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Capillary gel electrophoresis and the analysis of DNA phosphorothioates

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ABSTRACT

Capillary gel electrophoresis (CGE) is recognized as an effective method for the analysis of normal phosphodiester deoxyoligonucleotides (DNA). However, it was found that when the same electrophoretic principles were applied to the analysis of phosphorothioate DNA, peak broadening due to the phosphorothioate moiety made CGE's utility questionable. Therefore, optimization of several parameters is necessary for CGE to be effective in leading to the precise and accurate analysis of phosphorothioate DNA. Phosphorothioates are oligonucleotide analogs acknowledged as potential antisense therapeutics in the treatment of viral diseases and certain cancers. In order to improve a CGE approach to the analysis of this class of therapeutics, several parameters were investigated and improved upon. Three factors which proved critical in providing high resolution and good gel to gel reproducibility were: gel concentration, buffer additives, and pH.

INTRODUCTION

The advantages of capillary gel electrophoresis (CGE) in the analysis of synthetic oligonucleotides lie not only in its potential to resolve single base deletion products or failure sequences from the full-length oligonucleotide, but also in its capability to automate the electrophoretic analysis of these compounds in general [1,2]. However, in order for automated electrophoresis to occur via CGE, a gel-filled capillary must first prove itself resilient and at the same time, give reproducible results from run to run.

It is generally recognized that 7 to 8 *M* urea used as a denaturant in gel electrophoresis will denature all intermolecular secondary structures [3]. In lieu of high urea concentration, conducting CGE at elevated temperatures in conjunction with a rigid gel matrix provides adequate denaturing for the analysis of normal phosphodiester oligonucleotides. However, it was found that in the analysis of phosphorothioates these measures were not enough to overcome peak broadening that results in the loss of resolution between thioates differing in length by one base.

It has since been found that the pH of the buffer system has significant effects on the migration properties of oligonucleotides within a capillary gel [4,5]. Therefore, in the absence of a denaturing agent like urea, it is believed that by simply raising the pH of the buffer system, an effective denaturing gel could be achieved. We shall demonstrate that optimization of gel buffer pH, buffer concentration, and gel composition can lead to improved CGE performance in the separation of phosphorothioate DNA differing only by a single base in length, and to longer gel working lifetimes.

MATERIALS AND METHODS

Synthesis and purification of oligos

Phosphorothioate oligonucleotides were chemically synthesized using proprietary methods developed by Lynx Therapeutics on an Applied Biosystems (ABI, Foster City, CA, USA) 380B DNA synthesizer [6]. Truncating particular mixed base sequences by one base increments at the 3′ end created representative "failure" sequences. After these sequences were

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synthesized, cleaved off the solid support, and deprotected, the resulting crude solutions were purified on a Perkin-Elmer (Norwalk, CT, USA) HPLC system (Series 4) using an ABI 1783A detector and a Polymer Labs. (Amherst, MA, USA) PLRPS reversed-phase column (150 × 4.6 mm I.D., 8 μm). Chromatographic conditions were as follows: buffer A was 2% acetonitrile, 0.1 *M* triethylammonium acetate (TEAA) pH 7.5; buffer B was 100% acetonitrile; flow-rate of 1 ml/min. A linear gradient of 5–35% buffer B over 35 min was applied, and Trityl-on DNA (phosphorothioate), monitored at 300 nm, was collected at approximately 25 min. The purified DNA was detritylated with 80% acetic acid (*ca.* 5 ml total volume) for 15 min, dried to a residue under vacuum, and reconstituted in 1–2 ml NH_4OH (10%). An extraction was then carried out with ethyl acetate (2–4 ml) to remove free trityl group. The aqueous layer was retained, evaporated under a vacuum to a residue, and reconstituted in 1 *M* NaCl (1–2 ml). Three ethanol precipitations were done [7 parts ethanol to 3 parts NaCl (1 *M*)] to rid DNA of all organic solvents used in its synthesis and HPLC purification. The resulting purified "failure" sequences were mixed together in equimolar amounts along with their parent full-length oligo, and these "mixes" were used as standards to measure the resolution capabilities and gel-to-gel reproducibility of the different gel-filled capillaries.

Capillary electrophoresis

All capillary electrophoresis was conducted with an ABI 270A instrument. Capillaries (50 cm (30 cm to detector window) × 100 μm I.D. purchased from Polymicro Technologies (Phoenix, AZ, USA), were filled with ABI's Micro-Gel™ [7], and various buffer systems were used. All buffer reagents were purchased from Aldrich (Milwaukee, WI, USA). Runs were conducted with (−) polarity and a constant voltage of 22 kV was applied unless otherwise indicated. Injections were electrokinetic for 5.0 s (−8 kV). Detection was set at 260 nm, and temperature was kept at 55°C. Sample concentrations (total absorbance units/ml at 260 nm) were pre-determined and set at 0.5 absorbance units/ml unless otherwise indicated. The equation $R_s = 2(t_y - t_x)/w_y + w_x$ was used to determine resolution (R_s) between peaks, where y and x refer to any two peaks of interest, t is retention time in decimal min, and w is peak width in decimal min at the base.

RESULTS AND DISCUSSION

We evaluated several CGE parameters, including (1) buffer concentration, (2) buffer pH, (3) gel concentration, and (4) organic additive, and these systems are summarized in Table I. Figs. 1 and 2 illustrate the results of two different gel concentrations and two different buffer systems. In system I (Fig. 1), methanol is used as a preservative; however, these gels gave results that varied from gel to gel, and they often did not last beyond a few runs. It was soon discovered that the relatively high buffer concentration (75 m*M*) was contributing to excessive Joule heating within the gel, which resulted in premature gel failure. Thus, the buffer concentration was diluted to 35 m*M* and methanol was replaced with ethylene glycol (EG) (system II, Fig. 2). EG served not only as a preservative but, due to its viscosity, also produced a more rigid gel that was less likely to form voids or air bubbles. Neither of these systems gave sufficient resolution ($\bar{R}_s = 0.84$), and they were deemed inefficient gel capillaries for the analysis of phosphorothioates.

After it was discovered that lowering the buffer concentration increased the lifetime of the gel, improving the resolution of the gel was the next area of focus. It was felt that increasing the gel concentration would enhance peak resolution, and Fig. 3 is an example of a 10% gel (system III). Gels of 11 and 12% were also prepared, but they were prone to instability and air bubbles often developed. Although resolution ($\bar{R}_s = 1.07$) did improve with gels of higher concentration, the goal was to obtain gel-filled capillaries that would at least give near baseline resolution ($R_s \geq 1.2$).

As mentioned earlier, the pH of the buffer system has a direct effect on capillary-gel performance [4,5]. It has also been found that intramolecular secondary structure within single stranded DNA is not easily denatured even with

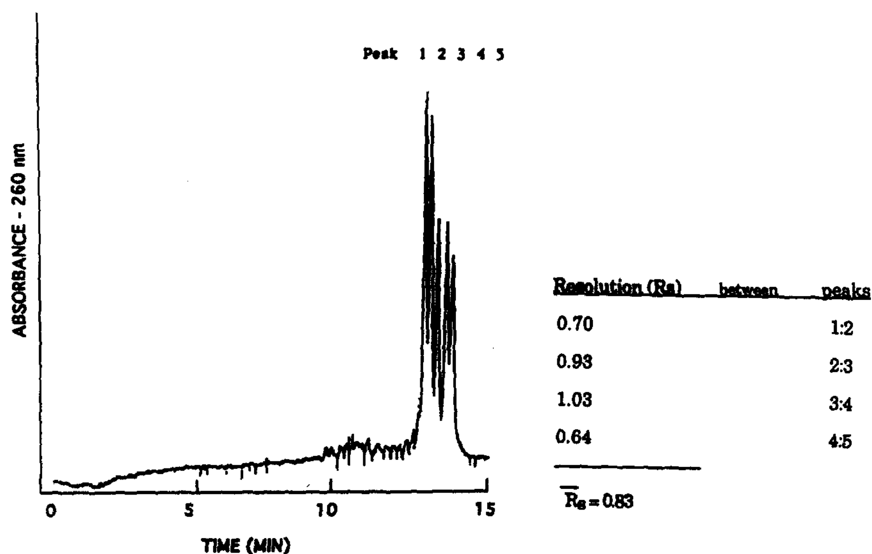


Fig. 1. Electropherogram of a mixture of different length phosphorothioates (22–26-mer). Sample concentration was 0.1 absorbance units/ml. Electrophoresis was conducted with an electrokinetic injection at −5 kV for 5 s, a running voltage of −10 kV, and buffer system I.

7 to 8 *M* urea [8,9]. Conceivably, intramolecular secondary structure, caused primarily by H-bonding, contributes to peak broadening and the loss of resolution which was illustrated in Figs. 1, 2 and 3. It was concluded that buffers of higher pH (>8.0) should help to denature intramolecular secondary structure caused by H-bonding.

The first attempt at a higher pH gel involved

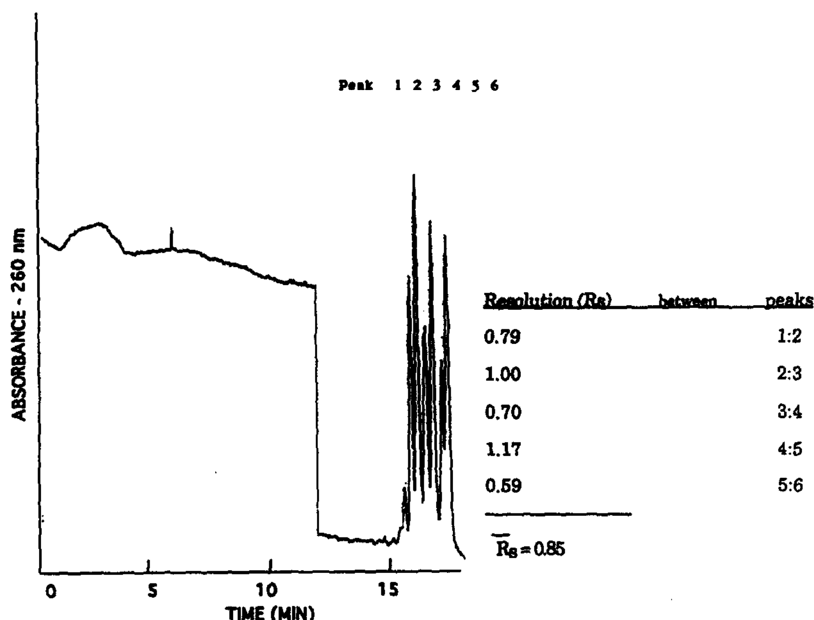


Fig. 2. Electropherogram of a mixture of different length phosphorothioates (16–21-mer). Sample concentration was 0.5 absorbance units/ml. Electrophoresis was conducted with an electrokinetic injection at −8 kV for 5 s, a running voltage of −22 kV, and buffer system II.

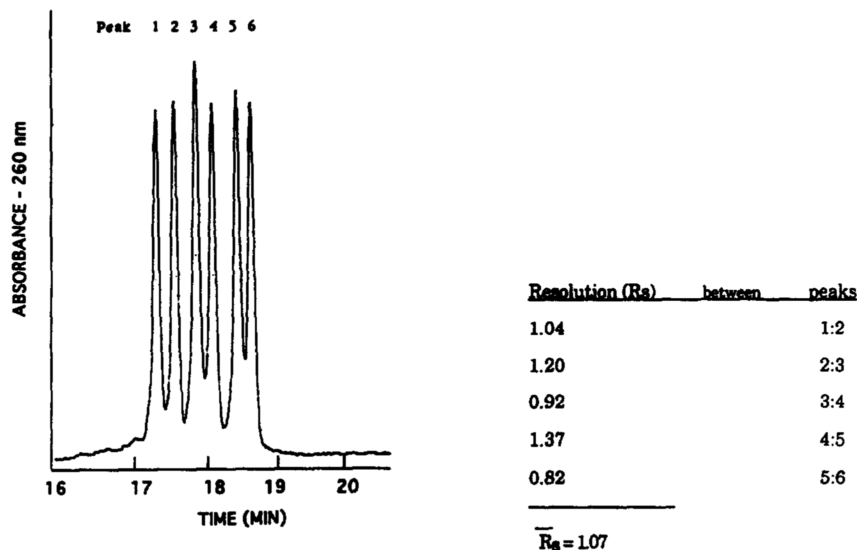


Fig. 3. Electropherogram of the phosphorothioate mixture from Fig. 2. Buffer system III was used, and other running conditions were the same as in Fig. 2.

Tris alone (system IV) as the buffer (Fig. 4). In this electropherogram, peaks are more uniform in shape and size, and resolution was slightly improved. However, at pH 10, Tris loses its buffering capacity, since it has a pK_a of 8.3. Consequently, these gels did not give consistent results, which can be attributed to a marked decrease in the pH of the Tris solution over time. Several other high pH buffer systems were tried, but the best results utilized a Tris–borate system (system V, Fig. 5A, B). It was found that a pH of 9.5 was still not low enough to maintain a true buffer involving Tris, and consequently, the gels utilizing 1.5 m*M* borate (Fig. 5A) did not give reproducible data. Gel system VI (10% Micro-Gel, 35 m*M* Tris, 5.6 m*M* borate, pH 9.0) exhibited good resolution ($\overline{R}_s = 1.95$) and, more importantly, reproducible data, and is the system shown in Fig. 5B. These gels continue to give consistent data and exhibit good gel to gel reproducibility; each gel lasts for approximately 30–40 injections.

Table I summarizes the results from the electropherograms of Figs. 1–5. Although system VI

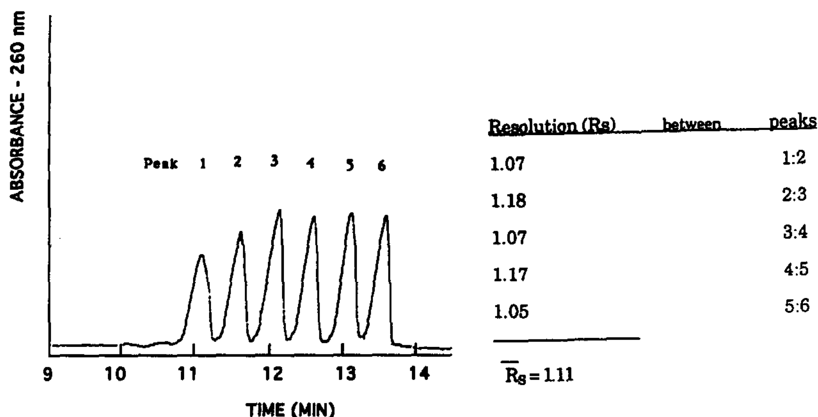


Fig. 4. Electropherogram of the same phosphorothioate mixture from Fig. 2. Buffer system IV was used, and other running conditions were the same as in Fig. 2.

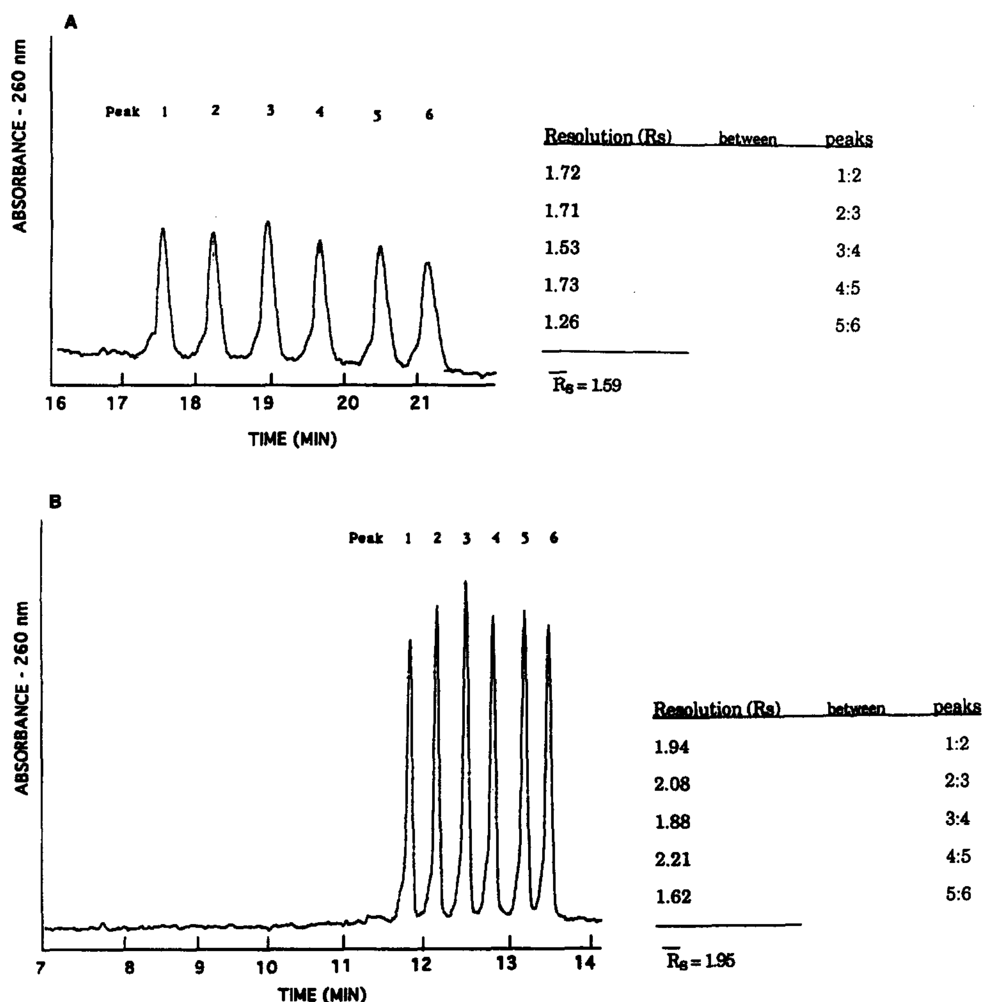


Fig. 5. Electropherogram of the phosphorothioate mixture from Fig. 2. The buffer system parameters were: (A) system V and (B) system VI. Other running conditions were the same as in Fig. 2.

(Fig. 5B) gave the best resolution, the gels used for Fig. 5A gave baseline resolution ($R_s = 1.59$), but as stated earlier, these gels proved unstable. In all these gel systems, resolution varied slightly from gel to gel.

Table II shows the resolution data from three representative capillaries using system VI. Average resolution varied from 1.47 to 1.70; however, all resolutions were 1.20 or greater. These gel-filled capillaries proved to not only give superior resolution but also exhibited the resilience to last several runs.

Fig. 6A is an example of a 24-mer of unknown purity co-injected with the thioate-mix (21–16-mer) seen in previous figures. The mix is acting like an internal marker. Since the oligonucleotide lengths of the mix are known, the unknown product is checked for full-length integrity. Counting up from the 16-mer in the mix verifies that a 24-mer is indeed the main constituent of the analysis.

Fig. 6B depicts the analysis of the 24-mer (phosphorothioate) seen in Fig. 6A. Presently, it is difficult to always obtain baseline resolution for the $n-1$-mer and $n+1$-mer when present in an analysis. This loss in resolution is due to peak broadening caused not only by the phosphorothioate moiety, but also from base composition.

TABLE I

RESOLUTION DATA FROM 6 DIFFERENT BUFFER SYSTEMS

Capillary 50 cm × 100 μm I.D., constant running voltage (−22 kV), sample injection at −8 kV for 5 s, UV detection at 260 nm.

System	Gel parameters	Average resolution ($\overline{R}_s$)
I	10% Micro-Gel, 75 m*M* Tris–phosphate (TP), 10% methanol, pH 7.5	0.83
II	8.5% Micro-Gel, 35 m*M* TP, 15% ethylene glycol, pH 8.0	0.85
III	10% Micro-Gel, 35 m*M* TP, 15% ethylene glycol, pH 8.0	1.07
IV	10% Micro-Gel, 35 m*M* Tris, 15% ethylene glycol, pH 10.0	1.11
V	10% Micro-Gel, 35 m*M* Tris, 1.5 m*M* borate, 15% ethylene glycol, pH 9.5	1.59
VI	10% Micro-Gel, 35 m*M* Tris, 5.6 m*M* borate, 15% ethylene glycol, pH 9.0	1.95

TABLE II

RESOLUTION DATA FROM 3 GEL-FILLED CAPILLARIES MANUFACTURED ON DIFFERENT DATES

Capillary 50 cm × 100 μm I.D., 10% Micro-Gel, 35 m*M* Tris, 5.6 m*M* borate, 15% ethylene glycol, pH 9.0, constant running V (−2.2 kV), sample (thioate-mix 16–21-mer) injection −8 kV for 5 s, UV detection at 260 nm.

Gel	Resolution ($\overline{R}_s$)	between peaks
1	1.67	1:2
	1.78	2:3
	1.60	3:4
	1.97	4:5
	1.49	5:6
Average	1.70	
2	1.54	1:2
	1.59	2:3
	1.40	3:4
	1.61	4:5
	1.20	5:6
Average	1.47	
3	1.52	1:2
	1.68	2:3
	1.52	3:4
	1.86	4:5
	1.36	5:6
Average	1.59	

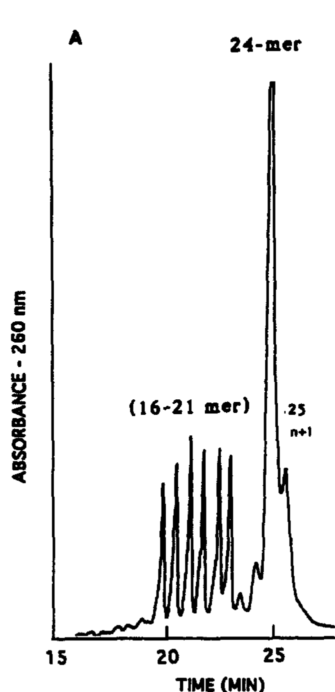
Overall $\overline{R}_s$ for above data = 1.59

It is known that base composition plays a role in the electrophoretic mobility of DNA [3]. For the gel-filled capillaries utilized here, oligos with high guanine content, like the oligonucleotide analyzed in Fig. 6, often give broader peaks that move at slightly slower rates when compared to oligonucleotides of the same length with relatively less guanine in their base composition.

Fig. 7 shows the analysis of a 21-mer identical to the 21-mer used in the thioate-mix. A 21-base phosphorothioate DNA is representative of those commonly synthesized for biological studies. This particular compound exhibits a typical $n-1$ mer and is used as a gel performance control. If the 20-mer does not resolve from the 21-mer in this sample, it is often a sign that the gel is breaking down and needs replacing. Most gel-filled capillaries of this type last approximately 30–40 runs.

CONCLUSIONS

The analytical results of phosphorothioates by CGE have always been unsatisfactory when compared to results obtained from phosphodiester DNA analysis. Phosphorothioates typically give broader peaks which cause poor resolution between oligos that differ only by one base. Gel concentration was shown to be a critical factor in reducing peak broadening. While a rigid gel is



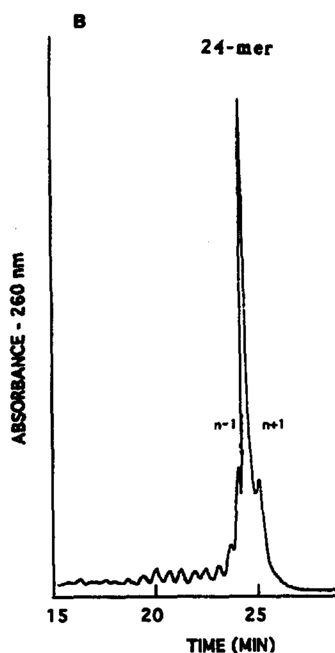


Fig. 6. (A) Analysis of the phosphorothioate mixture from Fig. 5A and B combined with a mixed base phosphorothioate (24-mer), and (B) the analysis of the 24-mer by itself. Gel parameters and running conditions were the same as in Fig. 5B.

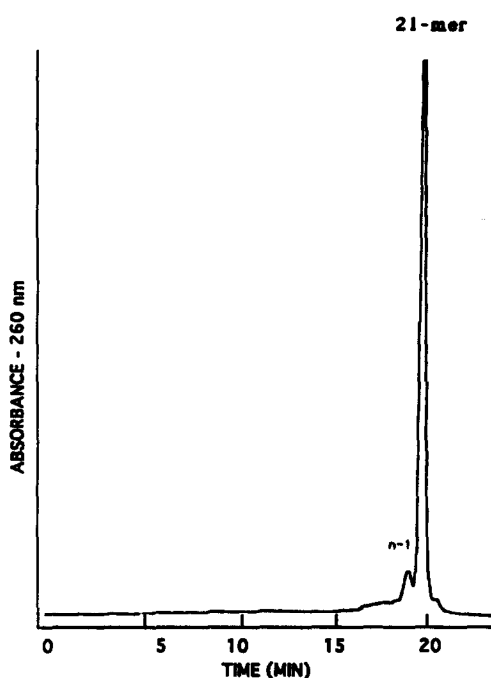


Fig. 7. Analysis of a phosphorothioate 21-mer. This oligo is used as a control sample because of the prominent 20-mer ($n-1$ mer) it contains. Gel parameters and running conditions were the same as in Fig. 5B.

crucial in obtaining resolutions higher than 1.0, gel concentrations higher than 10% give capillaries that often form air bubbles and fail prematurely. The use of ethylene glycol as a buffer additive was also found to increase the rigidity of the gel which served to prolong its lifetime on the instrument.

Since peak broadening could be a consequence of typical intramolecular secondary structures that neither high temperature (55°C) nor denaturing agents, *i.e.* 7–8 *M* urea, can completely disrupt, it was also demonstrated that by raising the pH of the buffer, markedly better electrophoretic conditions are achieved for the phosphorothioates. A pH of 9.0 gave the best resolution when compared to lower pH; however, a stable buffering system with a pH $\geq$ 10.0 would be more suitable since this would insure complete denaturation.

Finally, in data not shown here, it was found that buffer systems that contained Na^+ greatly reduced the chances of a gel surviving for more than a few runs. This is directly related to high

currents and joule heating, a consequence of high ionic strength within sodium buffers. For this reason, buffer systems that utilized dibasic or tribasic sodium phosphate failed. Therefore, organic buffers are most desirable, since their ionic strength is often relatively lower than buffers that contain high amounts of inorganic salt.

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