Journal of Chromatography, 389 (1987) 334–338
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 282

Note

High-performance liquid chromatographic method using a C_{18} column for the simultaneous separation of the products of decomposition and oligomerization of guanosine 5′-phospho-2-methylimidazolide

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(Received October 30th, 1986)

Interest in the separation of oligonucleotides has recently been stimulated by the introduction of template-directed reactions which result in the synthesis of oligomeric products up to *n* bases long[1,2]. The most efficient template-directed reaction is the oligomerization of guanosine 5′-phospho-2-methylimidazolide (2-MeImpG) with polycytidylate [(poly(C)] acting as the template[3]. Under optimal conditions this reaction results in the synthesis of oligoguanylates [$(pG)_n$] up to 40 bases long. This chemical system represents the most efficient non-enzymatic RNA-type semi-replication model known to date[4].

Fractionation of the products of the above reaction was achieved by high-performance liquid chromatography (HPLC) on an RPC-5 column using a sodium perchlorate gradient at pH 12.0[3,5]. The basic medium was necessary for the separation probably because in neutral medium oligoguanylates, in analogy to polyguanylates, self-associate into a four-stranded helical structure[6,7].

Although RPC-5 chromatography[8] has several useful applications, it is not widely used because the adsorbed quarternary ammonium ion (Adogen) is readily stripped off causing great loss in resolution. In addition, the RPC-5 column is not commercially available and the support materials, Kel-F or Plaskon, are not readily available. Due to the shortcomings of RPC-5 chromatography, other approaches have been taken for the fractionation of various oligonucleotides[9–13] but not for oligoguanylates.

In our own work we have been interested in the reactions of chemically activated guanosine and recently completed a study of the hydrolysis of 2-MeImpG in dilute solutions[14]. This type of substrate is known to yield up to 10% of dimer in relatively concentrated solutions (0.05 *M*) and even higher yields of longer oligomerization products in the presence of poly(C)[15]. Since decomposition and oligomerization occur concurrently, it is desirable to have a single method of analysis for both types of products. For this type of work RPC-5 chromatography did not seem the optimal method to use, because of the limitations mentioned previously, but also because it does not separate the products of 2-MeImpG decomposition. For example, 2-MeImpG and guanosine 5′-phosphate (GMP), which is the major product of decomposition, cannot be resolved on an RPC-5 column at pH 12.0. Therefore another chromatographic system was sought.

0021-9673/87/$03.50

We report on a new reversed-phase HPLC method using a commercially available silica-based C_{18} column for the separation of the products of 2-MeImpG decomposition and oligomerization to products up to four bases long. The chromatography uses a gradient with a 0.02 *M* potassium dihydrogen phosphate buffer (pH 6.5), as the weak and acetonitrile–water, as the strong solvent. A similar system was used for purity evaluation and preparative separation of synthetic deoxyribooligonucleotides[16].

EXPERIMENTAL

Materials

Analytical reagent grade materials were used throughout. Solvents were filtered through 0.45-μm Millipore filters under reduced pressure. Deionized water 18 mΩ · cm quality was obtained from a Lab one/Technic water purifier (Seattle, WA, U.S.A.). HPLC grade acetonitrile was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetonitrile–water mixtures were purged with helium prior to use. Guanosine derivatives were purchased from Sigma (St. Louis, MO, U.S.A.) or P-L Biochemicals (Milwaukee, WI, U.S.A.). $(pG)_n$, n = 2–15, were formed using known conditions for 2-MeImpG oligomerization on poly(C)[3]. 2-MeImpG sodium salt was synthesized following a literature procedure[17]. The major products of the oligomerization reaction were 3′–5′ internucleotide linked isomers which were analyzed by RPC-5 chromatography and the fractions with n = 2–4 were manually isolated. These fractions were neutralized using Tris–HCl buffer at pH 7.6 and kept at −20°C before analysis. Fractions were analyzed using the C_{18} column without further purification.

Equipment

The analyses were carried out on a Hewlett-Packard 1084B gradient model liquid chromatograph, equipped with a variable-wavelength detector set at 254 nm with 430 nm as a reference wavelength and interphased with a Hewlett-Packard 79850B LC terminal. The solvent flow was set at 1.0 ml/min for both chromatographic systems.

RPC-5 Chromatography

The RPC-5 column, 250 × 4.6 mm I.D., was packed with RPC-5 packing <35 μm using a stirred slurry packer of 6-g capacity supplied by Micromeritics (Norcross, GA, U.S.A.). Chromatographic conditions were as follows. Mobile phase: solvent A, 0.01 *M* sodium hydroxide; solvent B, 0.01 *M* sodium hydroxide, 0.1 *M* sodium perchlorate; gradient, 0 to 40% B in 53 min.

C_{18} Chromatography

Analysis was performed using a reversed-phase column Adsorbosphere HS C_{18}, 7 μm, 250 × 4.6 mm I.D. supplied by Alltech (Los Altos, CA, U.S.A.) protected with a guard column filled with C_{18} pellicullar material. Chromatographic conditions were as follows. Mobile phase: solvent A, 0.02 *M* potassium dihydrogen phosphate (pH 6.5); solvent B, acetonitrile–water (15:85). Gradient elution, 0 to 10% in 3 min; 10–40% B in 12 min; isocratic at 40% B for 5 min.

2-MelmpG reaction mixtures

Samples contained 0.05 *M* 2-MelmpG in a 0.4 *M* lutidine buffer (pH 7.8), containing 0.2 *M* magnesium nitrate and 1 *M* sodium chloride. EDTA (0.22 *M*) was used to chelate Mg^{2+} and to quench the reaction. Samples were diluted with water 500-fold prior to analysis.

RESULTS AND DISCUSSION

The only available method for the analysis of the decomposition products of 2-MelmpG was introduced by one of us in another paper[14]. It was based on a similar

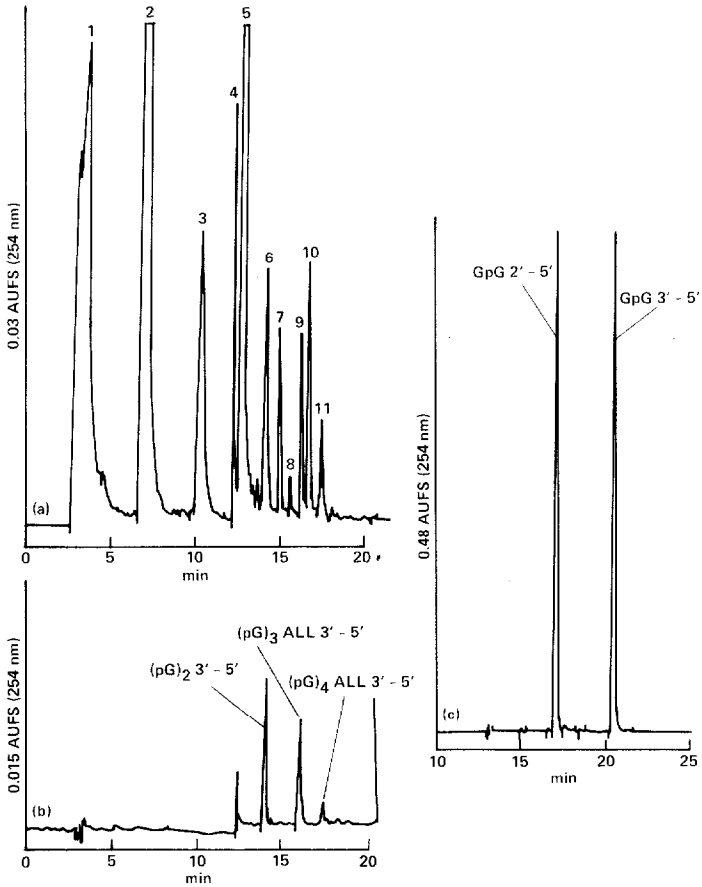

Fig. 1. HPLC elution profile of guanosine derivatives and oligoguanylates. Column, Adsorbosphere HS C_{18}; mobile phase: solvent A, 0.02 *M* potassium dihydrogen phosphate (pH 6.5); solvent B, acetonitrile–water (15:85); gradient elution 0–10% B in 3 min, 10–40% B in 12 min, isocratic at 40% B for 5 min. Chart speed was set at 0.5 cm/min. (a) 2-MelmpG reaction mixture incubated 24 h at 37°C in 0.05 *M* lutidine buffer (pH 7.80) containing 0.2 *M* Mg^{2+} and 1 *M* sodium chloride. Identification of peaks: 1, lutidine and EDTA buffers; 2, GMP; 3, unidentified; 4, thought to be $(pG)_2$ 2′–5′; 5, GppG; 6, $(pG)_2$ 3′–5′; 7, thought to be $Gp(pG)_2$; 8, 2-MelmpG; 9, $(pG)_3$ all 3′–5′; 10, guanosine 3′:5′-cyclic monophosphate; 11, guanosine. (b) Mixture of oligoguanylates $(pG)_n$ n = 2–4, all 3′–5′ internucleotide linked. (c) Standards GpG 2′–5′ and 3′–5′.

C_{18} column Adsorbosphere HS 5 μm with a 0.01 *M* potassium dihydrogen phosphate (pH 5.65) elution and a methanol–water gradient. Later we discovered that this chromatographic system did not separate guanosine from guanosine 3′:5′-cyclic monophosphate. However, we were able to achieve excellent separation of these compounds by replacing methanol with acetonitrile.

We now report that the method (as described under C_{18} chromatography) also separates GpG and $(pG)_n$, n = 2-4, according to base length and internucleotide linkage. Fig. 1a shows the HPLC elution profile of a reaction mixture of 2-MeImpG. Fig. 1b illustrates the profile of a mixture containing dimer, trimer and tetramer of oligoguanylates $(pG)_n$ all 3′–5′ internucleotide linked ones. Fig. 1c shows the profile of a mixture containing the two possible isomers of GpG, *i.e.* it suggests that separation of 2′–5′ and 3′–5′ internucleotide linked $(pG)_n$ with n = 2–4 may also be possible. Positive identification of the peaks was performed by co-elution with the standards. Identification of 2-MeImpG was also done by complete hydrolysis of the sample which results in removal of the corresponding peak. Less acetonitrile results in increased retention times for all compounds tested. Identified peaks were GMP, guanosine(5′)-pyrophosphate-(5′)guanosine (GppG), 2-MeImpG, guanosine 3′:5′-cyclic monophosphate, guanosine, $(pG)_n$, n = 2–4, all 3′–5′. We were not able to identify peak No. 3, but we suspect, based on the anticipated products of the oligomerization reaction and the proposed mechanism[18], that peak No. 4 may be GppGpG and No. 7 $(pG)_2$ 2′–5′ (Fig. 1a).

It is interesting that oligoguanylates were separated by elution with a neutral solvent. We believe that this was possible for the following reasons: (a) HPLC analysis of samples was carried out using a gradient of acetonitrile, which is a solvent known to destabilize base-stacking and oligonucleotide association; (b) samples were fairly dilute; and (c) only short oligoguanylates were tested. Association is expected to be stronger for longer oligomers. Indeed, longer oligomers than $(pG)_4$ could not be fractionated with this method.

This is the first time guanosine derivatives and short oligoguanylates have been fractionated simultaneously. We are currently using the described method of analysis to quantitatively evaluate the oligomerization products of 2-MeImpG in the presence of poly(C) at an early stage of the reaction, *i.e.* before longer products accumulate[18].

In summary, the method reported in this paper has proved to be suitable for the analysis of the decomposition products of 2-MeImpG as well as its oligomerization products $(pG)_n$, n = 2–4. It is tempting to suggest that this simple chromatographic method may also be applicable for fractionation of short, high in guanine content, oligonucleotides as has been observed with HPLC on RPC-5 column[19].

ACKNOWLEDGEMENTS

We are indebted to Prof. D. H. White and Dr. S. Chang for providing the facilities and to Dr. L. E. Orgel for the RPC-5 material. The National Research Council is greatfully acknowledged for supporting A. K. This research was supported in part by the NASA Planetary Biology program.

REFERENCES

1 T. Haertle and L. E. Orgel, *J. Mol. Biol.*, 188 (1986) 77.
2 G. F. Joyce and L. E. Orgel, *J. Mol. Biol.*, 188 (1986) 433.
3 T. Inoue and L. E. Orgel, *J. Mol. Biol.*, 162 (1982) 201.
4 T. Inoue and L. E. Orgel, *Science*, 219 (1983) 859.
5 E. Selsing, J. E. Larson and R. D. Wells, *Anal. Biochem.*, 99 (1979) 213.
6 J. F. Chantot, T. Haertle and W. Guschelbauer, *Biochimie*, 56 (1974) 501, and E. A. Lesnik, R. N. Maslova and Ya. M. Varscharskii, *J. Mol. Biol.*, 10 (1976) 114.
7 F. B. Howard, J. Frazier and H. T. Miles, *Biopolymers*, 16 (1977) 791.
8 R. L. Pearson, J. F. Weiss and A. D. Kelmers, *Biochim. Biophys. Acta*, 228 (1971) 770.
9 D. A. Usher, *Nucl. Acid Res.*, 6 (1979) 2289.
10 J. M. Flanagan, R. K. Fujimura and K. B. Jacobson, *Anal. Biochem.*, 153 (1986) 299.
11 T. R. Floyd, S. E. Cicero, S. D. Fazio, T. V. Raglione, S.-H. Hsu, S. A. Winkle and R. A. Hartwick, *Anal. Biochem.*, 154 (1986) 570.
12 R. R. Drager and F. E. Regnier, *Anal. Biochem.*, 145 (1985) 47.
13 J. B. Crowther, S. D. Fazio and R. A. Hartwick, *J. Chromatogr.*, 282 (1983) 619.
14 A. Kanavarioti, *Origins Life*, 17 (1986) 85.
15 R. Lohrman and L. E. Orgel, *J. Mol. Biol.*, 142 (1980) 555.
16 H.-J. Fritz, R. Belagaje, E. L. Brown, R. H. Fritz, R. A. Jones, R. G. Lees and H. G. Khorana, *Biochemistry*, 17 (1978) 1257.
17 G. F. Joyce, T. Inoue and L. E. Orgel, *J. Mol. Biol.*, 176 (1984) 279.
18 A. Kanavarioti, D. L. Doodokyan and D. H. White, unpublished results.
19 G. von Kiedrowksi, personal communication.