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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE PHOTOPRODUCTS OF NUCLEIC ACID COMPONENTS

I. THYMINE DIMERS IN SHORT OLIGONUCLEOTIDES

V. V. DEMIDOV
Drug Assay Department, Research Institute for Biological Testing of Chemicals, Moscow (U.S.S.R.)
and
V. N. POTAMAN*
Institute of Molecular Genetics, USSR Academy of Sciences, Moscow (U.S.S.R.)
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SUMMARY

Reversed-phase high-performance liquid chromatography in a gradient of acetate or phosphate buffer and methanol was used for separating short (n = 2–4) oligodeoxyribonucleotides and the products of their photomodification which contained thymine dimers. The modified oligomers have shorter retention times and are readily separated from the original ones. The modified oligonucleotides differing in the type (cyclobutane and non-cyclobutane) of the thymine dimer and its position in the molecule are effectively resolved. To establish the type of thymine photodimers in the modified oligomers, the use of their spectral properties, *viz.*, detection at several wavelengths, is sufficient.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has been used successfully for the separation of nucleic acid components: bases[1–6], nucleosides[1,3–7], mono-[4–6,8–10] and oligonucleotides[11–14]. A number of authors have reported the RP-HPLC separation of chemically modified monomeric components of nucleic acids[2–4,7,8,10]. Yet, the possible uses of RP-HPLC for the separation of modified oligonucleotides have not been extensively studied what has been described is the separation of protected oligonucleotides[11,15,16], oligonucleotides with non-canonical phospodiester (2′-5′) bonds[17,18] or etherified internucleotide phosphate groups[19,20], and oligonucleotides containing O^6-methylguanine[21] and C^2-aminoadenosine[22].

It is worthwhile trying to obtain oligonucleotides with a modified chemical structure, since they can be used as convenient models for mutation and repair studies and to investigate the effect of the chemical structure of nucleic acids on their conformational properties.

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One of the most interesting and biologically significant types of modification is the photochemical modification of nucleic acids resulting in various photoproducts of bases most of which are represented by cyclobutane[23] and non-cyclobutane[24] dimers of pyrimidines. The key rôle of these photoproducts in UV mutagenesis has been demonstrated with the help of modern sequencing techniques[25,26]. The RP-HPLC separation of some photomodified bases and nucleosides including thymine dimers has been described in several papers[27–29]. Photomodified oligonucleotides containing thymine dimers were for the most part separated by means of paper chromatography[30–33], size-exclusion chromatography[34] and paper electrophoresis[30,31]. However, size exclusion and electrophoresis do not ensure sufficient resolution. Electrophoresis and paper chromatography require radioactively labelled samples and a long time. Moreover, since it is impossible to establish directly the characteristic spectral and other features using the latter two methods, additional procedures, such as the elution of the substance from the carrier, are required for the preparation and identification of photoproducts. Time-consuming elution is also required in the case of preparative separation.

The present study deals with the RP-HPLC separation of short (n = 2–4) oligodeoxyribonucleotides* and their photomodification products containing various types of thymine dimers. The separation is fast, and the UV detection at several wavelengths enables the type of separated photoproduct to be identified directly, but requires only a small sample (less than 1 μg).

MATERIALS AND METHODS

Equipment

The chromatographic separation was based on a gradient system (Model 850; DuPont, Wilmington, DE, U.S.A.) comprising a pump, a microprocessor, a Zorbax ODS column (4.6 × 250 mm) with a Permaphase ODS precolumn (4.6 × 50 mm), a UV detector with wavelength fixed at 254 nm, a UV spectrophotometer and a two-channel recorder. Samples were introduced into the column by means of a Rheodyne 7125 injector with a 50-μl loop. A germicidal resonance low-pressure mercury lamp (254 nm, 15 W) was used for the UV irradiation of oligonucleotides.

Chemicals and oligonucleotides

Methanol, monoammonium phosphate and ammonium acetate were products of Reachim (U.S.S.R.). Methanol was purified further as described[35] to give the UV cut-off of 210 nm. The water–salt solutions were passed through a short Permaphase ODS column (4.6 × 100 mm) prior to chromatography.

Oligonucleotides synthesized by standard di- and triester procedures were obtained from the laboratories of Dr. V. D. Smirnov (Institute of Virology, Moscow), Professor Z. A. Shabarova (Moscow State University) and Dr. V. N. Nezavibatko (Institute of Molecular Genetics). The oligonucleotides were purified by RP-HPLC prior to the assays. Snake venom phosphodiesterase I from *Crotalus admanteus* (Worthington, Freehold, NJ, U.S.A.) was used.

* We shall denote oligonucleotides using IUPAC–IUB notation. The symbol d (deoxy) is omitted for sake of brevity.

Preparation of eluents and samples

For mobile phases, 50 m*M* $NH_4H_2PO_4$ (pH adjusted to the desired value with 40% water–ammonia) and pure methanol (system 1) or 0.12 *M* ammonium acetate (pH 6.8) and 0.1 *M* ammonium acetate in water–methanol (1:1) (system 2) were used. The oligonucleotides were dissolved in water or water–salt solution to a concentration of several OU/ml and UV irradiated in quartz cells at about 50 kJ/m^2. The solutions were not deaerated prior to the irradiation. The $(pT)_3$ oligonucleotides containing photoproducts were separated in an acetate buffer and then hydrolyzed with snake venom phosphodiesterase in a buffer containing 0.11 *M* Tris–HCl (pH 8.9), 0.11 *M* NaCl and 15 m*M* $MgCl_2$ as described[36].

Chromatographic conditions

The chromatographic separation was performed at room temperature. A linear gradient of the B solvent containing methanol in the A solvent ($NH_4H_2PO_4$ or CH_3COONH_4) was generated at a flow-rate of 1 ml/min. Gradient profiles are shown as dashed lines in the figures.

RESULTS AND DISCUSSION

The simplest oligonucleotide which can form thymine dimers is thymidylyl-(3′-5′)-thymidine (TpT). The UV irradiation of oligonucleotides is known to yield two types of thymine dimers as major photoproducts: cyclobutane and non-cyclobutane ones[23,24,30,31]. Both types show[23,24] considerable absorption at 230 nm (comparable to thymine absorption), insignificant absorption at 254 nm and only non-cyclobutane dimers show a noticeable absorption at wavelengths above 310 nm.

Fig. 1 shows a chromatogram of a TpT sample after UV irradiation (254 nm) recorded at three wavelengths. At 230 nm one observes several peaks corresponding to photoproducts and the original substance. We attribute the two major photoproduct peaks (1 and 2 in Fig. 1) to oligonucleotides with different types of dimers (see below). The minor peaks may be due to other minor photoproducts. In particular, one of them may be the photolysis product of the non-cyclobutane dimer[31]. At 254 nm only the original substance gives a clear-cut peak (3). The absence of peaks 1 and 2 from Fig. 1b is consistent with the dimeric nature of the photoproducts concerned; it also demonstrates that the widely used detection at 254 nm is inadequate in this case. At 320 nm only one of the photoproduct peaks can be clearly observed (peak 2). Since the substance shows absorption both at 230 nm and at 320 nm, we attribute this peak to the non-cyclobutane type of dimer. Consequently, peak 1 must correspond to the cyclobutane dimers. To test this we made use of photoreversibility, which is exhibited by cyclobutane but not by non-cyclobutane dimers[23]. The isolated component corresponding to peak 1 was chromatographed under the same conditions after UV irradiation. Detection at 230 nm again revealed three peaks (not shown), peak 3 (the original TpT) resulting from the photoreversion of the cyclobutane dimers and peak 2 arising as a result of the formation of non-cyclobutane dimers from photoreversed TpT.

Thus, we have used the characteristic properties of the dimers to identify the peaks corresponding to them in the chromatogram. It should be noted that the spectral properties of the dimers are sufficient for the correct identification of their peaks.

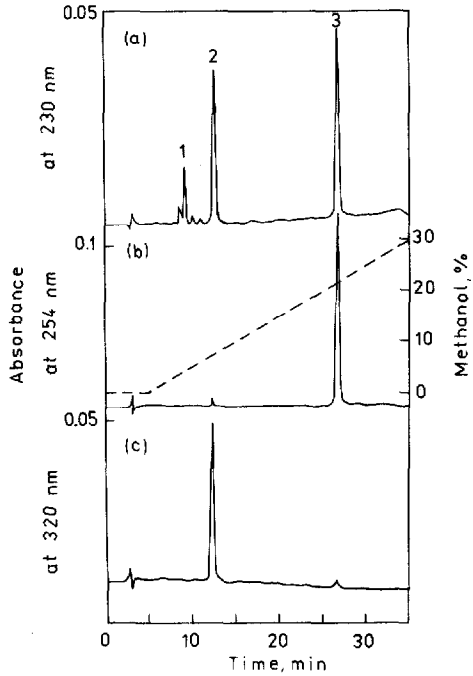


Fig. 1. Chromatogram of TpT after UV irradiation at 254 nm. Column: Zorbax ODS (4.6 × 250 mm). Eluents: A, 50 m*M* $NH_4H_2PO_4$ (pH 5.8); B, methanol; gradient, 0–30% B as shown by the broken line in Fig. 1b; flow-rate 1 ml/min. Peaks: 1 = cyclobutane dimer of TpT; 2 = non-cyclobutane dimer of TpT; 3 = unmodified TpT.

Separation at several pH values has shown that the resolution and the retention time of the individual components vary only slightly (Table I). The reason for this is that dinucleoside monophosphate TpT does not change its charge in the range pH 4.0–7.3. It is interesting therefore to look at the effect of a terminal phosphate group, *e.g.*, at the 5′ position, on resolution, since such a group confers an additional charge at pH <6.5 and two charges at pH >6.5.

Fig. 2 shows the separation of $(pT)_2$ and its photoproducts at pH 4.0. The data on separation at two other pH values are given in Table I. As expected, a higher pH results in a reduction of the retention times both for the original dinucleotide and for its photoproducts. Note that the individual components are well resolved at all the pH values tested. The peak width on the time scale does not exceed 1 min, ensuring virtually baseline resolution even for closely located dimer peaks. The peaks were identified in this and subsequent cases in the same way as with TpT. We should note, however, that in this separation the order of appearance of dinucleotides containing cyclobutane and non-cyclobutane thymine dimers was reversed as compared with TpT at all pH values.

It seems that the terminal phosphate group of the $(pT)_2$ dinucleotide containing a non-cyclobutane dimer is more accessible to interaction with the solvent than in the case of a cyclobutane dimer. Therefore the introduction of a 5′-terminal phosphate group in TpT leads to a somewhat greater reduction of the retention time for the dinucleotide with a non-cyclobutane dimer than for one containing a cyclobutane

TABLE I

RETENTION TIMES (MIN) FOR OLIGONUCLEOTIDES AND THEIR PHOTOPRODUCTS CONTAINING THYMINE DIMERS

Eluents: A, 50 m*M* $NH_4H_2PO_4$ (different pH values); B, methanol; gradient, 0% B for 4.5 min, then B increased at a rate of 1% per min. The elution time of an unretained sample is 2.7 min. For other conditions see Materials and methods.

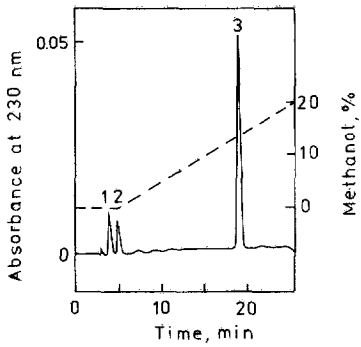
	pH 4.0			*pH 5.8*			*pH 7.3*		
	*a**	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
TpT	9.4	12.2	26.5	9.4	12.3	26.9	9.0	12.2	26.6
$(pT)_2$	5.0	4.2	19.9	4.6	3.6	18.1	4.2	3.4	16.9
$(pT)_3$	15.1	18.1	29.9	15.4	18.0	29.4	14.9	17.5	28.2
	16.5	19.9		16.8	19.8		16.1	19.2	
TpTpA	16.9	19.2	29.2	17.1	19.7	29.3	17.0	18.8	28.6
TpTpGpG	17.1	18.5	26.1	17.0	18.4	25.8	16.2	17.4	24.9
GpGpTpT	18.5	21.1	27.0	18.4	21.0	26.7	17.6	20.1	25.8

* a = Oligonucleotides with cyclobutane thymine dimers; b = the same with non-cyclobutane dimers; c = unmodified oligonucleotides.

thymine dimer, so that their elution order is reveersed. This order of photoproduct elution is characteristic only of $(pT)_2$; in all the other cases (see Tables I and II) the oligonucleotides containing cyclobutane dimers are eluted first.

As one goes from di- to longer oligonucleotides one gets an opportunity to change the separation of the original compound and its photoproducts. This is due, first, to the presence of the longer chains and, secondly, to the appearance of bases other than thymine. Table I lists the retention times for oligonucleotides having different sequences and for their photoproducts containing thymine dimers. In the tested pH range (4.0–7.3) the separation is complete in all cases. Figs. 1–3 are characteristic examples. One can detect photoproducts in these oligonucleotides at 254 nm since, apart from the highly dimerized pair of thymines with strong absorption only around 230 nm, they have additional bases which show appreciable absorption around 254 nm.

Fig. 3 shows the separation of $(pT)_3$ and its major photoproducts. Dimers of both types can form at either end of this trinucleotide. As the chain is polar, the photoproducts containing dimers of the same type at the 5′ and 3′ ends are not equivalent, so that with sufficient resolution the chromatogram must have five major peaks. Fig. 3 shows this to be the case; hence the change in the chromatographic mobility of an oligonucleotide upon thymine dimerization depends on the position of the dimer as well as its type. Trinucleotides containing cyclobutane dimers at different positions in the molecule (peaks 1 and 2 in Fig. 3) have the shortest retention times; trinucleotides with non-cyclobutane dimers (peaks 3 and 4) are eluted next. To identify the positions of thymine dimers relative to the chain ends of photomodified $(pT)_3$, we treated the isolated trinucleotides corresponding to the various peaks in Fig. 3 with snake venom phosphodiesterase, which hydrolyses oligonucleotides from the 3′ end to mononucleotides. The phosphodiester bond in thymine dimers is known to be resistant to this enzyme[31], so that only trinucleotides with the 5′-terminal position of the dimer are subject to hydrolysis. The results of the enzymatic



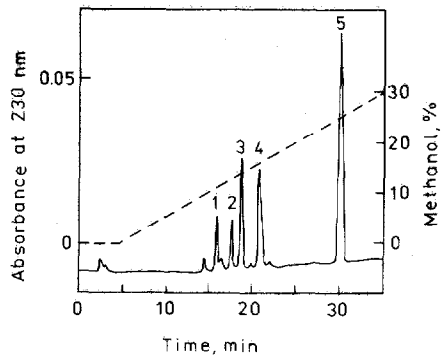


Fig. 2. Chromatogram of $(pT)_2$ after UV irradiation at 254 nm. Eluents: A, 50 m*M* $NH_4H_2PO_4$ (pH 4.0); B, methanol; gradient, 0–20% B as shown by the broken line. Column and other conditions as in Fig. 1. Peaks: 1 = non-cyclobutane dimer of $(pT)_2$; 2 = cyclobutane dimer of $(pT)_2$; 3 = unmodified $(pT)_2$.

Fig. 3. Chromatogram of $(pT)_3$ after UV irradiation at 254 nm. Conditions as in Fig. 2, except the gradient of 0–30% B (– – –). Peaks: oligonucleotides with cyclobutane dimers at the 5′ end (1) and at the 3′ end (2); the same with non-cyclobutane dimers at the 5′ end (3) and at the 3′ end (4); 5 = unmodified $(pT)_3$.

hydrolysis show that thymine dimers in the 5′-terminal position change the oligonucleotide's retention time to a greater extent than do 3′-terminal dimers of the same type.

Lines 4–6 in Table I refer to the separation of oligonucleotides containing bases other than thymine. By virtue of the greater stability of purines compared with pyrimidines[37], here again thymine dimers are the major photoproducts at the radiation doses used. The separation of photoproducts containing dimers of different types from each other and from the original oligonucleotide is again complete.

We should note that the shift of the photoproducts relative to the original substance in the chromatogram is not the same for TpTpGpG and GpGpTpT sequence isomers. For TpTpGpG, where the thymine dimer forms at the 5′ end, the change in the photoproduct retention time is greater than for GpGpTpT, where dimerization occurs at the 3′ end. This is consistent with the above data in that in $(pT)_3$ a thymine dimer forming at the 5′ end causes a greater reduction of the retention time than does a 3′-terminal dimer.

All the results reported so far refer to separation in a phosphate buffer at different pH values. Chromatography in an acetate buffer (pH 6.8), which allows the desalting of samples by lyophilization, is also characterized by a complete resolution of all components. Data on the separation of various oligonucleotides and their photoproducts in an acetate buffer are listed in Table II. The slight broadening of the peaks as compared with the separation in a phosphate buffer should be noted.

The results of this study show that the use of RP-HPLC allows a rapid analytical and preparative high-resolution separation of oligonucleotides containing thymine dimers of different types. The experiments performed on $(pT)_3$, TpTpGpG and GpGpTpT show the separation to be also sensitive to the dimer's position in the molecule. Simultaneous UV detection at several wavelengths obviates radioactive labelling of samples, and allows a direct identification of the photoproduct separated, only small sample amounts being required (less than 1 μg). Oligonucleotides containing dimers have shorter retention times than do the original substances, which

TABLE II

RETENTION TIMES (MIN) FOR OLIGONUCLEOTIDES AND THEIR PHOTOPRODUCTS CONTAINING THYMINE DIMERS

Eluents: A, 0.12 *M* CH_3COONH_4 (pH 6.8); B, 0.1 *M* CH_3COONH_4 in water–methanol (1:1); gradient 0% B for 4.5 min, then B increased at a rate of 2% per min. For other conditions and notation see Materials and Methods and Table I.

	a	*b*	*c*
TpT	6.8	10.3	25.4
$(pT)_2$	3.8	3.3	16.9
$(pT)_3$	13.8	16.0	28.1
	15.0	18.0	
TpTpA	16.8	18.1	28.2
TpTpGpG	16.2	17.3	25.2
GpGpTpT	17.4	20.2	26.3

is probably due to the decrease in hydrophobicity of a pair of thymines on dimerization. The retention times of the photoproducts differ considerably from that of the original oligonucleotides (by more than 10 min for dinucleotides and more than 5 min for tetranucleotides). As the chain length increases further, this difference must diminish. At the same time the peaks corresponding to individual components are about 1 min wide. One can hope therefore that the separation of the original and photomodified oligomers will be good enough for long oligonucleotides (about ten bases).

The change in chromatographic mobility on dimerization supplies information on the presence or absence, position and number of neighbouring thymines, which in some cases may suffice to identify the oligonucleotide without direct sequencing or the use of chromatographic standards.

The isolation of photomodified dimer-containing oligonucleotides is certainly worthwhile as oligonucleotides are convenient models for the study of normal-intensity UV radiation effects on nucleic acids, specifically with regard to thymine dimerization, which is one of the most important photochemical processes[23,38]. It can also be used in the study of the immune[39] and repair[32–34] responses of the cell to UV irradiation. The procedure described does not require a preliminary severe acid hydrolysis[29,39] for quantitation and enables the modified oligomers to be analysed directly. The photochemistry and photobiology of nucleic acids have recently received an impetus as double-quantum processes have been investigated[40–42]. One hopes that the results of the present study might also be useful in that area of research.

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