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Selectivity of anion exchange chromatography and capillary gel electrophoresis for the analysis of phosphorothioate oligonucleotides [1]

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Abstract

The complementary nature of anion exchange chromatography and capillary gel electrophoresis for oligonucleotide analysis is demonstrated by evaluating a comprehensive series of authentic deletion sequences and partial phosphodiester analogs of five phosphorothioate oligonucleotides of different base composition and sequence. While anion exchange HPLC is sensitive to differences in backbone length of phosphorothioate oligonucleotides, oligomers with length difference of one base unit are not resolved. Capillary gel electrophoresis, on the other hand, has excellent single-base resolution while being relatively insensitive to phosphate in the phosphorothioate backbone. The data definitively establish the necessity of employing both separation techniques for adequate characterization of lower order process-related impurities potentially found in synthetic phosphorothioate oligonucleotides. © 1997 Elsevier Science B.V.

Keywords: Anion exchange chromotography; Capillary gel electrophoresis; Oligonucleotide analysis

1. Introduction

Synthetic oligonucleotides represent a novel class of therapeutic agents currently undergoing preclinical and clinical development for a variety of indications. The naturally occurring phosphodiester oligonucleotides are rapidly degraded by serum nucleases, a problem which has been successfully overcome by incorporation of a sulfur atom in place of a non-bridging oxygen in the phosphate backbone [1]. These nuclease resistant phosphorothioates are now routinely synthesized in automated solid phase synthesizers. However, due to slightly less than quantitative coupling and/or thiation yields, the final product could contain analytically significant levels of process-related 'failure sequences' or 'partial phosphodiesters'. Traditional modes of characterizing oligonucleotides include gel electrophoresis, anion exchange chromatography, and ^{31}P NMR [2–11]. Anion exchange HPLC appears to offer selectivity

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on the basis of differences in phosphate backbone substitution as well as length, whereas slab gel or capillary gel electrophoresis has thus far only been shown to separate on the basis of length.

To assess the purity of any drug for clinical use, it is necessary to identify and quantitate the levels of all process-related impurities in the sample. For synthetic phosphorothioate oligonucleotides, impurities primarily constitute deletion sequences and thiation failures. Of these, failures such as *single* deletion sequences and monophosphodiesters are the most commonly found. If the purification processes are adequate, elongation or sulfurization failures at two or three locations resulting in (*n*-2) or (*n*-3) deletion sequences, di- or triphosphodiesters or a combination thereof, are less common. Currently, most purified phosphorothioate oligonucleotide drugs contain process-related impurities as a result of synthetic failures at a single location.

Single deletion sequences, also known as '(*n*-1) deletion sequences' result from failure to add a base at a single position. For a 20-base oligonucleotide without adjacent same-base nucleotides, 19 unique (*n*-1) deletion sequences are possible. Similarly, for a 20-base phosphorothioate oligonucleotide, 19 unique monophosphodiesters are possible. To date, no single separation technique has demonstrated the necessary analytical selectivity to simultaneously separate full-length, fully thioated phosphorothioate drugs from their fully thioated single-base deletion products and their full-length monophosphodiester analogs (see Fig. 1).

In an effort to fully characterize the analytical selectivity of anion exchange HPLC and capillary gel electrophoresis, specifically with respect to the more common lower order synthesis related failure products (single-base deletion sequences and monophosphodiesters), a comprehensive series of authentic deletion sequences [(*n*-1), (*n*-2) and (*n*-3) deletion sequences] and partial phosphodiester analogs (mono-, di- and triphosphodiesters) of five phosphorothioate oligonucleotides of varying base composition and sequence were synthesized and evaluated. In addition, the capacity of anion exchange HPLC and gel electrophoresis to detect not only quantity of failures i.e. (*n*-1) versus (*n*-2) sequence content or mono- or diphosphodiester content in a sample, but also the location of failure within the molecule, was evaluated. The methods used to perform the analyses reported in this manuscript represent the best analytical selectivities achieved to date upon optimization of common chromatographic and electrophoretic parameters such as pH, temperature, column/capillary chemistries, mobile phase composition/ionic strength/gradients and electrophoretic running buffers.

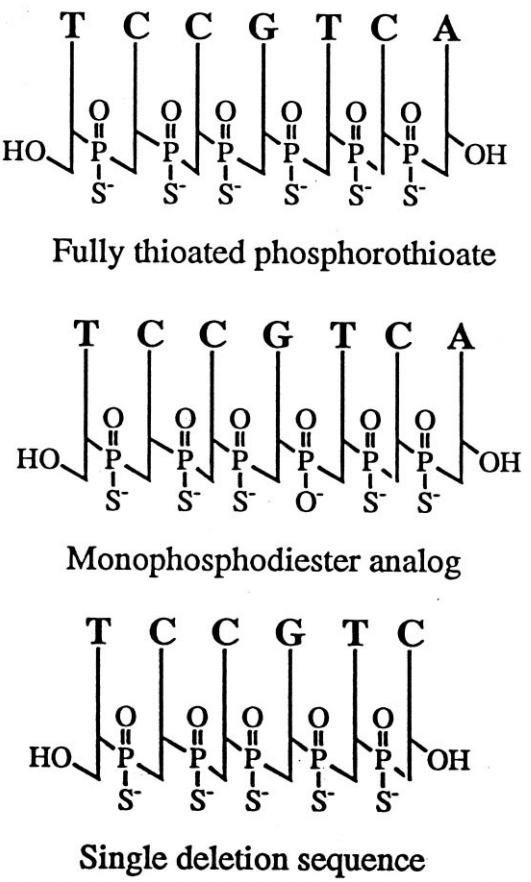

Fig. 1. An example of a seven-base full-length, fully thioated phosphorothioate, its monophosphodiester analog and a single deletion sequence resulting in a (*n*-1) mer.

Table 1
HPLC gradients used in anion exchange chromatography

Program	Column	Flow-rate and temperature		Time (min)	Mobile phase composition		
		(ml min^{-1})	°C		%A	%B	%C
I	GenPak Fax	0.8	80	0	100	0	0
		0.8		8	0	100	0
		0.8		15	0	100	0
		0.8		17	100	0	0
		1.0		19	100	0	0
		1.0		29	100	0	0
		0.8		30	100	0	0
II	HEMA-IEC	0.80	60	0	70	5	25
				7	45	30	25
				17	35	40	25
				20	35	40	25
				22	70	5	25
				30	70	5	25
III	HEMA-IEC	1.0	70	0	100	0	0
				20	0	100	0
				25	0	100	0
				28	100	0	0
				35	100	0	0
IV	Resource Q	1.0	60	0	100	0	0
				5	100	0	0
				40	0	100	0
				45	100	0	0
				55	100	0	0

2. Experimental

2.1. Chemicals and reagents

Phosphorothioate oligonucleotides were synthesized on a solid phase DNA synthesizer (Milligen 8800) and purified by preparative reversed phase chromatography. Oligonucleotide sequence, 5′-GCG TTT GCT CTT CTT CTT GCG-3′, was further purified by preparative anion exchange chromatography. The purified oligonucleotides were then desalted by reversed phase adsorption/elution, rotoevaporated and depyrogenated prior to lyophilization. The deletion sequences and partial phosphodiester analogs were similarly synthesized, ethanol precipitated and used without further purification. High purity water (Barnstead Nanopure, Newton, MA) was used for the preparation of analytical samples.

2.2. Anion exchange chromatography

The HPLC systems used were a HP 1090 Series II (Hewlett-Packard, Burlington, MA), a Waters system consisting of a 625 controller, 715 Wisp (autosampler), and 990 PDA detector (Waters, a division of Millipore, Milford, MA), and a Waters system equipped with a 625 controller, 715 WISP, 486 UV detector, and Chrom Perfect (data management) from Justice Innovations (Mountain View, CA). The anion-exchange column used for the analyses was either a GEN-PAK FAX (2.5 μm) 4.6 × 100 mm column (Waters), a HEMA-IEC BIO 1000 Q (10 μm), 4.6 × 150 mm column (Alltech Associates, Deerfield, IL, USA) or a Resource-Q (15 μm), 6.4 × 30 mm (1 ml) (Pharmacia Biotech, Piscataway, NJ). Mobile phases used for the separations were (I) A: 86 mM TRIS (pH 8.0)/86 mM TRIS, 2M NaCl (pH 8.0)/IPA

Table 2
Anion exchange HPLC retention times (min) of authentic full-length, phosphodiesters of a phosphorothioate oligonucleotide

Sequence (o represents P = O link)	T_7	RT	Normalized RT
Full-length, fully thioated sequence			
GCC CAA GCT GGC ATC CGT CA	12.98	20.83	1.60
Full-Length Monophosphodiester analogs:			
GoCC CAA GCT GGC ATC CGT CA	12.57	19.31	1.54
GC**o**C CAA GCT GGC ATC CGT CA	12.52	19.33	1.54
GCC**o** CAA GCT GGC ATC CGT CA	12.56	19.34	1.54
GCC C**o**AA GCT GGC ATC CGT CA	12.56	19.26	1.53
GCC CA**o**A GCT GGC ATC CGT CA	12.56	19.21	1.53
GCC CAA**o** GCT GGC ATC CGT CA	12.57	19.21	1.53
GCC CAA **Go**CT GGC ATC CGT CA	12.57	19.25	1.53
GCC CAA GC**o**T GGC ATC CGT CA	12.57	19.30	1.54
GCC CAA GCT**o** GGC ATC CGT CA	12.57	19.25	1.53
GCC CAA GCT **Go**GC ATC CGT CA	12.57	19.23	1.53
GCC CAA GCT GG**o**C ATC CGT CA	12.57	19.29	1.53
GCC CAA GCT GGC**o** ATC CGT CA	12.57	19.24	1.53
GCC CAA GCT GGC A**o**TC CGT CA	12.57	19.26	1.53
GCC CAA GCT GGC AT**o**C CGT CA	12.57	19.32	1.54
GCC CAA GCT GGC ATC**o** CGT CA	12.57	19.30	1.54
GCC CAA GCT GGC ATC C**o**GT CA	12.57	19.23	1.53
GCC CAA GCT GGC ATC CG**o**T CA	12.57	19.24	1.53
GCC CAA GCT GGC ATC CGT**o** CA	12.58	19.31	1.53
		Mean	1.53
		SD	0.005
		% RSD	0.33
Other partial phosphodiester analogs (Diphosphodiester)			
GCC CAA**o** GCT **Go**GC ATC CGT CA	12.56	18.12	1.44
(Triphosphodiester)			
GCC CAA**o** GCT **Go**GC ATC CGT C**o**A	12.58	16.98	1.35

(17:2:1, v/v/v), B: 86 mM TRIS, 2M NaCl (pH 8.0)/IPA (4:1, v/v); (II) A: 86 mM TRIS (pH 8.0)/B: 86 mM TRIS, 2M NaBr (pH 8.0)/C: MeOH; (III) A: 86 mM TRIS (pH 8.0)/86 mM TRIS, 2M NaCl (pH 8.0)/MeOH (17:2:1, v/v/v), B: 86 mM TRIS, 2M NaCl (pH 8.0)/MeOH (4:1, v/v) and, (IV) A: 100 mM Sodium Phosphate, Dibasic, 7-Hydrate/1 M NaCl (pH 11.5), B: 100 mM Sodium Phosphate, Dibasic, 7-Hydrate/3 M NaCl (pH 11.5). Data was collected at 266 nm for the gradient program I and 260 nm for programs II, III, and IV. Gradient programs are outlined in Table 1.

To allow determination of precise differences in retention times of the authentic analogs as a function of the position of their phosphodiester linkage, if any, an internal standard, 7-mer polythymidine phosphodiester, was added to each sample. Observed retention times were then normalized to that of the T_7 standard.

2.3. Capillary gel electrophoresis

Electrophoretic data were acquired using a Beckman 2000 or 5000 P/ACE instrument operated at an applied voltage of 14.1 kV. The column temperature was maintained at 30° or 50°C and detection was at 254 nm or the absorption maximum for the compound of interest, 47-cm polyacrylamide gel columns U100P (Catalog # 338480) with an effective column length of 40 cm (7 cm from the detector to the waste reservoir) and the running buffer tris-borate/urea (Catalog no. 338481) were purchased from Beckman In-

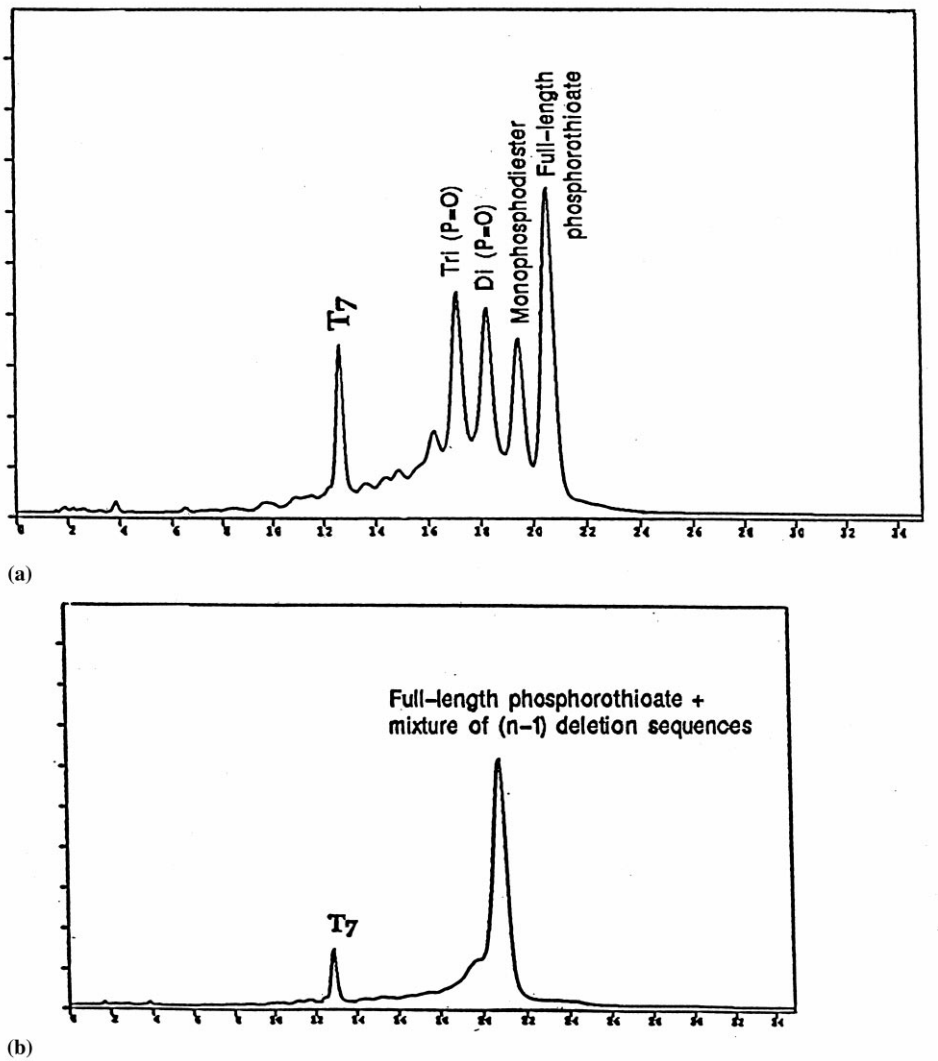

Fig. 2. Anion exchange chromatograms of (a) a mixture containing full-length, fully thioated, 20-base oligonucleotide, 5′-GCC CAA GCT GGC ATC CGT CA-3′, and its mono-, di-, and triphosphodiester analogs and (b) 1:1 solution of full-length 5′-GCC CAA GCT GGC ATC CGT CA-3′ sequence and a mixture of its (n-1) deletion sequences. T_7 is an internal standard.

struments, Fullerton, CA. Electrokinetic injections were made at an injection voltage of 7 kV for 20 s for 1 mg ml^{-1} samples.

To allow determination of precise differences in CGE migration times of the authentic impurities as a function of base deletion position within the oligonucleotide sequence, an internal standard, 23-mer (or 27-mer) polythymidine phosphodiester, was added to each sample. The observed migration times were then normalized to that of the internal standard.

3. Results

3.1. Selectivity of anion exchange chromatography

A series of authentic partial phosphodiesters of a phosphorothioate oligonucleotide of the se-

Table 3
Anion exchange HPLC retention times (min) of authentic deletion sequences of a phosphorothioate oligonucleotide

Sequence (represents a deletion)	T_7	RT	Normalized RT
Full-length, fully thioated sequence			
GCC CAA GCT GGC ATC CGT CA	12.98	20.83	1.60
Single deletion sequences			
_CC CAA GCT GGC ATC CGT CA	13.07	20.39	1.56
G_C CAA GCT GGC ATC CGT CA	13.10	20.64	1.58
GCC C_A GCT GGC ATC CGT CA	13.03	20.67	1.59
GCC CAA _CT GGC ATC CGT CA	13.08	20.62	1.58
GCC CAA G_T GGC ATC CGT CA	13.04	20.66	1.58
GCC CAA GC_ GGC ATC CGT CA	13.06	20.64	1.58
GCC CAA GCT _GC ATC CGT CA	13.00	20.58	1.58
GCC CAA GCT GG_ ATC CGT CA	12.86	20.56	1.60
GCC CAA GCT GGC _TC CGT CA	12.97	20.48	1.58
GCC CAA GCT GGC A_C CGT CA	12.99	20.54	1.58
GCC CAA GCT GGC AT_ CGT CA	13.01	20.71	1.59
GCC CAA GCT GGC ATC C_T CA	12.91	20.35	1.58
GCC CAA GCT GGC ATC CG_ CA	13.06	20.60	1.58
GCC CAA GCT GGC ATC CGT _A	12.95	20.64	1.59
Mean normalized retention time for single deletion sequences:	1.58 ± 0.01		
% RSD	0.63		
(n-2) Deletion sequences			
_CC CAA GCT GGC ATC CGT _A	13.02	20.01	1.53
_CC CAA GCT _GC ATC CGT CA	13.01	19.84	1.52
(n-3) Deletion sequences			
_CC CAA GCT _GC ATC CGT _A	13.05	19.41	1.49

quence, 5′-GCC CAA GCT GGC ATC CGT CA-3′, were analyzed by anion exchange chromatography. The data in Table 2 show resolution of the full-length monophosphodiester analogs from the full-length, fully thioated sequence. Furthermore, regardless of location of the phosphodiester in the backbone, all full-length monophosphodiester analogs elute 'as a class' and are well-resolved from the full-length, fully thioated sequence. For a typical oligonucleotide sample containing approximately 1 area-% full-length monophosphodiesters, the resolution between the full-length fully thioated molecule and the full-length monophosphodiesters was 0.6.

For a typical oligonucleotide sample containing approximately 1 area-% full-length monophosphodiesters, the resolution between the full-length fully thioated molecule and the full-length monophosphodiesters was 0.6.

Fig. 2(a) shows a chromatogram of a mixture of the full-length, fully thioated sequence, 5′-GCC CAA GCT GGC ATC CGT CA-3′ and a co-mix of a representative full-length monophosphodiester, diphosphodiester and triphosphodiester analogs. It should be noted that following synthesis, the authentic phosphodiester impurity analogs were ethanol precipitated and not purified further. Consequently, they contain low levels of failure sequences and other higher order phosphodiester impurities seen as small peaks in the base line. The tri- and diphosphodiesters elute well before the monophosphodiester analogs by anion exchange HPLC. The retention time data in Table 2 and the chromatogram shown in Fig. 2(a) confirm that the full-length mono-, di-, and triphosphodiesters are resolved from the full-length, fully thioated sequence. Similar data were obtained for the mono-, di- and triphosphodiester analogs of the other four phosphorothioate oligonucleotide sequences studied (see Table 7).

Similarly to full-length, partial phosphodiesters, a set of authentic deletion sequences of the phosphorothioate oligonucleotide sequence, 5′-GCC CAA GCT GGC ATC CGT CA-3′, was ana-

Table 4
Selectivity of alternate anion exchange HPLC columns

Selectivity of phosphodiesters	T_7	RT	Normalized RT
Gen-pak FAX column (4.6 × 100 mm)			
GCC CAA GCT GGC ATC CGT CA	10.52	22.90	2.18
Full-length monophosphodiester impurity		21.67	2.06
GCC CAA GC**o**T GGC ATC CGT CA	10.55	21.52	2.04
GCC CAA G**o**CT GGC ATC CGT CA	10.54	21.41	2.03
GCC CAA**o** GCT GGC ATC CGT CA	10.52	21.33	2.03
GCC CA**o**A GCT GGC ATC CGT CA	10.49	21.24	2.02
GCC C**o**AA GCT GGC ATC CGT CA	10.49	21.40	2.04
Mean normalized retention time for full-length monophosphodiester sequences		2.03 ± 0.007	
Alltech HEMA column (4.6 × 150 mm)			
GCC CAA GCT GGC ATC CGT CA	12.63	20.56	1.63
Full-length monophosphodiester impurity		19.45	1.54
GCC CAA GC**o**T GGC ATC CGT CA	12.57	19.30	1.54
GCC CAA G**o**CT GGC ATC CGT CA	12.57	19.25	1.53
GCC CAA**o** GCT GGC ATC CGT CA	12.57	19.21	1.53
GCC CA**o**A GCT GGC ATC CGT CA	12.56	19.21	1.53
GCC C**o**AA GCT GGC ATC CGT CA	12.56	19.26	1.53
Mean normalized retention time for full-length monophosphodiester sequences		1.53 ± 0.004	

Selectivity of deletion sequences	T_7	RT	RT/T_7
Gen-pak FAX column (4.6 × 100 mm)			
GCC CAA GCT GGC ATC CGT CA	10.51	22.83	2.17
Full-length monophosphodiester impurity		21.64	2.06
_CC CAA GCT GGC ATC CGT CA	10.49	22.09	2.11
G_C CAA GCT GGC ATC CGT CA	10.49	22.36	2.13
GCC C_A GCT GGC ATC CGT CA	10.49	22.43	2.14
GCC CAA _CT GGC ATC CGT CA	10.49	22.32	2.13
GCC CAA G_T GGC ATC CGT CA	10.49	22.52	2.15
Mean normalized retention time for fully thioated, single deletion sequences		2.13 ± 0.01	
Alltech HEMA column (4.6 × 150 mm)			
GCC CAA GCT GGC ATC CGT CA	12.98	20.83	1.60
Full-length monophosphodiester impurity		19.69	1.52
_CC CAA GCT GGC ATC CGT CA	13.07	20.39	1.56
G_C CAA GCT GGC ATC CGT CA	13.10	20.64	1.58
GCC C_A GCT GGC ATC CGT CA	13.03	20.67	1.59
GCC CAA _CT GGC ATC CGT CA	13.08	20.62	1.58
GCC CAA G_T GGC ATC CGT CA	13.04	20.66	1.58
Mean normalized retention time for fully thioated, single deletion sequences		1.58 ± 0.01	

lyzed. Table 3 summarizes the data from these experiments. The normalized retention times verify that the average retention time for all possible (n-1) deletion sequences of this phosphorothioate oligonucleotide are within a narrow range of 1.58 ± 0.01 min. Some resolution of the (n-2) and (n-3) deletion sequences from the (n-1) and full-length sequence is suggested by comparison of the average retention times listed in Table 3. The normalized retention time of the full-length sequence, 1.60, is only 0.02 relative units from the average range for the (n-1) mers confirming incomplete anion exchange HPLC resolution of the (n-1) deletion sequence impurities from the full-length sequence. This is illustrated in the chromatogram in Fig. 2(b) of a solution containing

Table 5
Capillary gel electrophoresis normalized migration times (min) of authentic phosphodiester analogs of a phosphorothioate oligonucleotide

Sequence (o represents P = O link)	T_{23}	MT	Normalized MT
Full-length, fully thioated sequence			
GCC CAA GCT GGC ATC CGT CA	19.392	18.649	0.9617
Monophosphodiester analogs of the full-length sequence			
G**o**CC CAA GCT GGC ATC CGT CA	23.931	22.95	0.9590
GC**o**C CAA GCT GGC ATC CGT CA	23.718	22.747	0.9591
GCC**o** CAA GCT GGC ATC CGT CA	12.008	11.517	0.9591
GCC C**o**AA GCT GGC ATC CGT CA	11.900	11.417	0.9591
GCC CA**o**A GCT GGC ATC CGT CA	21.679	22.613	0.9587
GCC CAA**o** GCT GGC ATC CGT CA	22.175	23.124	0.9590
GCC CAA G**o**CT GGC ATC CGT CA	22.390	23.356	0.9586
GCC CAA GC**o**T GGC ATC CGT CA	19.589	18.714	0.9553
GCC CAA GCT**o** GGC ATC CGT CA	19.493	18.632	0.9558
GCC CAA GCT G**o**GC ATC CGT CA	19.354	18.496	0.9557
GCC CAA GCT GG**o**C ATC CGT CA	23.356	22.39	0.9586
GCC CAA GCT GGC**o** ATC CGT CA	21.857	22.808	0.9583
GCC CAA GCT GGC A**o**TC CGT CA	23.124	22.175	0.9590
GCC CAA GCT GGC AT**o**C CGT CA	11.924	11.453	0.9605
GCC CAA GCT GGC ATC**o** CGT CA	21.086	20.202	0.9581
GCC CAA GCT GGC ATC C**o**GT CA	20.537	19.624	0.9555
GCC CAA GCT GGC ATC CG**o**T CA	22.747	23.718	0.9591
GCC CAA GCT GGC ATC CGT**o** CA	22.950	23.931	0.9590
Mean normalized migration time for full-length monophosphodiester analogs:		0.9581 ± 0.0016	
% RSD		0.167	
Diphosphodiester analog			
GCC CAA**o** GCT G**o**GC ATC CGT CA	13.387	12.839	0.9591
Triphosphodiester analog			
GCC CAA**o** GCT G**o**GC ATC CGT C**o**A	23.534	22.584	0.9596

equal concentrations of the full-length phosphorothioate oligonucleotide and an authentic (n-1)-mer mix. Coelution of the full-length sequence with all possible (n-1) deletion sequences is clearly observed. In summary, while there is some degree of length-based separation by anion exchange chromatography, for phosphorothioate oligonucleotides of approximately 20-base units in length, this selectivity is limited to differences of ≥ 2 base units.

During method development it was noted that the analytical selectivity of anion exchange chromatography for phosphorothioates was maintained regardless of the types of anion exchange column used. This is demonstrated for a representative phosphorothioate oligonucleotide sequence, 5′-GCC CAA GCT GGC ATC CGT CA-3′ and its authentic full-length monophosphodiester and single base deletion sequence analogs in Table 4. A similar trend has been noted using the Pharmacia Resource-Q anion exchange HPLC columns.

3.2. Selectivity of capillary gel electrophoresis

A series of authentic partial phosphodiesters of the sequence, 5′-GCC CAA GCT GGC ATC CGT CA-3′, were analysed by capillary gel electrophoresis. Table 5 summarizes the relative migration times of authentic full-length mono- and higher order phosphodiester analogs. The mean relative migration time measured for a series of authentic mono-, di-, and tri-phosphodiester analogs is 0.9572 ± 0.0015. This is essentially identical to the relative migration time recorded

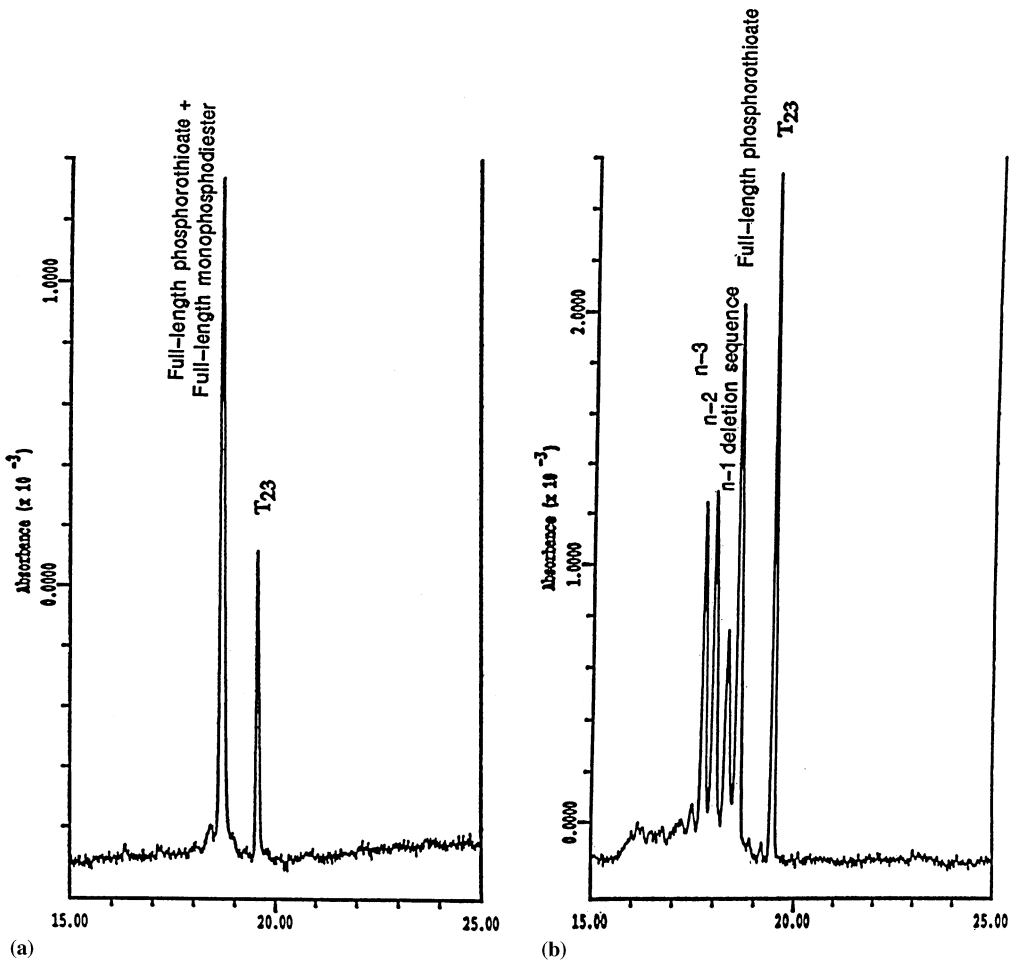

Fig. 3. Capillary gel electropherogram of (a) a 1:1 mixture of the phosphorothioate oligonucleotide, 5′-GCC CAA GCT GGC ATC CGT CA-3′, and a full-length monophosphodiester analog and (b) a mixture of the phosphorothioate oligonucleotide, 5′-GCC CAA GCT GGC ATC CGT CA-3′, and its (*n*-1); (*n*-2) and (*n*-3) deletion sequences. Samples contain T_{23} as the internal standard.

for the full-length, fully thioated phosphorothioate sequence (0.9601). Fig. 3(a) further demonstrates comigration of the full-length, fully thioated phosphorothioate sequence and a full-length monophosphodiester analog. It was also found that the di-, tri-, and the phosphodiester analogs comigrated with the full-length phosphorothioate.

Table 6 summarizes the relative migration times of authentic deletion analogs of the 5′-GCC CAA GCT GGC ATC CGT CA-3′ sequence. The relative migration times of all possible (*n*-1) deletion sequences fall within $\pm$ 0.0028 of the average MT, 0.9447, and are well-separated from authentic full-length, fully thioated sequence which has a relative migration time of 0.9601. This is further demonstrated in Fig. 3(b) in which the (*n*-1), (*n*-2), and (*n*-3) deletion sequences are well-separated from the full-length, fully thioated oligonucleotide. For a typical oligonucleotide sample containing approximately 2 area-% (*n*-1) deletion sequences, the resolution between the full-length molecule and the (*n*-1) deletion sequences was 1.0.

Table 6
Capillary gel electrophoresis normalized migration times (min) of authentic deletion sequences of a phosphorothioate oligonucleotide

Sequence (_ represents a deletion)	T_{23}	MT	Normalized MT
Full-length, fully thioated sequence			
GCC CAA GCT GGC ATC CGT CA	19.392	18.649	0.9617
Fully thioated single deletion sequences			
_CC CAA GCT GGC ATC CGT CA	19.864	18.728	0.9428
G_C CAA GCT GGC ATC CGT CA	19.995	18.904	0.9454
GCC C_A GCT GGC ATC CGT CA	21.876	20.655	0.9442
GCC CAA _CT GGC ATC CGT CA	20.097	10.002	0.9455
GCC CAA G_T GGC ATC CGT CA	24.017	22.773	0.9482
GCC CAA GC_ GGC ATC CGT CA	23.866	22.579	0.9461
GCC CAA GCT _GC ATC CGT CA	23.620	22.408	0.9487
GCC CAA GCT GG_ ATC CGT CA	21.037	19.951	0.9484
GCC CAA GCT GGC _TC CGT CA	21.156	19.991	0.9449
GCC CAA GCT GGC A_C CGT CA	21.358	20.165	0.9441
GCC CAA GCT GGC AT_ CGT CA	21.467	20.288	0.9451
GCC CAA GCT GGC ATC C_T CA	21.285	20.471	0.9397
GCC CAA GCT GGC ATC CG_ CA	21.802	20.496	0.9401
GCC CAA GCT GGC ATC CGT _A	NA	NA	NA
Mean normalized migration time for single deletion sequences:		0.9447 ± 0.003	
% RSD		0.296	
(n-2) Deletion sequences			
_CC CAA GCT GGC ATC CGT _A	22.005	20.422	0.9281
_CC CAA GCT _GC ATC CGT CA	21.881	20.241	0.9250
(n-3) Deletion sequences			
_CC CAA GCT _GC ATC CGT _A	24.034	22.071	0.9183

3.3. Selectivity of anion exchange HPLC and CGE: sequence dependence

Experiments similar to those summarized in Tables 2–4 and 6 were performed for authentic, fully thioated, (n-1) deletion sequences and full-length monophosphodiester analogs of four other phosphorothioate oligonucleotides of various base composition and sequence. As shown in Table 7, the analytical selectivity of anion exchange HPLC and Capillary Gel Electrophoresis are identical for the five sequences studied. The data demonstrate that for phosphorothioate oligonucleotides of approximately 20-base units in length, the selectivities exhibited by anion exchange HPLC and capillary gel electrophoresis are essentially independent of base composition or sequence.

4. Discussion

The complementary nature of anion exchange HPLC and capillary gel electrophoresis was demonstrated by evaluating a comprehensive series of authentic deletion sequences and partial phosphodiester analogs of five phosphorothioate oligonucleotides of varying base composition and sequence. It is clear that from the selectivity differences presented that a combination of both techniques is necessary for adequate assessment of the purity of synthetic phosphorothioate oligonucleotides.

Anion exchange chromatography, while demonstrating excellent selectivity for separating the full-length monophosphodiester from the fully thioated sequence, is not able to separate the (n-1) deletion sequences from the full-length analogs. The higher-order deletion sequences ($>n$-2) are,

Table 7
Anion exchange HPLC retention time (min) and capillary gel electrophoresis migration time (min) data of authentic analogs of phosphorothioate oligonucleotides as a function of oligonucleotide base composition

Oligonucleotide sequence	Base composition A:C:G:T	Anion exchange LC (Pharmacia Resource Q) retention times normalized to T_7 internal standard			Capillary gel electrophoresis migration times normalized to T_{23} or T_{27} internal standard		
		Full-length, fully thioated sequence	(n-1), all P = S sequences	Full-length mono P = O sequences	Full-length, fully thioated sequence	(n-1), all P = S sequences	Full-length mono P = O sequences
5′-TTG CTT CCA TCT TCC TCG TC-3′	1:8:2:9	11.05 [a,b]	Coelute with P = S peak	10.46[a,b]	28.2[b]	27.7[b]	Coelute with full-length sequence
5′-GCG TTT GCT CTT CTT CTT GCG-3′	0:6:5:10	4.50	Coelute with P = S peak	4.06	0.98	0.96	Coelute with full-length sequence
5′-GCC CAA GCT GGC ATC CGT CA-3′	4:8:5:3	1.60	Coelute with P = S peak	1.53[a]	0.96	0.94	Coelute with full-length sequence
5′-GTT CTC GCT GGT GAG TTT CA-3′	2:4:6:8	4.17	Coelute with P = S peak	3.84	0.85[c]	0.86[c]	Coelute with full-length sequence
5′-TCC CGC CTG TGA CAT GCA TT-3′	3:7:4:6	4.17	Coelute with P = S peak	3.84	0.91	0.89	Coelute with full-length sequence

[a] GenPak FAX anion exchange column.
[b] Retention and migration times not normalized to an internal standard.
[c] Normalized to a T_7 internal standard.

however, separated from the main peak, although they coelute with full-length monophosphodiesters. Selectivity of capillary gel electrophoresis, on the other hand, appears to be solely a function of oligonucleotide length as all full-length partial phosphodiesters comigrate with the full-length, full-phosphorothioate. This complex selectivity pattern makes accurate identification/quantitation of the levels of process-related oligonucleotides in a phosphorothioate oligonucleotide sample less than straightforward.

In order to accurately determine process-related oligonucleotides in a phosphorothioate drug sample, it is first important to assess full-length content by gel electrophoresis. If the sample contains primarily (n-1) deletion sequences, then, anion exchange HPLC may be used to determine its monophosphodiester content. But, if the sample contains appreciable amounts of (n-2) deletion sequences, there would be an overestimate of the amount of monophosphodiesters by anion exchange HPLC due to coelution of (n-2) deletion sequences with full-length monophosphodiesters. At this point, it is necessary to adjust the anion exchange area-% value to reflect the presence of (n-2) deletion sequences in the sample. Similarly, if the sample were to contain significant levels of (n-3) deletion sequences, they would logically be expected to coelute with the full-length diphosphodiesters by anion exchange HPLC and further analysis is necessary to delineate contributions to the observed area-% from these components.

In summary, the complicated selectivity pattern of anion exchange HPLC and capillary gel electrophoresis and the necessity of using both techniques has been demonstrated for the separation of commonly found process-related oligonucleotides in synthetic phosphorothioates. This selectivity pattern appears to be maintained for phosphorothioate oligonucleotides of approximately 20-base units in length regardless of base composition or sequence.

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