Improvements in Anion Exchange HPLC Purity Analysis of Double-Stranded Nucleic Acid Drugs

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Abstract

HPLC studies with a prototype duplex DNA oligonucleotide using a Dionex DNA-Pac PA100 anion exchange column demonstrated beneficial effects on oligonucleotide resolution when mobile phases were pretreated with a proprietary multivalent cation decontamination resin. Furthermore, it was shown that removal of contaminating multivalent cations significantly reduced the melting temperature (T_m) of the oligonucleotide duplex. Addition of a proprietary co-solvent to the mobile phase led to an improvement in resolution at high temperature (above 60˚C). These mobile phase treatments do not require specialized equipment and are easily employed in any laboratory. These mobile phase treatments improved the potential effectiveness of anion exchange HPLC for analyzing the purity of the prototype oligonucleotide drug.

Introduction

The advent of widespread oligonucleotide drug development spurred many advances in anion exchange HPLC chromatography at high temperature for purity analysis of nucleic acids. The structural basis of this method is the strong interaction between the negatively charged phosphate backbone of the oligonucleotide and the positively charged stationary phase, and the need to denature the oligonucleotide duplex on the column during chromatography. Oligonucleotides are eluted by increasing the salt concentration in the mobile phase. Many of these advances are based on the development of new HPLC column stationary phases that are designed to withstand these conditions better than matrices available previously. Although high temperature can improve resolution by melting secondary structure in single stranded nucleic acid drugs, potential oligonucleotide degradation resulting from the high temperature complicates the resolution analysis. While advancements in stationary phases have allowed for purity analysis of nucleic acids, there is room for improvement in terms of duplex melting (denaturing) temperature and resolution. These factors are also affected by mobile phase properties, such as organic co-solvent used and multivalent cation contamination.

In the current study, a synthesized 20 base pair DNA oligonucleotide with a randomly selected sequence (5'-ATGCAAACGTGGGTAGCTTG-3') was annealed with a synthesized complementary oligonucleotide to generate a model duplex drug for these studies. The duplex was analyzed by high temperature anion exchange HPLC using four different sets of mobile phases (A and B) to evaluate mobile phase effects on melting temperature and resolution. The column temperature was varied from 40˚C to 90˚C. For each set of mobile phases, the T_m—estimated to be the temperature midway between the temperature of complete on-column denaturation and the highest temperature at which no denaturing was observed by HPLC—was determined. For both co-solvents, the T_m dropped dramatically (80˚C to 45-50˚C) when mobile phases were decontaminated with the proprietary resin to remove divalent cations. Furthermore, the resolution between the two complimentary strands increased dramatically throughout all the temperature ranges evaluated (approximately five fold greater at 90˚C) when mobile phases were pretreated with the proprietary resin. At temperatures above 60˚C, the proprietary co-solvent enhanced the resolution when pretreated with the proprietary resin. The large magnitude of the differences observed in HPLC analysis (T_m and resolution) for decontaminated mobile phases was unexpected. The results above suggest that removal of divalent cations from mobile phases is an important factor in achieving optimized anion exchange HPLC analysis of nucleic acid drugs.

Experimental

Four sets of mobile phases (A and B) were used in the experiment. With each mobile phase set used, the column temperature was varied in 5°C increments from 40°C to 90°C. Only the mobile phases used and the column temperature were varied during the experiment; all other chromatographic parameters and samples injected remained constant.

A 0.4mg/mL double-stranded DNA sample was prepared by annealing two complimentary 20 base pair single-strands (named "Strand A" and "Strand B" arbitrarily) in a 16mM PO_4 solution. With each mobile phase set listed in Table 1, one injection of the 0.4 mg/mL oligonucleotide duplex was made at each column temperature. The extent of denaturation of the double strand was observed from 40°C to 90°C. 0.2mg/mL preparations of each single strand in 16mM PO_4 were also injected at 90°C for peak identification purposes.

Table 1: Mobile Phase Composition for Analytical Runs

20% ACN, No Resin	A: 20% ACN / 80% 20mM PO_4, pH 8.0
	B: 20% ACN / 80% 20mM PO_4, 1M NaBr, pH 8.0
20% ACN, Resin[1]	A: 20% ACN / 80% 20mM PO_4, pH 8.0
	B: 20% ACN / 80% 20mM PO_4, 1M NaBr, pH 8.0
20% PC[2], No Resin	A: 20% PC[2] / 80% 20mM PO_4, pH 8.0
	B: 20% PC[2] / 80% 20mM PO_4, 1M NaBr, pH 8.0
20% PC[2], Resin[1]	A: 20% PC[2] / 80% 20mM PO_4, pH 8.0
	B: 20% PC[2] / 80% 20mM PO_4, 1M NaBr, pH 8.0

[1]60 mL of resin was added per liter of mobile phase. Incubated with magnetic stirring such that resin was in contact with the entire volume of mobile phase for approximately 15 hours. Filtered through a 0.45μm nylon filter membrane prior to use.

[2]PC = Proprietary Co-solvent

Table 2: Mobile Phase Gradient

Time (min.)	% Mobile Phase A	% Mobile Phase B
0.0	90	10
30.0	0	100
35.0	0	100
36.0	90	10
40.0	90	10

Table 3: Chromatographic Parameters

HPLC System:	Agilent 1200 series equipped with DAD detector		
Column:	Dionex, DNAPac PA-100, 4 x 250 mm		
Flow Rate:	1.0 mL/min	Column Temp:	Varied (40°C to 90°C)
Detection:	UV @ 260 nm	Injection Volume:	10 μL
Sample Temp:	5°C	Run Time:	40 minutes

The basic set of mobile phases (A and B) consisted of 20% ACN, 20mM PO_4, pH= 8.0 and 20% ACN, 20mM PO_4, 1M NaBr, pH= 8.0. A second set of mobile phases was prepared with a proprietary co-solvent at 20% v/v instead of ACN. A portion of each set of mobile phases was then incubated with a proprietary resin to remove contaminating divalent cations. Oligonucleotide peaks were eluted by linear gradient from 10% B to 100% B over 30 minutes.

Results

Table 4: Summary of Results

Mobile Phase	Strand Resolution at 90°C	T_m	Strand Resolution at T_m	Strand Resolution at Lowest Fully Resolved Temperature, (°C)
20% ACN, No Resin	1.58	80°C	0.63	1.58 (90°C)
20% ACN, Resin	7.13	45°C	1.80	4.99 (50°C)
20% PC[1], No Resin	1.64	80°C	0.60	1.64 (90°C)
20% PC[1], Resin	8.14	50°C	1.54	4.44 (55°C)

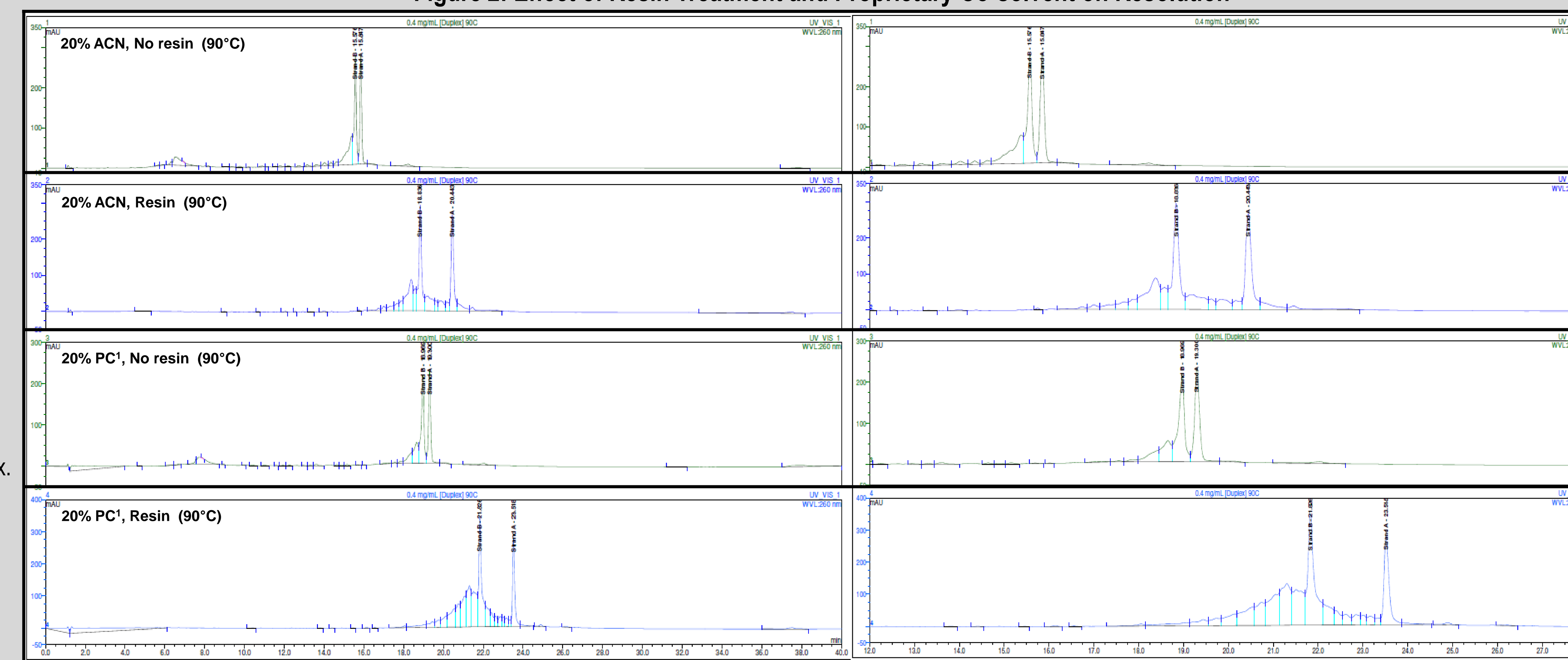
[1]PC = Proprietary Co-solvent

Both resin-treated mobile phase sets yielded a much lower T_m for the oligonucleotide duplex. The T_m decreased by 35°C with the use of the resin-treated 20% ACN mobile phases, and by 30°C with the 20% proprietary co-solvent mobile phase set treated with resin. Figure 1 illustrates the melting range and T_m for the duplex when run with 20% ACN mobile phases with and without resin treatment.

Treatment of the 20% ACN and 20% proprietary co-solvent mobile phases with the proprietary multivalent cation decontamination resin increased resolution between the oligonucleotide strands by a factor of 4.5 and 5.0, respectively, at 90°C (Figure 2). At T_m, the resolution was also tripled with the use of the resin treatment.

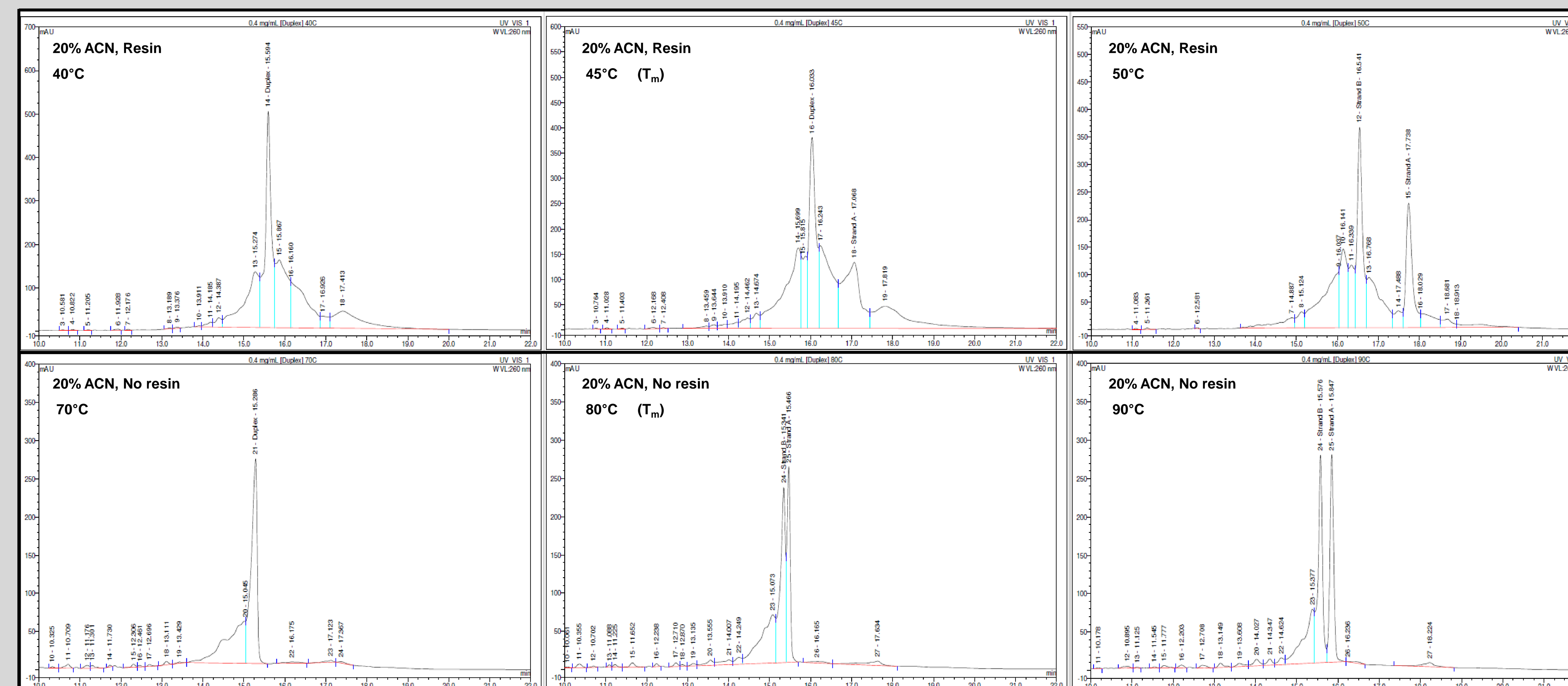
Above 60°C, the resin-treated mobile phase set containing proprietary co-solvent yielded a higher resolution between the oligonucleotide strands than the resin-treated mobile phases containing ACN.

Figure 2: Effect of Resin Treatment and Proprietary Co-solvent on Resolution



[1]PC = Proprietary Co-solvent

Figure 1: Effect of Resin Treatment on Duplex Melting Temperature



Conclusion

For the model nucleic acid duplex evaluated, significant improvements in anion exchange HPLC resolution were achieved by treating mobile phases with a resin designed to remove contaminating divalent cations. This mobile phase treatment also significantly decreased the T_m (observed on-column denaturing temperature), regardless of the organic co-solvent used. The presence of contaminating divalent cations in the water and buffer salts used was expected in relatively low levels. It was hypothesized that these contaminating divalent cations stabilize the oligonucleotide duplex by binding to phosphate groups, raising the T_m. It was envisioned that removing these cations would lower the T_m observed during HPLC analysis, facilitating lower temperature analysis of oligonucleotide duplexes.

The dramatic improvement in resolution observed for decontaminated mobile phases was unexpected. One possible explanation is that the contaminating divalent cations bind to the phosphate groups on the denatured oligonucleotide strands so that the negatively charged phosphates are partially neutralized. This could interfere with the strong interactions between the positively charged anion exchange resin and the negatively charged oligonucleotides. Based on this model, resolution would be improved by removing these contaminants and strengthening the interaction between the phosphate groups on the oligonucleotide and the anion exchange resin.

It should be noted that although the effects described in this report are ascribed to contaminating divalent cations, the removal of these cations was not verified by direct assay (measuring divalent cation levels in mobile phase before and after decontamination). Rather, these effects are assigned to divalent cations based on the expectation that the decontamination resin is designed to bind to and remove divalent cations. Further studies will need to be carried out to verify the mechanism by which mobile phase treatment with decontamination resin affects HPLC analysis of oligonucleotides.