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Journal of Chromatography A, 958 (2002) 167–182

JOURNAL OF CHROMATOGRAPHY A

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Ion-pair reversed-phase high-performance liquid chromatography analysis of oligonucleotides: Retention prediction

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Received 31 December 2001; received in revised form 19 March 2002; accepted 25 March 2002

Abstract

An ion-pair reversed-phase HPLC method was evaluated for the separation of synthetic oligonucleotides. Mass transfer in the stationary phase was found to be a major factor contributing to peak broadening on porous C_{18} stationary phases. A small sorbent particle size (2.5 μm), elevated temperature and a relatively slow flow-rate were utilized to enhance mass transfer. A short 50 mm column allows for an efficient separation up to 30mer oligonucleotides. The separation strategy consists of a shallow linear gradient of organic modifier, optimal initial gradient strength, and the use of an ion-pairing buffer. The triethylammonium acetate ion-pairing mobile phases have been traditionally used for oligonucleotide separations with good result. However, the oligonucleotide retention is affected by its nucleotide composition. We developed a mathematical model for the prediction of oligonucleotide retention from sequence and length. We used the model successfully to select the optimal initial gradient strength for fast HPLC purification of synthetic oligonucleotides. We also utilized ion-pairing mobile phases comprised of triethylamine (TEA) buffered by hexafluoroisopropanol (HFIP). The TEA–HFIP aqueous buffers are useful for a highly efficient and less sequence-dependent separation of heterooligonucleotides.

Keywords: Retention prediction; Oligonucleotides; DNA

1. Introduction

Synthetic oligonucleotides are utilized in many molecular biology applications including polymerase chain reaction (PCR), DNA sequencing [1,2], and genotyping [3–5]. The dramatic proliferation of oligonucleotide-based genetic assays was facilitated by an equivalent increase in oligonucleotide synthesis capacity. Although state-of-the-art oligonucleotide synthesis is a reliable, fast and highly efficient process, the multi-step nature of synthesis (subsequent coupling of mononucleotides into a linear oligonucleotide chain) results in a limited yield of target product [6]. The yield decreases with the oligonucleotide length [7]. Oligonucleotides designed as PCR primers are typically 15–35 nucleotides long (15–35mer) [8] with a typical yield rom syntheis of 60–80%. The byproducts of synthesis are

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00306-0

shorter oligonucleotides (termed failed sequences), where one or several nucleotides failed to be incorporated into the target sequence [6]. Certain applications (e.g. DNA sequencing) do not require purification of synthetic oligonucleotides [8,9], however, some applications including genotyping assays depend strongly on the oligonucleotide probe purity [5,10–12].

The technology of phosphoroamidite oligonucleotide synthesis as well as throughput has improved considerably in the last 10 years. In contrast, the methodology of oligonucleotide purification still relies on the traditional separation techniques such as polyacrylamide gel electrophoresis (PAGE), cartridge purification [6], or high-performance liquid chromatography (HPLC) [7].

Oligonucleotides purified by PAGE are considered to be of premium quality (>98%). However, the method is lengthy, laborious and the yield is limited. Ion-exchange HPLC separates oligonucleotides according the number of charged groups (phosphate linkages) [7,13]. The separation selectivity decreases with oligonucleotide length. In order to achieve a desirable product purity (>90%), part of the oligonucleotide is usually sacrificed by heart-cutting of the target peak. The collected product has to be desalted prior the use in bio assays.

Reversed-phase (RP)-HPLC purification has become a popular technique for oligonucleotide purification with the introduction of the ''trityl on'' (''DMT on'') method [14–18]. This method utilizes the hydrophobicity of the dimethoxytrityl (DMT) protecting group employed in oligonucleotide synthesis. The DMT group is not cleaved from the oligonucleotide 5′-end after the very last synthesis step to assist in RP-HPLC ''trityl on'' purification. The resulting synthetic mixture contains the full length ''trityl on'' product and shorter ''trityl off'' failure sequences. The separation selectivity between the target product and failure sequences is greatly enhanced due to the presence of trityl group, allowing for convenient purification of oligonucleotides up to ~100mer [18].

Notwithstanding the usefulness of the ''trityl on'' method, it has several drawbacks. The synthesis process generates a certain amount of failure sequences carrying the trityl group. As a result, ''trityl on'' purification delivers a product of limited purity. Also, the trityl group needs to be cleaved from the oligonucleotide after purification, either on-column [19] or off-column. The additional steps reduce yield and add to the total time and complexity of purification.

The ''trityl on'' RP-HPLC method is often combined with ion-exchange to purify oligonucleotides to greater than 95% purity for specific applications. The combination of two HPLC steps offers a product of high quality, but it is nearly as laborious as slab gel electrophoresis purification.

Reversed-phase high-performance ion-pair chromatography HPLC (RP-HPIPC) was used for the separation and purification of ''trityl off'' oligonucleotides [16,20–25]. Similar to ion-exchange HPLC, the separation selectivity decreases with increasing length of the oligonucleotides, which makes separation of longer oligonucleotides (>20mer) difficult. Both ion-exchange and RP-HPLC suffer from slow mass transfer of oligonucleotides in the stationary phase [26–28]. Although improved performance was achieved using non-porous sorbents and monolith columns (separation of >50 mer, sometimes up to the ~100 mer is feasible) [24,29], the limited mass capacity of these sorbents limits their usefulness for preparative applications.

The goal of this work was to develop a ''trityl off'' ion-pair reversed-phase HPLC method useful for fast analysis and purification of synthetic oligonucleotides. We utilized columns packed with a small particle size (2.5–5.0 μm) and a porous sorbent for the analysis and purification of 10–30mer oligonucleotides. We systematically studied the effect of particle size, column length, mobile phase flow-rate, separation temperature, and ion-pairing buffer composition on the oligonucleotide separation. We evaluated the impact of the oligonucleotide sequence on chromatographic retention and separation. An empirical mathematical model is proposed which predicts the elution behavior from oligonucleotide length and base composition.

2. Experimental

2.1. Chemicals and samples

Triethylamine, 99.5%; glacial acetic acid, 99.99%; 1,1,1,3,3,3-hexafluoro-2-propanol, 99.8% (HFIP);

HPLC-grade methanol and acetonitrile (ACN) were purchased from Aldrich (Milwaukee, WI, USA). Polyethylene glycol, M_r 35 000, was purchased from Fluka (Milwaukee, WI, USA). Tris(hydroxymethyl)aminomethane (Tris), urea, and boric acid were purchased from Sigma (St. Louis, MO, USA). A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water for HPLC mobile phases. DNA oligonucleotides were purchased from Hybridon (Milford, MA, USA), Midland Certified Reagents (Midland, TX, USA), and Ransom Hill Bioscience (Ramona, CA, USA). The ~10–30mer homooligonucleotide ladders $(dC)_{10-30}$, $(dA)_{10-30}$, $(dT)_{10-30}$ and CCG motif 10–30mer oligonucleotides were generated by pooling 15, 25 and 30mer in an approximately equimolar ratio and digesting the mix with 3′-exonuclease [30]. We used bovine intestinal mucosa, or snake venom phosphodiesterase I (Sigma). The heterooligonucleotide ladder was generated by digesting the following oligonucleotides: 15mer TCC CTA GCG TTG AAT; 20mer TCC CTA GCG TTG AAT TGT CC; 25mer TCC CTA GCG TTG AAT TGT CCC TTA G, and 30mer TCC CTA GCG TTG AAT TGT CCC TTA GCG GGT (5′–3′). The digestion progress was monitored by HPLC and terminated at an appropriate time by heating the sample for 10 min at 94 °C. Oligonucleotides were purified prior to digestion by RP-HPIPC.

2.2. HPLC instrumentation and columns

HPLC experiments were carried out using the following instruments: model 2690 Alliance HPLC system with a 996 photodiode array detector (Waters, Milford, MA, USA). The column was operated at 50–70 °C; the temperature was controlled by either a built-in column heater or a Nestlab Endocal water bath (Nestlab Instruments, Newington, NH, USA). HPLC conditions are given in the figure captions. HPLC columns used in this study were packed in house with XTerra MS C_{18} sorbent. We used following column dimensions: 30×4.6 mm, 50×4.6 mm, 75×4.6 mm and 150×4.6 mm.

2.3. Capillary electrophoresis

Electrophoretic separations were performed using a Waters Quanta 4000E capillary electrophoresis system. The separation was carried out in 34.5 cm long DNA Analysis Capillary (Bio-Rad, Hercules, CA, USA) 365 μm O.D.×75 μm I.D. (effective capillary length to detector was 27.5 cm). The capillary was filled manually with a sieving polymer solution prior to each analysis. Replaceable sieving matrix was prepared by dissolving 1.8 g of polyethylene glycol (M_r 35 000) in 7.2 ml buffer. The 0.1 *M* Tris–boric acid, 1.25 m*M* EDTA·2Na, 1 *M* urea buffer was prepared fresh prior to use. CE separation was performed at 15 kV. Separation temperature was 30 °C. Samples were injected into the capillary by applying a potential of 9 kV for 4 s.

2.4. HPLC peak resolution

Peak resolution was calculated as $R_S = 2(t_{R_2} - t_{R_1})/(w_{b1} + w_{b2})$. Retention time t_r and peak widths at base w_b are given in time units. The R_s value >1.5 indicates baseline resolution of adjacent peaks. The R_s criterion fails to describe the resolution of poorly resolved peaks where the values of the peak widths at the base cannot be obtained. In particular cases, we used the formula $d_0 = (h_p - h_v)/h_p$ for the description of 29/30mer resolution. The discrimination factor d_0 is calculated from the height of the smaller peak in the pair h_p (29mer) and the height of the valley between the separated peaks h_v. The discrimination factor value varies between 0 (coeluting peaks) and 1 (fully resolved peaks) [31].

3. Results and discussion

3.1. Isocratic vs. gradient separation of oligonucleotides

The analytical techniques for the separation of oligonucleotides are in principle based on the discrimination of the molecular size (length) or charge. It is known that 10mer and 11mer (10% difference in charge/length) will be separated more easily than 100mer and 101mer oligonucleotides (1% difference in charge/length) [32,33]. The decrease in separation selectivity with the increase in oligonucleotide length has been described for both ion-exchange [13] and

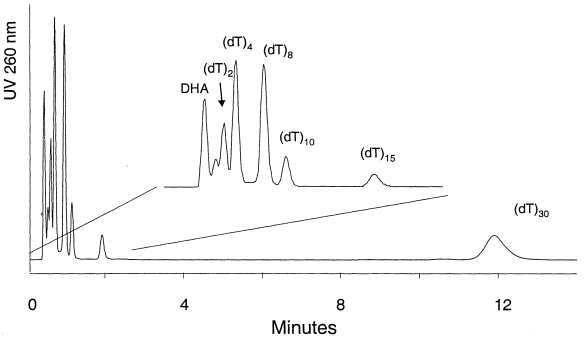

Fig. 1. Isocratic separation of oligodeoxythymidines. Inset shows an expanded view of the initial 2.5 min of chromatogram. The first peak is the void volume marker dihydroxyacetone (DHA). XTerra MS C_{18} 30×4.6 mm, 2.5 μm; flow-rate 1 ml/min, 60 °C. Mobile phase was acetonitrile–0.1 *M* TEAA, pH 7 (10:90, v/v).

ion-pairing RP-HPLC [21,24]; similar trends were observed in capillary gel electrophoresis [34–36].

Despite the fact that gradient elution reduces the separation selectivity, it is traditionally employed for the HPLC separation of biopolymers. Isocratic elution of biopolymers is not common [37], although it has been used as a part of the step gradient [38]. Due to differences in the chromatographic behavior of biopolymers and small molecules, many practitioners assume that the retention principles differ fundamentally. Some suggest that macromolecules are retained or precipitated at the column inlet, until they are desorbed by a gradient of mobile phase and eluted without any further interaction with the stationary phase. However, Snyder et al. successfully applied common theory to the description of biomolecules' chromatographic behavior [39]. The fact that the dynamic adsorption–desorption takes place during the analysis of oligonucleotides is underlined by isocratic separation of oligodeoxythymidines (Fig. 1). The crucial difference between small molecule and biopolymer analysis is the sharp change of the retention factor of biopolymers with a small change in mobile phase strength. Changes in the retention factor *k* with changes in the mobile phase strength were measured under isocratic conditions (Fig. 2). As seen from the plot, the changes in acetonitrile content have greater a effect on the retention of longer oligonucleotides than on shorter ones. For example, the *k* values of 15mer oligodeoxythymidine decreases from 100, 13.5, to 3.2 with the change of mobile phase composition from 8, 9 to 10% of acetonitrile, respectively.

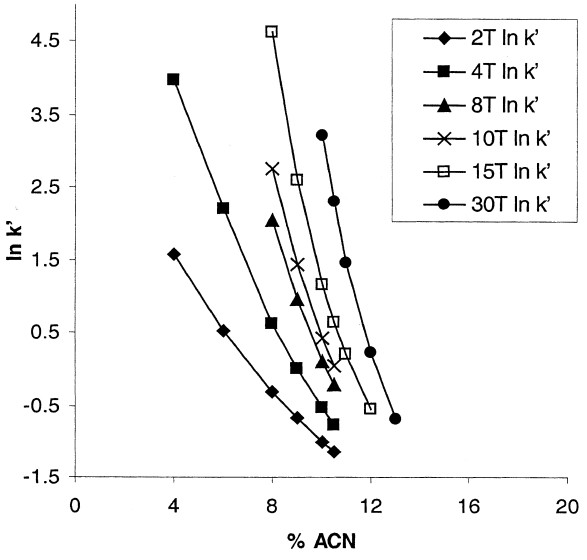

Fig. 2. Isocratic retention behavior of oligodeoxythymidines. Longer oligonucleotides show a sharper decrease in retention with the change of mobile phase strength. XTerra MS C_{18} 30×4.6 mm, 2.5 μm; flow-rate 1 ml/min, 60 °C. Mobile phase consisted of acetonitrile and 0.1 *M* TEAA, pH 7. Retention factor *k* for selected oligonucleotides was measured at 4, 6, 8, 9, 10, 10.5, 11, 12, and 13% (v/v) ACN.

This described retention behavior makes the use of isocratic elution for analysis of oligonucleotides impractical, as it would require a careful optimization of the mobile phase strength for every oligonucleotide length to achieve desirable retention.

3.2. Impact of mass transfer in stationary phase on oligonucleotide separation

Chromatographers developing oligonucleotide separation methods can choose from a wide selection of C_8 and C_{18} sorbents as well as sorbent particle sizes [16,20–22]. Judging from the published reports and our own evaluation [6], the choice of the reversed-phase sorbent type is not crucial, as many columns perform comparably well for oligonucleotide separation [21,24,40,41]. On the other hand, the impact of the particle size of the sorbent is often underestimated. We compared three columns packed with 2.5, 3.5 and 5 μm C_{18} sorbent using a mixture of 2–30mer oligodeoxynucleotides (Fig. 3). The

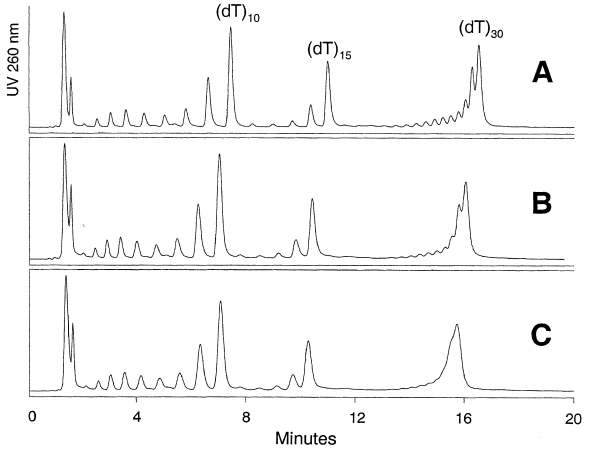

Fig. 3. Impact of sorbent particle size on the separation of a 2–30mer oligodeoxythymidine ladder. The separation was performed using a XTerra MS C_{18} 30×4.6 mm column packed with 2.5 μm (A), 3.5 μm (B), and 5 μm sorbent (C). Flow rate was 0.5 ml/min, and the column temperature 50 °C. Mobile phase A: acetonitrile–0.1 *M* TEAA, pH 7 (5:95, v/v). Mobile phase B: acetonitrile–0.1 *M* TEAA, pH 7 (20:80, v/v). Gradient starts at 26.7% B (9% ACN); at 20 min it reaches 53.3% B (13% ACN). The gradient slope was 0.2% of acetonitrile per minute (0.4% per milliliter).

separation conditions were identical, using a shallow gradient for elution. For separation conditions see the figure captions. The shorter oligonucleotides, including the 14mer and 15mer, are successfully separated on all three columns (Fig. 3), although noticeable peak broadening can be observed on the columns packed with 3.5 and 5 μm particles when compared to the 2.5 μm material. A substantial loss of separation is clearly visible in the region of 25–30mer oligonucleotides, where peak broadening degrades the resolution of closely adjacent peaks. One may expect that the gradient elution would sufficiently compensate for peak broadening and mask the loss of resolution, but this is apparently not the case.

As mentioned above, slow mass transfer of biopolymers in the stationary phase contributes significantly to peak broadening. Generally, for the separation of oligonucleotides (peptides, proteins) one should to use a column packed with a small particle size in order to shorten the diffusion path and improve the separation of slowly diffusing molecules. To evaluate the importance of mass transfer for oligonucleotide separation, we con-

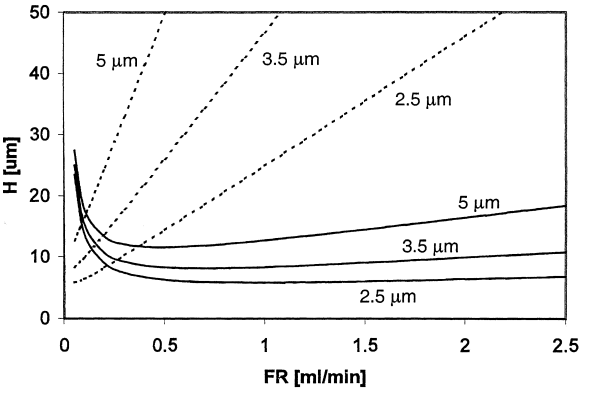

Fig. 4. Calculated Van Deemter curves for a 50×4.6 mm column. Parameters *A*, *B* and *C* were set to 1.5, 1 and 0.167, respectively. The change of the theoretical plate height *H* with the mobile phase flow-rate was calculated for two diffusion coefficients. (A) $D_m = 1.25 \cdot 10^{-9}$ m^2/s; full lines and (B) $D_m = 1 \cdot 10^{-10}$ m^2/s; dotted lines. The particle size of the column packing is indicated in the graph.

structed theoretical Van Deemter curves for two different analytes: benzene and a hypothetical oligonucleotide. Fig. 4 shows calculated Van Deemter curves for 50×4.6 mm columns packed with 5, 3.5, and 2.5 μm sorbent. The term values used for the Van Deemter curve construction were $A = 1.5$, $B = 1$, $C = 0.167$ [31]. Solid line curves in Fig. 4 representing benzene with diffusion coefficient $D_m = 1.25 \cdot 10^{-9}$ m^2/s (30 °C) exhibit the classical shape with a local minimum indicating the optimal flow-rate for a given column. When the diffusion coefficient value of D_m $1 \cdot 10^{-10}$ m^2/s is used for the calculation (D_m of hypothetical oligonucleotide [42]), the shape of the Van Deemter curve changes dramatically (dotted lines). The *C* term contribution to the theoretical plate height (*H*) becomes predominant, while the *A* and *B* terms' contribution is less significant. As a result, the Van Deemter curve assumes a nearly linear shape without a local minimum in an analytically useful flow-rate range. Fig. 4 suggests several important conclusions:

(i) The mass transfer in the stationary phase (*C* term of Van Deemter equation) has a major impact on oligonucleotide separation.

(ii) A small particle size of the chromatographic sorbent can, to a certain extent, reduce the impact of slow diffusion. Separation performance of 2.5 μm columns is dramatically better

than that of columns packed with 5 μm particles. The effect is more apparent for longer oligonucleotides that are separated with lower selectivity and have lower D_m values.

(iii) The oligonucleotide separation benefits from slow mobile phase flow-rate.

The impact of the flow-rate was evaluated experimentally. We measured the resolution of oligonucleotides using a 50·4.6 mm XTerra MS C_{18}, 2.5 μm column at a flow-rate range of 0.5–2 ml/min. The gradient time table was adjusted accordingly, so the gradient slope per mobile phase volume was identical for all experiments. For other HPLC conditions see the figure captions. In agreement with the theoretical prediction (Fig. 4) we observed an improvement in separation at low flow-rates. Retention time and resolution values are shown in Table 1. The resolution of 29 and 30mer oligonucleotides at a flow-rate of 2 ml/min is poor. However, fast flow-rates can be utilized for a rapid separation of oligonucleotides shorter than 25mer.

One possible strategy to minimize the negative impact of mass transfer is to use an elevated column temperature. The practical limitation is the sorbent stability. XTerra hybrid-silica sorbent is known to have greater stability at higher pH and temperature than conventional silica based sorbents [43]. We chose 60 °C as a standard working condition for further experiments. We performed about 1000 analyses under these conditions without detecting column performance deterioration.

Mass transfer can also be enhanced by using non-porous RP-HPLC sorbents [24,29,44] or Poroshell particles with improved mass transfer in the stationary phase [45]. However, porous sorbents have approximately an order of magnitude greater mass load capacity, which is important for preparative applications.

The data presented in Table 1 show that oligonucleotide separation benefits from the use of slow flow-rates. However, the use of slow flow-rates for improved separation requires longer analysis time. To remedy that, the faster gradients can be used. We evaluated the gradient slopes of 0.4, 1 and 2% of acetonitrile per milliliter of mobile phase. In order to design a gradient slope for different flow-rates, the slope is given in percent per milliliter. As one would expect, the steeper gradient shortened the analysis, however, it also reduced the separation selectivity. The gradient slope has greater effects on the separation of longer oligonucleotides (29/30mer) than the separation of shorter (14/15mer and 9/10mer) oligonucleotides (Table 2). We found that the separation of ~30mer oligonucleotides required a shallow gradient (0.25% acetonitrile/ml of mobile phase or slower). Such a gradient, however, may result in an excessively long analysis time. In order to minimize analysis time, the proper choice of initial mobile phase strength is important. Table 3 shows that adjusting the initial mobile phase strength (7, 8, and 9% ACN) while keeping the gradient slope constant one can shorten the analysis time without the loss of

Table 1
Impact of the mobile phase flow-rate on oligonucleotide separation

Mobile phase flow-rate[a] (ml/min)	Retention time (min)			Resolution[b]		
	9mer 10mer	14mer 15mer	29mer 30mer	9/10 mer	14/15 mer	29/30 mer
0.5	6.04	11.67	25.49			
	7.01	12.91	26.10	3.02	2.87	0.87
1.0	3.17	6.06	12.75			
	3.69	6.66	13.04	2.57	2.31	0.69
1.5	2.03	4.35	8.79			
	2.68	4.76	8.98	2.08	1.80	0.42
2.0	1.63	3.17	6.51			
	1.90	3.48	6.81	1.82	1.64	0.33

[a] Other HPLC conditions: 50×4.6 mm XTerra MS C_{18}, 2.5 μm column, A: 0.1 *M* TEAA, pH 7; B: acetonitrile–0.1 *M* TEAA, pH 7 (20:80, v/v), gradient starts at 26.7% B (9% ACN), gradient slope 0.1% acetonitrile/ml, column temperature 60 °C.

[b] Resolution 9/10mer and 14/15mer was calculated as $R_S = 2(t_{R_2} - t_{R_1})/(w_{b1} + w_{b2})$. Resolution 29/30mer was calculated as discrimination factor $d_0 = (h_p - h_v)/h_p$. For details see Experimental section.

Table 2
Impact of the gradient slope on oligonucleotide separation

Gradient slope[a] (% ACN/min)	Gradient slope (% ACN/ml)	Retention time (min)			Resolution[b]		
		9mer 10mer	14mer 15mer	29mer 30mer	9/10 mer	14/15 mer	29/30 mer
0.2	0.4	17.21	21.29	26.08			
		18.26	21.80	27.06	3.59	1.91	0.35
0.5	1.0	10.08	11.62	13.79			
		10.46	11.84	13.79	1.94	1.09	0
1.0	2.0	7.23	8.02	8.91			
		7.40	8.02	8.91	1.03	0	0

[a] Other HPLC conditions: 50×4.6 mm XTerra MS C_{18}, 2.5 μm column, A: 0.1 *M* TEAA, pH 7; B: acetonitrile–0.1 *M* TEAA, pH 7 (20:80, v/v), 0.5 ml/min, gradient starts at 13.3% B (7% ACN), column temperature 50 °C.

[b] Resolution 9/10mer and 14/15mer was calculated as $R_S = 2(t_{R_2} - t_{R_1})/(w_{b1} + w_{b2})$. Resolution 29/30mer was calculated as discrimination factor $d_0 = (h_p - h_v)/h_p$. For details see Experimental section.

oligonucleotide resolution. The exception is the resolution of shorter oligonucleotides, which are not well retained in 9% of acetonitrile.

We also evaluated the impact of column length on the oligonucleotide separation. The 30, 50, 75 and 150 mm columns (4.6 mm internal diameter) were packed with 2.5 μm sorbent. Analysis was performed using identical gradient. In agreement with theoretical prediction [39], we observed no significant improvement in resolution for 14/15mer and 29/30mer pairs (data not shown). This demonstrates the rational of using the short columns for separation of biopolymers. We chose a 50×4.6 mm column as a standard format for further experiments.

3.3. Impact of oligonucleotide sequence on RP-HPIPC retention and separation

The proper choice of mobile phase has a profound impact on the quality of the oligonucleotide separation. Some earlier published reports utilized mobile phase buffers comprised of ammonium acetate or other non-ion-pairing buffers and acetonitrile [46]. The RP-HPLC separation was therefore based purely on oligonucleotide hydrophobicity. A similar approach was later employed for LC–MS oligonucleotide analysis, since the buffer components are compatible with electrospray ionization (ESI) MS detection [47,48]. Fig. 5 shows an example of a RP-

Table 3
Effect of initial gradient strength on oligonucleotide retention and separation

Initial mobile phase strength[a] (% ACN)	Retention time (min)			Resolution[b]		
	9mer 10mer	14mer 15mer	29mer 30mer	9/10 mer	14/15 mer	29/30 mer
7	17.21	21.29	26.08			
	18.26	21.80	27.06	3.58	1.91	0.35
8	12.57	16.53	22.11			
	13.58	17.09	22.35	3.51	1.99	0.34
9	6.65	10.50	16.56			
	7.51	11.13	16.81	2.94	2.13	0.41

[a] Other HPLC conditions: 50×4.6 mm XTerra MS C_{18}, 2.5 μm column, A: 0.1 *M* TEAA, pH 7; B: acetonitrile–0.1 *M* TEAA, pH 7 (20:80, v/v), 0.5 ml/min, gradient slope 0.2% acetonitrile/min (0.4%/ml), column temperature 50 °C.

[b] Resolution 9/10mer and 14/15mer was calculated as $R_S = 2(t_{R_2} - t_{R_1})/(w_{b1} + w_{b2})$. Resolution 29/30mer was calculated as discrimination factor $d_0 = (h_p - h_v)/h_p$. For details see Experimental section.

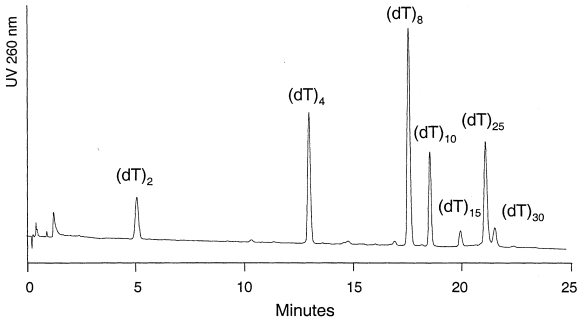

Fig. 5. Separation of 2, 4, 8, 10, 15, 25 and 30mer oligodeoxythymidines by RP-HPLC in non-ion-pairing conditions. XTerra MS C_{18} 50×4.6 mm column packed with 2.5 μm sorbent. The flow-rate was 1 ml/min, and the column temperature 60 °C. Mobile phase A: 25 m*M* NH_4HCO_3, 2 m*M* EDTA·2Na, pH 7.6. Mobile phase B: acetonitrile. The gradient starts at 2% B. Gradient slope was 0.15% of acetonitrile/min (milliliter).

HPLC separation of a homooligodeoxythymidine ladder in the range 2–30mer length. The separation performance is sufficient up to ~10mer, but sharply declines for longer oligonucleotides.

The separation of heterooligonucleotides in a RP-HPLC system could be challenging due to the different hydrophobicities of the A, C, T, and G bases. In fact, oligonucleotides of the same length but different sequence may exhibit different retention. This feature of RP-HPLC could be advantageous as well as challenging for some applications.

The introduction of ion-pairing buffers for oligonucleotide separation follows two basic goals: (i) to improve separation selectivity so that the longer (10–30mer) oligonucleotides can be resolved, (ii) to introduce charge–charge interaction in the separation mechanism to achieve a regular retention of oligonucleotides according to their chain length. Triethylammonium acetate (TEAA) is the most common ion paring mobile phase, but others have been employed [16,21,41,49,50]. The triethylammonium acetate gained popularity because it allows for good separation of oligonucleotides, and it is inexpensive, and volatile so there is no need for desalting the collected fractions. On the other hand, TEAA is a weak ion-pairing system that does not completely eliminate the impact of the oligonucleotide sequence on its retention. Oligonucleotides of the same length but with different base composition have different retention times. This is demonstrated by the analysis of four different homooligonucleotide ladders in Fig. 6. The separation of G homooligonucleotides is generally a problem for HPLC [13,51,52] because of the strong inter- and intramolecular interactions of G-rich DNA sequences. Therefore, to study the impact of G's contribution to the retention, we used a sequence with a repetitive $(CCG)_n$ motif.

From the experiment shown in Fig. 6 we can draw several conclusions. First, the base composition plays a role in oligonucleotide retention despite the use of the TEAA ion-pairing mobile phase. Second, the hydrophobicity contribution to the oligonucleotide retention increases in order C<G<A<T, which is in agreement with earlier published data [23,24]. Interestingly, the presence of G nucleotides in the overall C oligomer (the CCG motif oligonucleotide) only slightly increased the retention of the 30mer oligonucleotide (Fig. 6B). The retention of the 29mer CCG motif oligonucleotide was in fact lower than that of the all-C 29mer oligonucleotide (Fig. 6). Most interestingly, the G nucleotides had a pronounced effect on the spacing of the peaks in the CCG motif oligonucleotide ladder. Even though the overall presence of guanidine bases does not change the 30mer retention dramatically suggesting similar hydrophobicity of C and G, loss of a single G nucleotide from the 3′end enhances the separation of the 29/30mer peaks. Presumably, not only the hydrophobicity of the nucleobases but also steric effects (secondary structure, charge accessibility) contribute to the retention of oligonucleotides. The impact of the sequence is noticeable despite the elevated separation temperature (60 °C) and the fact that the oligonucleotide is not self-complementary.

The impact of a sequence on retention complicates the development of an HPLC method for the routine analysis of oligonucleotides. As was discussed earlier, our strategy for successful oligonucleotide sepration relies on the combination of a shallow gradient (Table 2) and the proper choice of initial mobile phase strength (Table 3). The mixed-sequence oligonucleotides with average content of C, G, T and A all have similar retention. Therefore, we used generic HPLC conditions with starting mobile phase strength of 7% acetonitrile, a slope of 0.25% ACN per milliliter, and a flow-rate of 1 ml/min (column temperature is set to 60 °C). However, the C-rich or T-rich oligonucleotides may elute outside

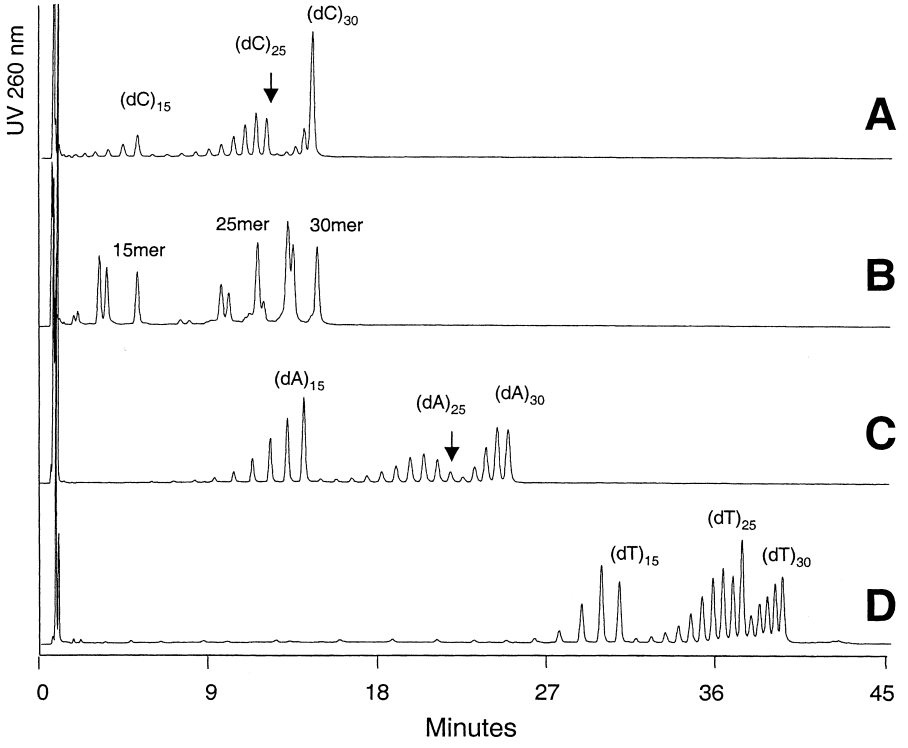

Fig. 6. Separation of a 10–30mer deoxycytidine ladder (A), 10–30mer oligonucleotide ladder comprised of a repeating CCG motif (B), 10–30mer deoxyadenosine ladder (C) and 10–30mer deoxythymidine ladder (D). Separation conditions were identical. XTerra MS C_{18} 50×4.6 mm column was packed with 2.5 μm sorbent. Flow rate was 1 ml/min, column temperature 60 °C. Mobile phase A: acetonitrile–0.1 *M* TEAA, pH 7 (5:95, v/v). Mobile phase B: acetonitrile–0.1 *M* TEAA, pH 7 (15:85, v/v). Gradient starts from 0% B (5% ACN), at 45 min it reaches 67.5% B (11.75% ACN); gradient slope was 0.15% of acetonitrile/min (milliliter).

of a useful time range (either in void volume, or in extensively long time). Modification of these generic separation conditions is then required.

Semi-empirical case-to-case optimization of the separation conditions for unusual oligonucleotides is feasible, but not practical. The large-scale routine purification of synthetic oligonucleotides would require reliable guidelines for choosing suitable separation conditions. Earlier attempts to predict oligonucleotide HPLC behavior from length and sequence [16,32,33,41,53] did not provide a useful solution. No method applicable for heterooligonucleotide retention prediction in HPLC is available to date. We propose a method employing a mathematical regression of empirically measured retention data for a series of homooligonucleotides. This method is base on several assumptions:

1. The oligonucleotide secondary structure does not affect its retention and is effectively eliminated under the HPLC conditions (60 °C, acetonitrile content).
2. The oligonucleotide is completely adsorbed on the stationary phase in a mobile phase weaker than the certain critical value. When the gradient reaches the critical mobile phase strength, oligonucleotide retention is infinitely small and the oligonucleotide is eluted.
3. This critical mobile phase strength (% of ACN) is equal to the mobile phase strength at the moment of the elution of oligonucleotide peak.
4. Ion-pairing efficiency does not change during the gradient elution.

Using empirical mathematical modeling, we found that the dependence of elution strength of the mobile phase versus the homooligonucleotide length can be accurately described by a logarithmic function (Eq.

(1)), where y is the mobile phase strength (in this case acetonitrile%), z and w are constants and N is the number of the nucleotides in the oligonucleotide chain.

$$y = z \ln (N) + w \quad (1)$$

Since the retention in RP-HPIPC is comprised of the hydrophobic and charge–charge interactions, the constants z and w combine the contribution of both retention mechanisms.

The values of the elution mobile phase strength (% of ACN) calculated from the retention time of peaks for homooligonucleotide ladders $(dC)_{10-30}$, $(dA)_{10-30}$, and $(dT)_{10-30}$ (Fig. 6) were plotted against the chain length N. A logarithmic trend line was fitted to experimental data (10–30mer) to obtain the constant values from Eq. (1). The homo-oligoguanidine equation parameters were obtained by modifying the fit from the homooligocytidine series. We added 0.18% of acetonitrile to the G series, which is a difference in the elution strength of the 30mer C oligonucleotide and the 30mer CCG motif oligonucleotide multiplied by three (CCG motif oligonucleotide has only 1/3 of G's in the sequence). We assume that the value 0.18% represents the difference between all G 30mer and all C 30mer oligonucleotides. The experimental data fitted with logarithmic trend lines are shown in Fig. 7. Because of the overall similar retention trend, the constant g in Eq. (5) is equal to constant c. Eqs. (2)–(5) with the corresponding parameters derived from the mathematical fit are shown below. The r^2 value for Eq. (5) is 1.0 because the G series equation was mathematically derived from the y_C equation (Eq. (4)) as described above.

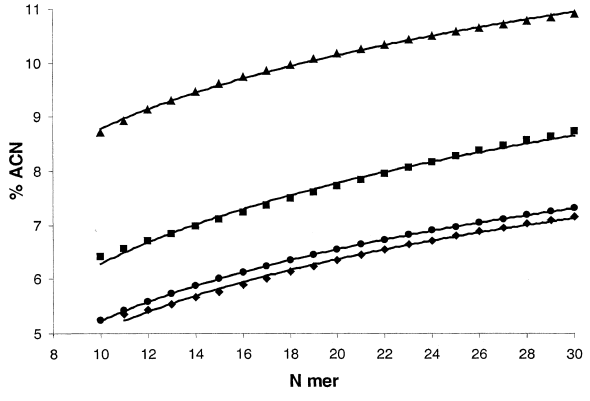

Fig. 7. Experimentally measured retention data for homooligonucleotide ladders (Fig. 6) were accurately described by logarithmic function (Eq. (1)). The constants derived from the mathematical fit as well as regression coefficients are shown in the text (Eqs. (2)–(5)). Oligo dT data (triangles), oligo dA data (squares), oligo dG data (circles), oligo dC data (diamonds).

$$y_A = a \ln N + e = 2.109 \ln N + 1.47$$
$$r^2 = 0.9928 \quad (2)$$

$$y_T = t \ln N + f = 2.125 \ln N + 3.77$$
$$r^2 = 0.9947 \quad (3)$$

$$y_C = c \ln N + h = 1.887 \ln N + 0.71$$
$$r^2 = 0.9951 \quad (4)$$

$$y_G = g \ln N + i = 1.887 \ln N + 0.89$$
$$r^2 = 1.0000 \quad (5)$$

For mononucleotides the N value is equal to 1. The logarithm component of the equations then equals zero and the e, f, h and i constant values should represent the elution order of the C, G, A and T mononucleotides, respectively. The mononucleotide retention data published in the literature C<G<T<A are not in agreement with the predicted order [7,54]. However, the retention of dinucleotides $(dC)_2 < (dG)_2 < (dA)_2 < (dT)_2$ follows the predicted pattern [55].

Eqs. (2)–(5) can be used to predict the HPLC retention of homooligonucleotides, however, our goal is to predict the retention behavior of heterooligonucleotides as well. For this purpose we combined the parameters and rearranged the equations into Eq. (6). The n_c, n_g, n_a, and n_t values are the number of C, G, A, and T nucleotides in a heterooligonucleotide sequence where $N = n_c + n_g + n_a + n_t$.

$$y = \frac{n_a}{N} \cdot [a \ln N + e] + \frac{n_t}{N} \cdot [t \ln N + f] + \frac{n_c}{N} \cdot [c \ln N + h] + \frac{n_g}{N} \cdot [g \ln N + i] \quad (6)$$

Eq. (6) can be presented in a simple logarithmic form (Eq. (7)) with the constants essentially repre-

Table 4
List of oligonucleotides used for evaluation of the prediction model

Oligo-nucleotide	Length	Sequence (5'-3')	A	T	C	G	Predicted[a] %ACN	Experimental[b] %ACN	Δ %ACN
1	16	GTAGCAGCAGCCAGAC	5	1	5	5	6.66	7.12	0.46
2	16	GTCTGGCTGCTGCTAC	1	5	5	5	7.25	7.66	0.42
3	16	GACATGGGCTCCGCTC	2	3	6	5	6.87	7.22	0.36
4	18	GAGATCTAGGTGATGTGAC	4	5	2	7	7.59	7.84	0.25
5	18	ACAGTATTGCTGGCGGAG	4	4	3	7	7.38	7.92	0.55
6	19	GAAGAATTGGTAGGAGTCC	6	4	2	7	7.57	7.72	0.15
7	20	ACTGGGAACGAGGGCGACGT	5	2	4	9	7.18	7.77	0.59
8	20	TCAGACGAGAAGGACCTGAC	7	2	5	6	7.29	7.78	0.48
9	20	TTCGCCAGCAACAATGTGAC	6	4	6	4	7.58	8.47	0.89
10	22	GTCCTTTGAATGCAGAAATGGC	6	6	4	6	8.02	8.57	0.55
11	22	ATGGTTCCTGTGCAACTGTGAC	4	7	5	6	8.06	8.75	0.69
12	23	CAGCCCCTTCCCTCTGCCGCCAG	2	4	13	4	7.45	7.89	0.44
13	25	ACCTCTGCACCCATCTCTCTCCTCA	4	7	13	1	8.10	9.02	0.92
14	26	GGGGTACCAGTATCGAAAGCACCTCC	7	4	8	7	7.90	8.47	0.57
15	26	GCGAATTCCAATACCGATATTTCGGA	8	7	6	5	8.38	9.26	0.88
16	26	GCGAATTCTTATTTAGGATTGATAGC	7	10	3	6	8.77	9.42	0.65
17	27	TGGGAATTCAGTGCTTGGTCACTTCCG	4	9	6	8	8.49	9.20	0.71
18	30	GGGCTTCATGATGTCCCCATAATTTTTGGC	5	11	7	7	8.84	9.48	0.63
19	30	AGCGTTGGCTACCCGTGATATTGCTGAAGA	7	8	6	9	8.57	9.37	0.80
20	32	CTTACCCAACAAGTGCGGCCGATCGAATTGAG	9	6	9	8	8.45	9.22	0.77
21	32	CTCAATTCGATCGGCCGCACTTGTTGGGTAAG	6	9	8	9	8.68	9.37	0.69
22	32	GCCAGGTGCCAGGCTGGGTTGCCATTGATGGC	4	7	8	13	8.37	8.82	0.46
23	32	CCGCTCGAGGGTTCTCTGGATAACATCTCAGC	6	8	10	8	8.55	9.19	0.63
24	33	CTCCAACGGGAACCTGGCCCCCTGGGCAGGGGG	5	3	12	13	7.97	8.75	0.78
25	33	CCCCCTGCCCAGGGGGCCAGGTTCCCGTTGGAG	3	5	13	12	8.10	8.88	0.78
26	34	CCTGCGCATCATTTAATCAGAATGGCATTCACCG	9	9	10	6	8.84	9.71	0.87
27	34	CGGTGAATGCCATTCTGATTAAATGATGCGCAGG	9	9	6	10	8.86	9.53	0.67
28	37	AATGGATCCGGAGGAGGAGGGGACACCTTCATCCGTC	9	6	9	13	8.60	9.56	0.95
29	39	CCCAAGCTTATGGGAAACGCGGCGGCGGTGGGCCAGGCG	7	4	11	17	8.39	9.06	0.67
30	39	CCATCCAGGGGGGAGCGGCCGCCCCTCGACCTCAGAGCC	6	3	17	13	8.23	8.88	0.65
31	39	CATCCTCAGGTAGGCACTGAGCATCCCCCCATACGCTCC	8	7	17	7	8.68	9.37	0.69
32	39	CCTCAGGTAGGCACTGAGCATCCCCCCATAAGCTCCACC	9	6	17	7	8.62	9.29	0.67
33	41	CATCCTCAGGTAGGCACTGAGCATCCCCCCATAACTTCCTC	9	9	17	6	8.96	9.34	0.38
34	48	GGAGCCACTGGCCACGCCGCCGCTTTGGATGCGCGGGAGGTGATTCCC	6	8	16	18	8.95	9.56	0.61
35	54	TATAAGCTTGAAGGGGAACGAAAGATGGCGGCGGAAACACTGCTGTCCAGTTTG	16	11	9	18	9.60	10.08	0.48
36	56	AGAGAATTCCCACCATGGACTACAAGGACGACGATGACAAGTCTCAGAGCAACCGG	20	7	15	14	9.44	9.87	0.42
37	57	ATTTCTAGATTACTATTTGTCATCGTCCTTATAGTCAAAGTCAAATTCGTCCAGGTC	15	22	12	8	10.35	10.72	0.37
38	57	CAATATTTTACATGAACTGGAGGTCCGTCAATGACAGTGTAGGCTGGAGCTGCTTCG	14	16	11	16	9.93	10.17	0.24
39	60	GACTTAGACTTAGACTTAGACTTAGACTTAGACTTAGACTTAGACTTAGACTTAGACTTA	20	20	10	10	10.37	10.57	0.20

[a] Predicted values of acetonitrile% were calculated according to formula 7.

[b] Experimental values of acetonitrile% were measured using 50×4.6 mm XTerra MS C_{18}, 2.5 μm column, flow-rate 1 ml/min, 60 °C. Mobile phase A: acetonitrile–0.1 *M* TEAA, pH 7 (5:95, v/v). Mobile phase B: acetonitrile–0.1 *M* TEAA, pH 7 (15:85, v/v). Gradient starts at 5% acetonitrile, gradient slope was 0.25% acetonitrile/min (milliliter).

senting the averaged parameters derived for the homooligonucleotide retention.

$$y = \left(\frac{n_a a + n_t t + n_c c + n_g g}{N}\right) \cdot \ln N + \left(\frac{n_a e + n_t f + n_c h + n_g i}{N}\right) \quad (7)$$

The constructed model predicts that oligonucleotides of the same base composition should be identically retained without an impact of the nucleotide sequence. According to the literature data, such heterooligonucleotides in fact exhibit very similar, but not identical retention [21]. This does not undermine the utility of the model for the design of mobile phases for oligonucleotide purification.

We assume that the proposed model describes the oligonucleotide retention with acceptable accuracy in the range of 10–60mers. The exception may be oligonucleotides shorter than 10mer, which do not fulfill the above mentioned assumption of a sharp change in retention with an infinitely small change in mobile phase strength (Fig. 2). We expect that the applicability of the model for longer than ~60mers may be limited, as they are more likely to form strong secondary structures or intramolecular complexes.

To confirm the validity of the model, we correlated theoretically predicted data with the experimentally measured retention of thirty nine different heterooligonucleotide sequences. Samples were chosen to cover the length range of 16–60mers. Sequences and retention values are given in Table 4. Fig. 8 illustrates a strong correlation between predicted and experimentally measured mobile phase strength. The correlation coefficient $r^2 = 0.946$ suggests that the model describes the retention behavior of the oligonucleotide set with good accuracy. It should be noted that the real values of the mobile phase strength are systematically higher than predicted (Table 4). The average difference (Δ ACN%) is about 0.6%. This systematic error should be taken into account when designing gradient conditions for the purification of synthetic oligonucleotides.

3.4. Impact of sequence on heterooligonucleotide separation

The oligonucleotide sequence has an impact not only on retention, but also on the success of the target product separation from failure sequences. Fig. 6 demonstrates that homooligonucleotide ladders are generally separated with even spacing between peaks. However, as is case with the heterooligonucleotide ladder (CCG motif oligonucleotide), the spacing between peaks is affected by the sequence. According to the experimental data, the hydrophobic contribution of C and G mononucleotides to the oligonucleotide retention is less significant than A and T. Therefore, peak retention for a heterooligonucleotide ladder may be affected by its nucleotide sequence. Some fragments may not be well resolved, while others are separated with better than average selectivity. More extensive discussion of the sequence-dependent separation rules was published earlier [56].

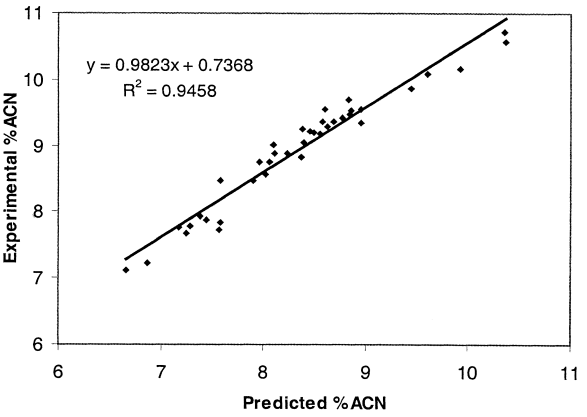

Fig. 8. Correlation between the predicted and experimentally measured mobile phase strength for the elution of oligonucleotides. Thirty-nine heterooligonucleotides of chain length 16–60mer were used for the experiment. For oligonucleotide sequences and base composition, see Table 4.

Fig. 9 shows the separation of a heterooligonucleotide ladder in three different ion-pairing buffers. The ladder was prepared by 3′-exonuclease as described in the experimental section and spiked with 20, 25 and 30mer oligonucleotides. Fig. 9A illustrates that certain oligonucleotides are not well separated in the TEAA ion-pairing mobile phase. In order to improve the separation, we prepared an alternative ion-pairing buffer comprised of triethylamine–hexafluoropropanol (TEA–HFIP) [57]. This buffer–methanol mobile phase was described to be compatible with ESI-MS detection [41,49,58], allowing for LC–MS analysis of oligonucleotide purity

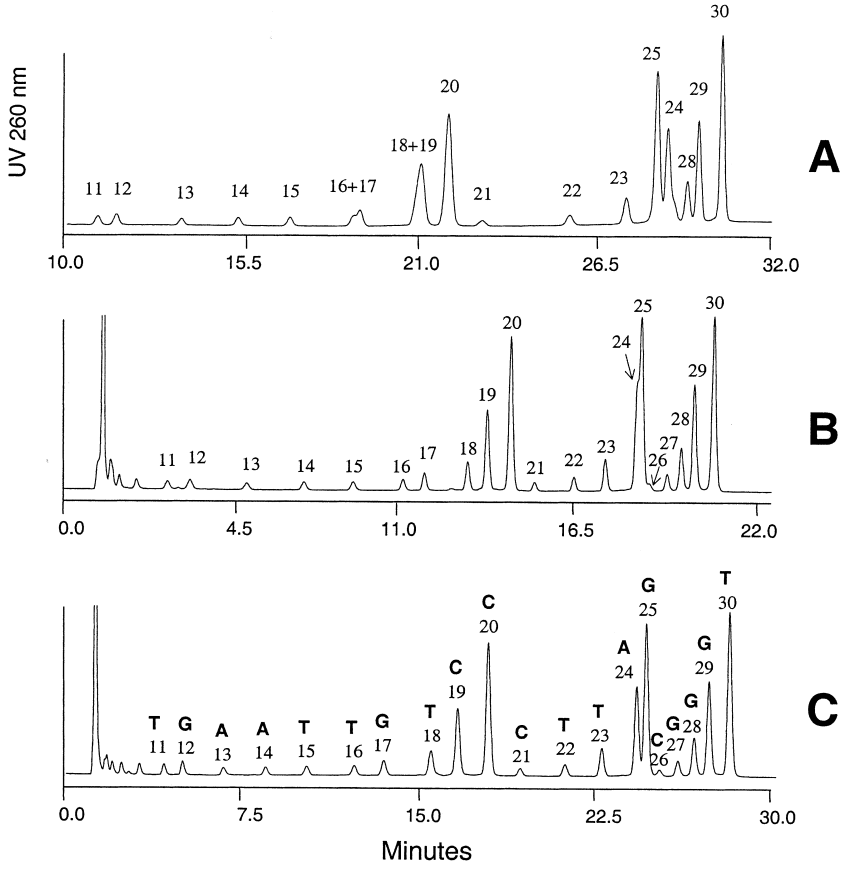

Fig. 9. Separation of a 10–30mer heterooligonucleotide ladder using three different ion-pairing buffers. (A) 0.1 *M* TEAA, pH 7, ion-pairing system. Mobile phase A: acetonitrile–0.1 *M* TEAA, pH 7 (5:95, v/v). Mobile phase B: acetonitrile–0.1 *M* TEAA, pH 7 (15:85, v/v). Gradient starts from 5% acetonitrile; gradient slope was 0.25% acetonitrile/min. (B) 4.1 m*M* TEA, 100 m*M* HFIP ion-pairing buffer. Mobile phase A: 10% methanol, 90% of aqueous buffer of 4.1 m*M* TEA, 100 m*M* HFIP buffer, pH 8.2. Mobile phase B: 40% methanol, 60% of 4.1 m*M* TEA, 100 m*M* HFIP buffer, pH 8.2. Gradient starts at 10% methanol. Gradient slope was 0.25% methanol/min. (C) 16.3 m*M* TEA, 400 m*M* HFIP ion-pairing buffer. Mobile phase A: 10% methanol, 90% of 16.3 m*M* TEA, 400 m*M* HFIP buffer, pH 7.9. Mobile phase B: 40% methanol, 60% of 16.3 m*M* TEA, 400 m*M* HFIP buffer, pH 7.9. Gradient starts at 16% methanol and the gradient slope was 0.23%/min. The length of the oligonucleotide fragments is indicated above peak apex along with the 3′-terminal nucleotide. All separations were performed using an XTerra MS C_{18} 50×4.6 mm column packed with 2.5 μm sorbent. Flow rate was 1 ml/min, and the column temperature 60 °C.

[56]. Despite the fact that the concentration of the triethylammonium ion-pairing agent in the TEA–HFIP buffer is lower than in TEAA, we observed an improvement in separation (Fig. 9B). A complete separation of all peaks in the oligonucleotide ladder was achieved with a 16.3 m*M* TEA, 400 m*M* HFIP, pH 7.9, ion-pairing buffer (Fig. 9C). The oligonucleotide length and 3′ end nucleotide is indicated at the peak apex. The separation using a buffer comprised of 4.1 m*M* TEA, 100 m*M* HFIP, pH 8.2, (Fig. 9B) indicates a more pronounced effect of the sequence on oligonucleotide retention. Noticeably, the 24 and 25mer coelute and the separation of some other peaks shows lower than average selectivity (for example 16/17mer, 18/19mer and 11/12mer). The most significant contribution of the sequence was observed with the TEAA (Fig. 9A). Although the 30mer is still successfully resolved from the 29mer, the 24–27mer peaks, as well as the 18/19mer and 16/17mer peaks coelute. The proposed retention model can, to a certain extent, predict the peak spacing taking into account that the change of

oligonucleotide retention with the addition (or loss) of a single mononucleotide correlates with the nucleotide hydrophobicity C<G<A<T. In other words, the addition of C or G does not increase oligonucleotide retention as dramatically as the addition of A and particularly T.

From Fig. 9A, it appears that the 24mer and 25mer switched retention order. This was confirmed by spiking additional 25mer oligonucleotide into the sample. This surprising behavior is not in agreement with the proposed retention model. The data published in the literature suggest that terminal nucleotides may have a stronger impact on oligonucleotide retention than other nucleotides within the sequence [17,59]. Fig. 9A indicates that the separation of a crude synthetic oligonucleotide from N-1 and N-2 failure fragments may be difficult for some particular sequences [56]. TEA–HFIP ion-pairing buffers may offer a better separation performance.

The 16.3 m*M* TEA, 400 m*M* HFIP buffer, pH 7.9, with methanol gradient was employed for the separation of homooligonucleotide ladders. Fig. 10 shows a less pronounced impact of the oligonucleotide hydrophobicity on its retention than was observed in the TEAA mobile phase (Fig. 6). Presumably, the retention is mainly due to ion-pairing interaction. Interestingly, the CCG motif oligonucleotide is slightly less retained than the $(dC)_{10\text{-}30}$ ladder and shows regular peak spacing (compare Figs. 6B and 10B). Despite the lower concentration of TEA^+ pairing ion in TEA–HFIP buffers, the separation selectivity of ~10–25mer oligonucleotides is superior compared to the TEAA. It is known that the charge-to-charge interaction strength in ion-pairing

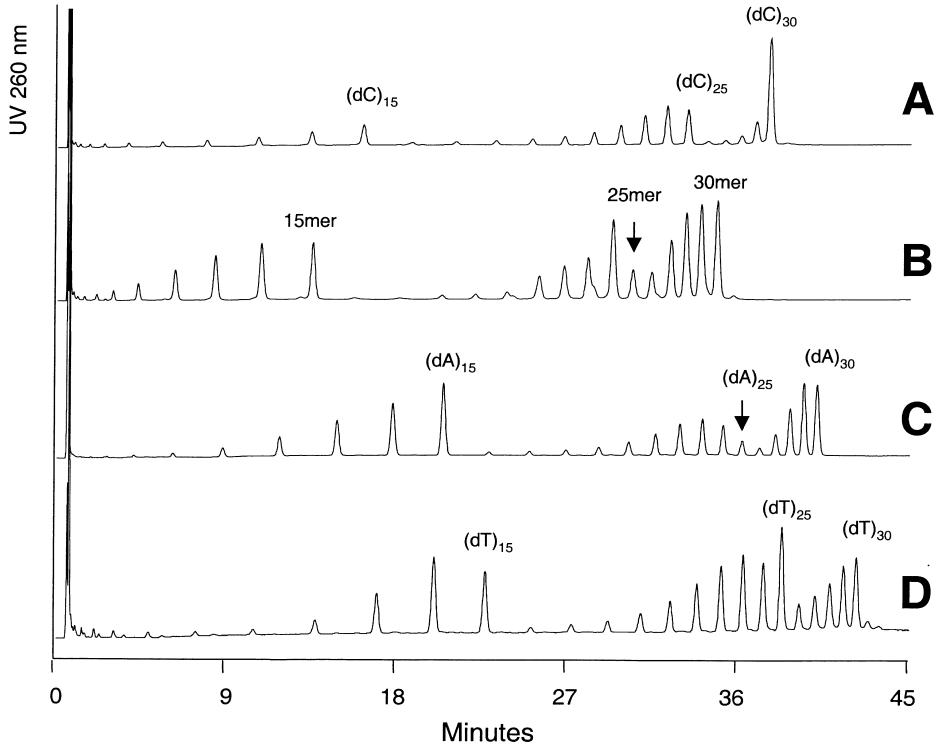

Fig. 10. Separation of a 10–30mer deoxycytidine ladder (A), 10–30mer oligonucleotide ladder comprised of a repeating CCG motif (B), 10–30mer deoxyadenosine ladder (C) and 10–30mer deoxythymidine ladder (D). All separations were performed using identical conditions on an XTerra MS C_{18} 50×4.6 mm column packed with 2.5 μm sorbent. Flow rate was 1 ml/min, and the column temperature 60 °C. Mobile phase A: 10% methanol, 90% of aqueous buffer consisting of 16.3 m*M* TEA and 400 m*M* HFIP, pH 7.9. Mobile phase B: 40% methanol, 60% of aqueous buffer consisting of 16.3 m*M* TEA and 400 m*M* HFIP, pH 7.9. The gradient starts at 12.5% methanol and the gradient slope was 0.25% of methanol/min (milliliter).

chromatography depends on the concentration of the pairing ion adsorbed on the sorbent surface [60,61] rather than the concentration of the pairing ion in the mobile phase. We believe that the TEA–HFIP buffer is an efficient ion pairing system because of the limited solubility of TEA^+ in the HFIP buffer (16.3 m*M* is the highest concentration of TEA we were able to dissolve in a 400 m*M* HFIP aqueous solution) [56]. The poor TEA^+ solubility effects its distribution between the mobile and the stationary phase. Taking into account that methanol dramatically improves the TEA^+ solubility, the increase of its concentration during the chromatographic separation results in a decrease of ion-pairing efficiency. This can be observed as a substantial decline of the separation selectivity for oligonucleotides longer than 25mer (Fig. 10).

Due to the substantial changes in ion-pairing interaction strength during the gradient, the oligonucleotide retention (% of methanol versus N) could not be accurately fitted by the logarithmic Eq. (1) when using the TEA–HFIP buffer system. However, Fig. 10 suggests that a generic gradient is applicable for all oligonucleotides independent of their sequence. The TEA^+ adsorption on the stationary phase in the TEAA buffer system does not seem to be significantly affected by the acetonitrile gradient [54]. The proposed retention model is valid for the TEAA ion-pairing system in a range of acetonitrile concentrations used for oligonucleotide separation.

4. Conclusion

The ion-pair reversed-phase HPLC method was evaluated for fast analysis and purification of synthetic oligonucleotides. We developed a mathematical model for the prediction of oligonucleotide retention from nucleotide composition. The prediction is based on the assumption that different types of nucleotides add differently to the retention. Retention behavior of heterooligonucleotides was mathematically extrapolated from the retention of homooligonucleotide ladders. The model is useful for a rational choice of the initial mobile phase strength, which is crucial to reduce the analysis time. We applied the model to the fast purification of 0.01–0.1 μmol synthetic oligonucletides in a single injection.

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