucrose gradient centrifugation, caesium chloride buoyant density, melting point, T_m, and nucleoside ratio determination, as described in Materials and Methods.

	Batch C	*Batch B*	*Batch A*
Mean sedimentation constant	6S	7S	7–9S
Density in CsCl	1.84	1.84	1.85
Melting point, T_m (°C)	56	57	60
Hyperchromicity (%)	49	56	57
Uridine/adenosine molar ratio	1.1	1.03	1.06

Poly(A) · Poly(U) solution was confirmed by buoyant density centrifugation analysis in caesium chloride (Table I).

Reversed-phase HPLC analysis of Poly(A) · Poly(U)

The three batches of Poly(A) · Poly(U) were analyzed by reversed-phase HPLC. Although they were prepared using the same protocol, the analysis showed significant differences, as seen in Fig. 2. The behaviour of each batch was very different. For example, batch A exhibited only one peak with an elution time of 27 min, whereas batch B and especially batch C were eluted as three different components, each appearing heterogeneous.

These differences between the three batches cannot be attributed to the quantity of Poly(A) · Poly(U) loaded on the column, first because the same amount of material was used in each case that is 60 μg of polynucleotide in 200 μl of buffer (Fig. 2). Furthermore we have studied the influence of the quantity applied to the column on the elution profile. There was a very small change in the appearance of the profile, less

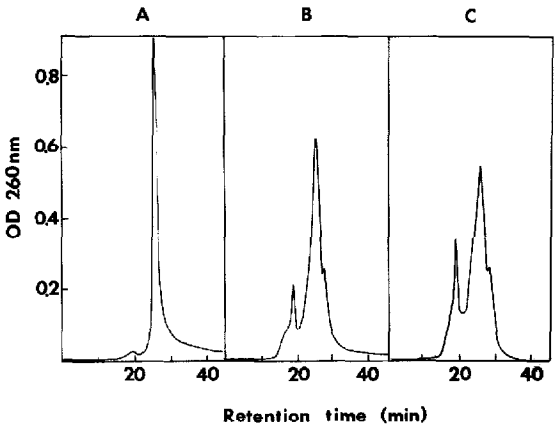

Fig. 2. Elution profiles of representative preparations of Poly(A) · Poly(U). Three representative preparations were analyzed by reversed-phase chromatography on a C_{18} column as described in Materials and Methods. In each case 60 μg of lyophilized Poly(A) · Poly(U) were dissolved in 200 μl of 0.15 *M* sodium chloride. In order to obtain a good dissolution, the preparation was kept for at least 16 h at 4°C before loading. OD = Absorbance. Retention times: (A) 27 min; (B) 19, 27 and 29 min; (C) 19, 27 and 29 min.

than 5% when doubling the quantity. This result is in accord with an equilibrium between the single stranded and double stranded portions proposed below.

Several other hypotheses can be proposed to account for these results. The first is a possible contamination by products other than Poly(A) · Poly(U), particularly the precursors in their synthesis (ADP and UDP, or degradation products of the polymers, AMP, UMP, nucleotides, nucleosides and oligonucleotides). Consequently we analyzed, under the same conditions, ADP, UDP and the products obtained from degradation by alkaline hydrolysis (*i.e.*, adenosine 3′-phosphate and uridine 3′-phosphate) or the nucleosides obtained by further phosphatase digestion. Nuclease-degraded Poly(A) · Poly(U) was also analyzed. The retention times of these products are shown in Table II. They are very different from those of Poly(A) · Poly(U). Only adenosine exhibited a retention time close to that of Poly(U), *i.e.*, 18 min compared to 19 min, but they differed slightly when analyzed together.

We also analyzed the behaviour of Poly(A) and Poly(U) separately (Table II), and were very surprised to find that their retention times were exactly the same as those of peaks 1 and 2 of Poly(A) · Poly(U). It is thus possible that under the chromatographic conditions the two strands are dissociated and eluted separately. However, this hypothesis does not take two facts into account: (i) the melting point of these molecules is around 57°C and (ii) the different batches gave different results (see Fig. 1). Therefore we further analyzed the products obtained after separation by HPLC.

Furthermore when two different batches were mixed and analyzed by HPLC the resulting chromatogram was the sum of the chromatograms obtained for each batch analyzed separately.

Analysis of fractions separated by HPLC

The three fractions separated by HPLC were precipitated with 2 volumes of ethanol and purified twice by HPLC. The materials corresponding to peaks 1 and 2 were easily separated but molecules corresponding to peak 3 were not totally pure. This unsuccessful purification may have been due to the very small amounts and

TABLE II

RETENTION TIMES OF SOME OF THE MOLECULES THAT MAY CONTAMINATE THE PREPARATION

To examine whether molecules used during the synthesis of the Poly(A) · Poly(U) or degradation products could produce the complex Poly(A) · Poly(U) elution profile, we determined their retention times under the conditions of analysis used in Fig. 2 and described in Materials and Methods.

Molecule analyzed	*Retention time (min)*
ADP	8.75
UDP	3.5
Uridine	9
Adenosine	18.25
Digest	3–15
Poly(A)	27
Poly(U)	19

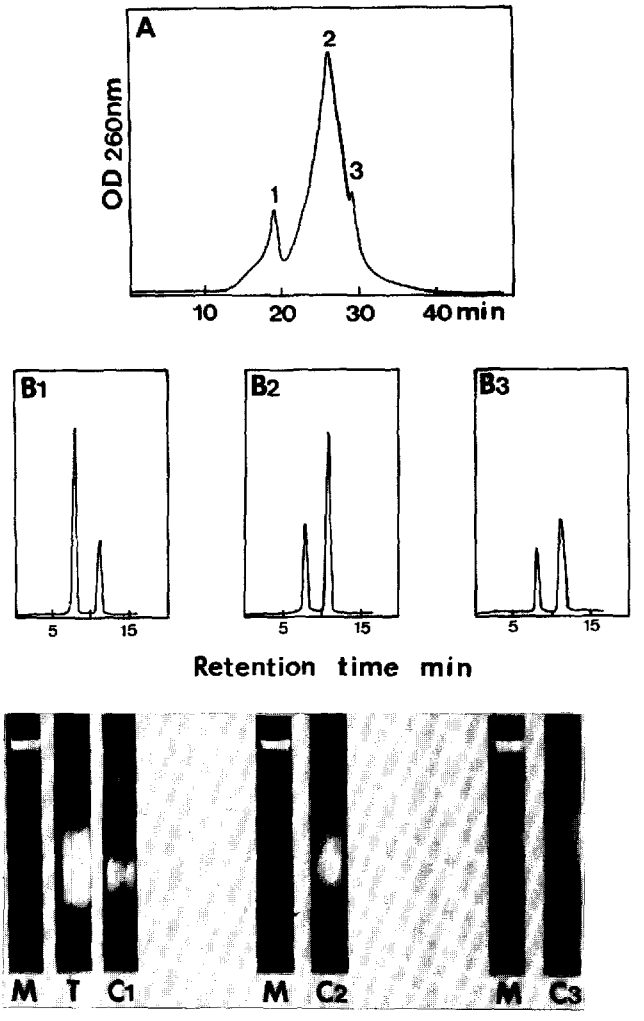

Fig. 3. Analysis of the materials separated by HPLC. Poly(A) · Poly(U) (batch B, 0.5 mg) was chromatographed as described in Fig. 2 (Fig. 3A). Fractions corresponding to the three peaks were pooled and precipitated with 75% ethanol. A second round of purification was performed on each fraction. They were then analyzed for base composition (Fig. 3B) or for size determination by gel electrophoresis (Fig. 3C) as described in Materials and Methods. (B) The HPLC profile obtained after hydrolysis and thus depicts the the base composition of the different peaks: 1 = peak 1; 2 = peak 2 and 3 = peak 3. (C) The size analysis determination by agarose gel electrophoresis. In each case M represents for the size markers; there were obtained by digestion of lambda phage with restriction enzyme Hind III. T is the Poly(A) · Poly(U) before separation by HPLC and is thus given as a reference. The three components separated by HPLC in (A) were analysed: 1, peak 1, 2, peak 2 and 3, peak 3.

minimum differences in the retention times of the contaminating products, or to the apparent conversion of this peak into peak 2 during purification. In any case, we analyzed the size agarose gel electrophoresis and the base composition (alkaline hydrolysis, HPLC) of the material separated in peaks 1, 2 and 3 (Fig. 3). Peak 1 contained material of small size, with a mean of around 250–300 base pairs. Base

composition analysis revealed that uridine was the major component, but adenosine was also present, in a molar ratio A/U of 0.6. Peak 2 contained molecules of a mean size close to that of the starting material and a molar ratio (A/U = 1.2) showing a slight increase in the A base. Peak 3 contained molecules of large size and a molar ratio A/U of around 1.

Interpretation of the results

These results can be explained by the theory of multiple interactions and the behaviour of nucleic acids in reversed-phase HPLC as described previously[7–9,12]. Assuming that Poly(A) · Poly(U) molecules have gaps in their secondary structures, *i.e.*, single-stranded regions, these portions would interact strongly with the stationary phase as previously shown[8]. Thus, there are three possibilities, *i.e.*, the molecule contains: (i) stretches of single-stranded Poly(U), (ii) single-stranded Poly(A) or (iii) both. In the case where the single-strand stretchers are only on Poly(U), the double-stranded part would detach from the stationary phase and the molecule would be attached by the single-stranded Poly(U) (see ref. 8); the whole molecule would consequently be eluted with Poly(U) and exhibit the same retention time. In other words, in this case, the retention time of the molecule would be determined by the single-stranded region and the whole molecule would exhibit the elution profile of Poly(U). In contrast, if a molecule contains both single-stranded Poly(U) and single-stranded Poly(A) [where higher hydrophobicity of Poly(A) would dominate], the retention time of the whole molecule would be that of Poly(A). None of these molecules contains two perfectly matching strands, or they would be eluted earlier[8]. It can be assumed that when the molecules of Poly(A) and Poly(U) are small, the fraction of molecules containing only single-stranded Poly(U) increases, as does the fraction containing only single-stranded Poly(A), but the latter is eluted with molecules also containing single-strand Poly(U). Thus the smaller the molecules are, the greater is the ratio of peak 1 to peak 2, and thus reversed-phase HPLC measures the mean size of the polymers. If this interpretation is correct, single strands should be detectable in the molecule. To test for the presence of such single strands we have digested the molecules with single-strand-specific S1 nuclease. Fig. 4 shows that Poly(A) · Poly(U) was digested by this nuclease, implying the presence of single-stranded regions. Analysis by HPLC was also performed under the conditions used for native Poly(A) · Poly(U). Degradation products corresponding to oligonucleotides and finally to nucleotides have been observed (results not shown). However a peak corresponding to genuine Poly(A) · Poly(U) was still observed but disappeared when digestion was performed for 3 h. Thus Poly(A) · Poly(U) contains single-stranded parts that are sensitive to S1 nuclease.

The nature of peak 3 is more difficult to explain. It may represent an unidentified complex structure, or could be the result of a contaminant protein, but such a protein was not detected. We have not detected protein contamination by colorimetric protein content analysis, gel electrophoresis, nor using a radioimmunologic assay directed against PNPase the enzyme used for synthesis of Poly(A) · Poly(U).

CONCLUSIONS

We have shown the advantages of reversed-phase HPLC in analyzing synthetic

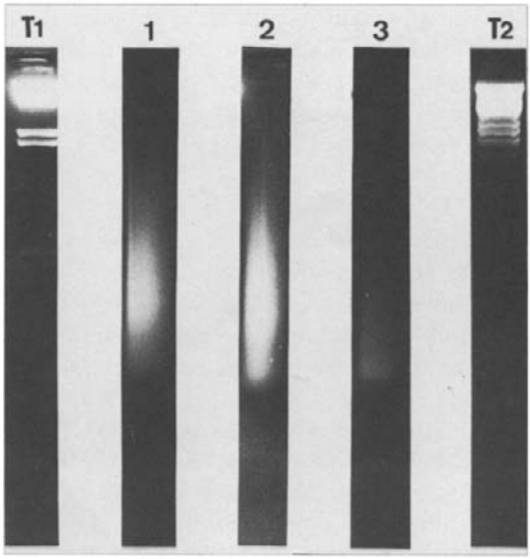

Fig. 4. Degradation of Poly(A) · Poly(U) by S1 nuclease. Batch B of Poly(A) · Poly(U) (0.1 mg) was incubated with 30 units of S1 nuclease for 1 h at 37°C in the buffer recommended by the supplier. The reaction was stopped at 0°C, the mixture diluted 10 times in sucrose buffer and immediately deposited on the gel and electrophorized as described in Fig. 1. Lanes: 1, Poly(A) · Poly(U) control; 2, Poly(A) · Poly(U) incubated at 37°C without nuclease; 3, Poly(A) · Poly(U) digested by S1 nuclease; T, lambda DNA digested by restriction enzyme Hind III (T1) or Pst I (T2) as the size marker. Lane 3 reveals that Poly(A) · Poly(U) has been digested by the single-strand-specific enzyme: first because the mean molecular weight has decreased and secondly because the quantity revealed by ethidium bromide coloration, which binds only long double-stranded molecules, has considerably decreased.

Poly(A) · Poly(U) preparations. A single chromatographic analysis revealed most of the biochemical properties of the preparations. The method is rapid, reproducible and easy to perform. It is particularly suitable for analyzing preparations for industrial or pharmacological use, as well as those used in biological experiments.

Based on the results, we hypothesized that these molecules contain stretches of single strands, and this was confirmed by enzymatic digestion with the S1 nuclease known to degrade only single strands. The result may be of interest in explaining the biological properties of the molecule.

These results complement the analysis of nucleic acids previously reported[7–9,12]. They show that large nucleic acids can be separated by their secondary and tertiary structures. This property has potential applications in the field of molecular biology and genetic engineering.

ACKNOWLEDGEMENTS

This work was mainly supported by a contract between Expansia and the Université de Montpellier I which financed a fellowship for one of us (J.L.). This work was also supported by INSERM and CNRS.

REFERENCES

1 G. Felsenfeld, D. Davies and A. Rich, *J. Am. Chem. Soc.*, 78 (1956) 2023–2028.
2 R. Blake, J. Massoulie and J. R. Fresco, *J. Mol. Biol.*, 30 (1967) 291–308.
3 A. Michelson, J. Massoulie and W. Guschlbauer, *Prog. Nucleic Acid Res. Mol. Biol.*, 6 (1967) 83–141.
4 S. Broitman, D. Im and J. R. Fresco, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 5120–5124.
5 W. Braun and M. Nakano, *Science (Washington, D.C.)*, 157 (1967) 819–821.
6 F. Laceau, G. Delage and C. Chianale, *Science (Washington, D.C.)*, 187 (1975) 256–259.
7 S. Garcia and J. P. Liautard, *J. Chromatogr. Sci.*, 21 (1983) 398–404.
8 J. P. Liautard, *J. Chromatogr.*, 285 (1984) 221–225.
9 S. Colote, C. Ferraz and J. P. Liautard, *Anal. Biochem.*, 154 (1986) 15–20.
10 D. R. Mc Ewen, *Anal. Biochem.*, 20 (1967) 114–149.
11 H. Sober (Editor), *Handbook of Biochemistry*, CRC Press, Boca Raton, FL, 2nd ed., 1970, p. H19.
12 S. Garcia and J. P. Liautard, *J. Chromatogr.*, 296 (1984) 355–362.