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Base composition analysis of phosphorothioate oligomers by capillary gel electrophoresis

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Abstract

The base composition analysis of synthetic oligodeoxyribonucleotides (ODN) by high-performance capillary gel electrophoresis (HPCGE) is reported here. This newly developed method is fast, sensitive and highly reproducible. It involves the separation of deoxyribonucleotides monophosphate (dNMPs) by capillary gel electrophoresis using a 47 cm×100 μm I.D. capillary and the samples were run at constant voltage (isoelectrostatic) and constant temperature (isothermal). We evaluated this technique by using commercially available dNMPs as well as dNMPs generated from enzymatic digestion of phosphorothioate ODNs. Our results indicated (a) picomoles of sample are sufficient, (b) the migration times of each of the four bases is less than 25 min, (c) the calculated empirical formula values of parent compound are matched up with the expected values, and (d) the results are highly reproducible. Thus we have developed an alternative method for the base composition analysis of synthetic ODN which we believe has the potential to become the method of choice in the future.

Keywords: Phosphorothioate oligomers; Oligodeoxyribonucleotides

1. Introduction

Synthetic oligodeoxyribonucleotides (ODN) and their analogs have attracted considerable interest in recent years due to the possibility of their practical application as a potential diagnostic tool as well as selective therapeutic agents [1–3]. Due to increased number of ODN based drug candidates entering late stage clinical trials (phase II–III), there is a need for a method which is faster, sensitive and reproducible compared to traditional sequencing [4,5] and HPLC [6] methods, as well as newly improved versions of them [7,8] to identify its components. Three drawbacks of the present techniques are, (a) need for large amounts of samples, (b) reagents and (c) turn around time. In this report, we show the preliminary results of base composition analysis of phosphorothioate ODNs which were subjected to enzymatic digestion to generate deoxyribonucleotides monophosphate (dNMPs) and subsequently analyzed using high-performance capillary gel electrophoresis (HPCGE). In our continuing effort to developing analytical methods for identification and quantitation of dNMPs, we have successfully employed HPCGE which has become a very powerful analytical tool in biochemistry and molecular biology [9–13]. It has a number of similarities to HPLC but can be performed without the use of large amounts of samples and high purity solvents. By using HPCGE, the base composition analysis of six different digested ODNs showed the calculated empirical formula values are in reasonably good agreement with expected values. This method

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has demonstrated a promising way to routinely analyze synthetic ODNs' components.

2. Experimental

2.1. Apparatus

The experiments were done on the P/ACE System 5000 capillary electrophoresis apparatus (Beckman Instrument, Fullerton, CA, USA) with reversed polarity (cathode on inlet and anode on outlet). The samples were separated on eCap ssDNA 100 (Beckman) capillary with 47 cm (effective length at 40 cm)×100 μm I.D. and monitored at a fixed wavelength of 260 nm. The capillary was immersed in 5 ml of filtered (0.2 μm pore) buffer (Tris–boric acid, 44:56 (v/v) 7 *M* urea, pH=8.0, obtained from Beckman) and run at constant temperature of 30°C by using the P/ACE cooling system. The buffer was replaced every ten runs to obtain reproducible results. The data were collected by IBM 433Dx/si and analyzed by System Gold software (Beckman).

2.2. Procedures

Synthesis of ODNs

Phosphorothioate (PS) ODNs were synthesized on Expedite using betacyanoethyl phosphoramidite (Perseptive Biosystems, Framingham, MA, USA) chemistry and purified by HPLC as described [14,15]. Note that the sulfurizing reagent, Beaucage, was obtained from American International Chemical (Natick, MA, USA).

Enzymatic digestion of PS-ODN

First PS-ODN was converted to phosphodiester (PO)ODN by desulfurization as described [16]. After ether extraction, the aqueous phase was evaporated to 200 μl in Savant speed-vac and ethanol precipitated on dry ice for 20 min. After centrifugation at 15 800 *g* for 10 min at 4°C, the supernatant was removed, 225 μl water added to the pellet, followed by 25 μl of 3 *M* sodium acetate and 700 μl of ethanol to effect precipitation. The mixture was chilled, re-spun, decanted and put on a speed-vac to dry the pellet, then resuspended in 20 μl water. One A_{260} unit of ODN was used in enzymatic digestion as follows. In 55 μl total volume of reaction mixture, 3 μg of snake venom phosphodiesterase (1 mg/0.5 ml, Boehringer Manheim, Indianapolis, IN, USA), 32 m*M* Tris (Sigma) buffer at pH 7.5 and 18 m*M* of magnesium chloride (Sigma) were used and the digestion was incubated in a water bath at 37°C overnight. After digestion, precipitation of the nucleotides was carried out by adding 7 μl of 3 *M* sodium acetate and 155 μl of ethanol and chilled on dry ice for 10 min. The DNMPs were recovered by centrifugation at 15 800 *g*, 4°C for 10 min and the supernatants containing DNMPs were carefully removed and placed on a Savant speed-vac to dry, then resuspended in 125 μl of sterile water for CE analysis.

Quantitation of HPCE data for base composition

The empirical formulas were calculated for ODNs based on DNMPS standard curves as described in [8]. The DNMPs for standard solution were obtained from Peninsula Laboratory and standard solutions (from 0.625 to 10 pmol/μl) were prepared in water. The concentrations were calculated based on extinction coefficients reported by the manufacturer.

3. Results and discussion

3.1. Injection parameters

Recent study by McLaughlin et al. [17] on the effect of internal diameter (I.D.) of capillary concluded that larger I.D. resulted in increased concentration sensitivity as a consequence of large volumes of sample being handled. Therefore, a 47 cm length (effective length 40 cm)×100 μm I.D. capillary was chosen. The injection parameters were optimized and used at 5 kV, 20 s, 50 μl total volume (data not shown) throughout this report. To our knowledge, the exact amount of sample injected by electrokinetic injection has not been previously quantified. In an attempt to find out how much was loaded by electrokinetic injection, under the set of parameters used in this study, 1 pmol/50 μl of NUC2 digested sample was injected 15 times. Since the intensity of the signal at fifteenth run was as strong as the first run, it was concluded that less than 0.67 mol of sample was loaded onto the capillary.

3.2. Base resolution

Fig. 1 shows the electropherograms of a mixture of all four dNMPs at constant voltage and temperature. Isoelectrostatic and isothermal conditions are necessary to achieve fine resolution [18]. The electropherograms also show the order of migration of dNMP (C<G). The identity of the dNMPs were confirmed by running the dNNPs separately under the same set of parameters. The relative order of migration of nucleotides (C=307<T=322<A=331<G=347) depends on shape, size and charge [18] of nucleotides. In this case, it is interesting to note that the pyrimidine nucleotides migrate faster than the purine nucleotides. Since the charge on the dNMPs are the same, one possible explanation for

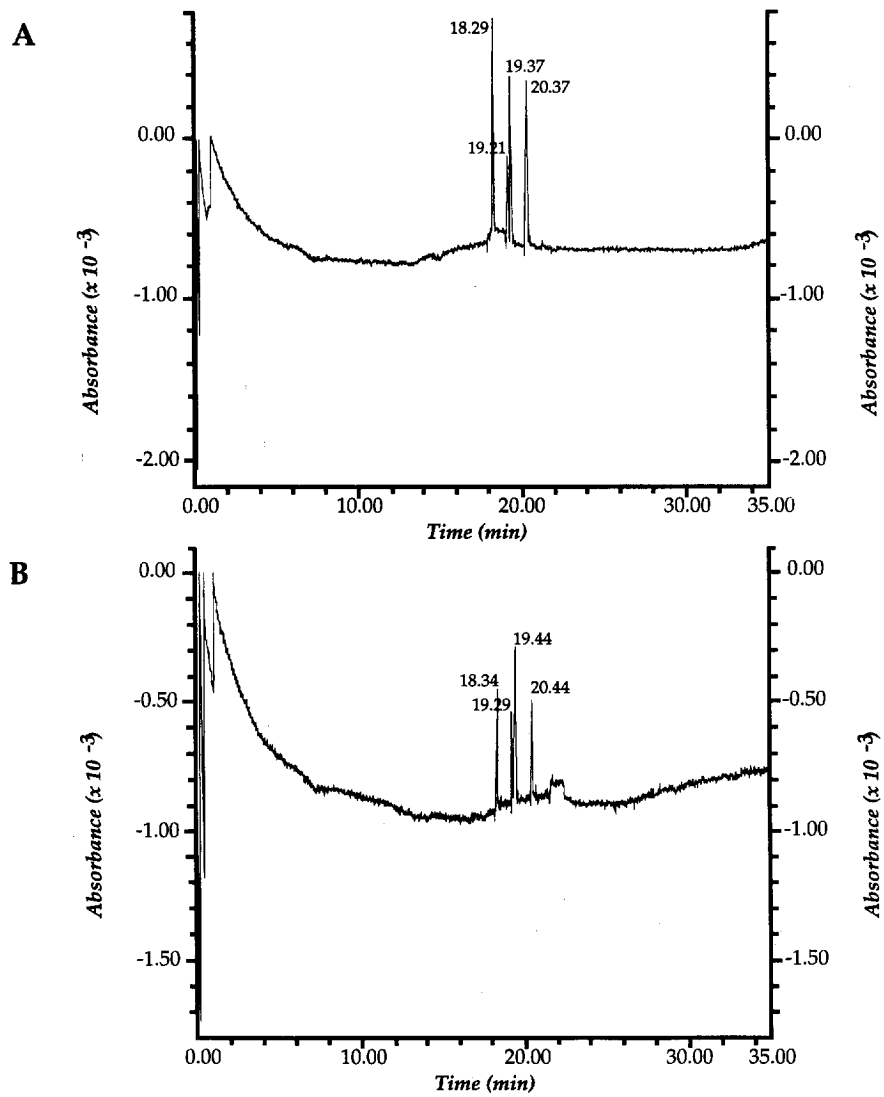

Fig. 1. Capillary gel electrophoretic separation of deoxynucleotide monophosphate (dNMP). (A) Mixture of dCMP, dTMP, dAMP and dGMP obtained from Peninsula Lab. Migration times are, dCMP=18.29, dTMP=19.21, dAMP=19.37, dGMP=20.37. (B) Digested phosphorothioate oligomer. Migration times are, dCMP=18.34, dTMP=19.29, dAMP=19.44, dGMP=20.44. Conditions: Isoelectrostatic (constant voltage) at 300 V/cm; isothermal (constant temperature) at 30°C; injection at 5 kV, 20 s; detection range (AU) at 0.005.

the observed migration pattern could be attributed to the difference in their molecular masses. It should also be noted here that a minimum of nine relative molecular mass difference between dTMP and dAMP is necessary for base resolution under the current set of parameters.

The comparison of the migration times of commercially available dNMPs (Fig. 1A) versus digested PS-ODN (Fig. 1B) is in good agreement (±0.07 min or 0.4%). Furthermore, the analysis of the nucleotides was completed in less than 25 min. In an effort to reduce the analysis time further, the constant voltage was increased to 20 kV (426 V/cm) and an additional 5 min was removed from the analysis time. Even though it was possible to reduce the analysis time by increasing the applied electric field, it is not recommended for routine analysis, because increasing the applied electric field causes the gel in the capillary to break down more frequently. As a result, fewer runs can be performed using the same capillary.

3.3. Standard curves

A standard curve for each of the dNMPs was obtained over 16-fold range of concentrations (0.625–10 pmol/μl). By using linear regression method to analyze the data, these standard curves are represented by the following equations (a) for dCMP ($y=-0.176+5.408x$, $R=0.9981$); (b) for dTMP ($y=-0.175+5.060x$, $R=0.9974$); (c) for dAMP ($y=-0.175+3.561x$, $R=0.9988$); and (d) for dGMP ($y=-0.164+3.148x$, $R=0.9986$). The R values (correlation coefficient) are approaching 1.0, which means the standard curves have a good correlation between the peak areas and the nucleotide concentrations. These curves were used to quantitate the amount of each nucleotide present in the sample obtained from the enzymatic digestion of phosphorothioate ODNs [8]. The slope of the standard curve multiplied by the peak area corresponding to each dNMP yields the amount of nucleotide in moles corresponding to that peak. The empirical formula was calculated by multiplying the length of the oligomer (26) by the ratio of the calculated number of moles of the given nucleotide to the calculated total number of moles of all nucleotides in the sample. The results are shown in Table 1.

Table 1
Summary of digested phosphorothioate oligomers base composition analysis

Sequence name	Empirical formula[a]							
	dCMP		dTMP		dAMP		dGMP	
	E	Ca	E	Ca	E	Ca	E	Ca
NUC3	13	12.98	6	5.11	6	6.70	1	1.20
NUC4	12	11.83	6	5.20	6	6.54	2	2.42
NUCS	11	10.74	6	5.11	6	6.49	3	3.66
NUC6	10	9.59	6	5.39	6	6.47	4	4.56
NUC2	7	6.81	6	5.24	6	6.33	7	7.62
Gps0193	10	9.51	6	5.57	0	0	10	10.92

[a] E=Expected; Ca=Calculated.

3.4. Analysis of base composition

Six PS-ODNs were first converted to PO-ODNs, then enzymatically digested with SVP to generate nucleotides. The recovery rates of nucleotides following the above protocol with 1, 2 and 3 A_{260} of PS-ODN were >90%, 60% and 65% respectively (data not shown). The low recovery from the enzymatic digestion of 2 and 3 A_{260} of PS-ODNs may due to insufficient amount of enzyme and/or time necessary for complete digestion were not optimized. However, the current method (HPCGE) is extremely sensitive and digestion of 1 A_{260} unit of an oligomer was sufficient to carry out the base composition analysis. A typical electropherograms of digested phosphorothioate oligomers are shown in Fig. 2. The detection of 2 G out of 26 bases (7.7%) and 7 G out of 26 bases (26.9%) are shown in Fig. 2A and B. The sensitivity of the current method is evidenced by sharp peaks and low noise level. Additionally, Table 1 shows that even a single nucleotide (G) out of 26 bases (3.8%) could be easily detected by this method. The data shown in Table 1 represents the average of 3 runs for each of digested ODN and is highly reproducible as indicated by very low standard deviation (from ±0.4 to 2.8%). Thus, the method shows the high reproducibility and sensitivity that are required for analytical methods. Though the calculated values in Table 1 show some discrepancies from the expected values, most are still within the acceptable range of ±0.5 base for each nucleotide component [8]. From Table 1, one could observe that there are definitely trends involved in the

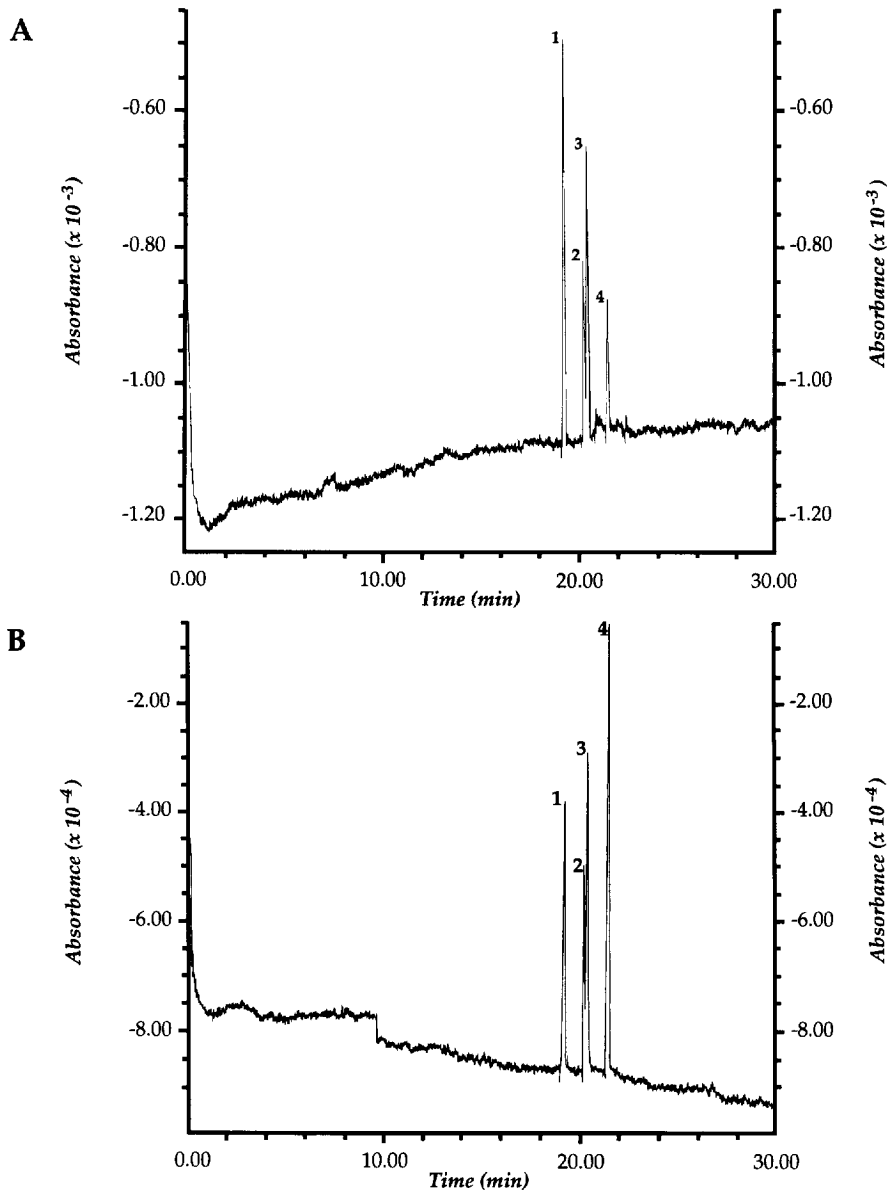

Fig. 2. Electropherograms of digested phosphorothioate oligomers (10 nmol/50 μl). Peak 1=dCMP, Peak 2=dTMP, Peak 3=dAMP, Peak 4=dGMP. (A) NUC4: 2 G out of 26 bases. (B) NUC2: 7 G out of 26 bases. Conditions as in Fig. 1.

calculated values. The trends show that dCMP, dTMP (pyrimidines) are underestimated where as dAMP, dGMP (purines) are overestimated. Further refinements in the assay protocol are currently under way to address the small differences seen between the calculated and expected values.

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