WHITE PAPER

Benefits of Daicel Columns in the Separation of Oligonucleotides

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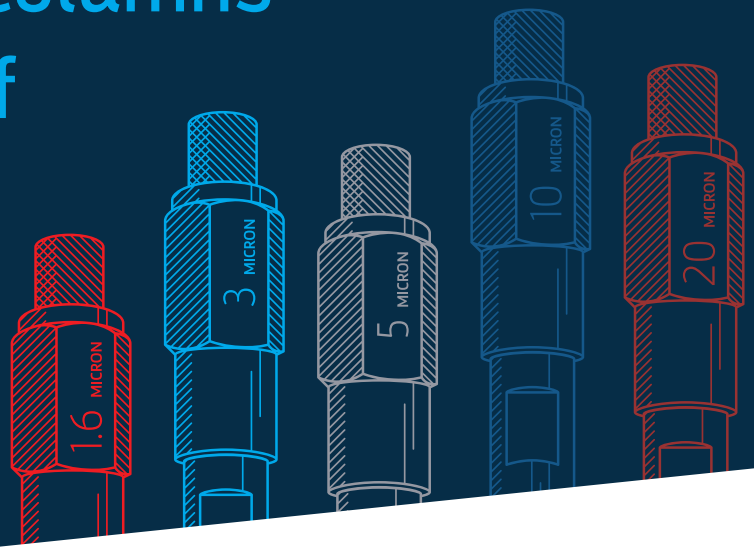

Research on therapeutic oligonucleotides started in the early 90s with a Phase I clinical trial of an antisense oligonucleotide. Less than 5 years later the FDA approved Fomivirsen as an innovative antisense antiviral drug [1]. Since then, many analytical challenges in the accurate characterization of these entities have been highlighted. Fortunately, progress involving liquid chromatography has been achieved in the past years. Due to their complex structure, different recognition mechanisms are considered such as Ion Pair Reversed-Phase Chromatography or Ion Exchange Chromatography [2] and an increasing number of applications are now published.

However, many challenges remain. Still to be addressed are:

1. Resolution of entities that have very close structure to the end-product (n-1, n+1)
2. Use of lower cost mobile phase components
3. Reduction in the level of salt
4. Analysis incorporating a substantial number of side components
5. Gain in sensitivity to analyze trace levels
6. Need for MS-compatibility
7. Universal chromatographic conditions.

Daicel's chromatography columns have been widely used for more than 40 years in various application areas. They are proven solutions for chiral analysis of molecules with a high degree of structural and functional diversity. Analysis of given therapeutic oligonucleotides involving chiral columns has already been reported [3].

Daicel has continuously innovated in the life sciences domain with new developments in achiral columns, genomics, labelled standards, method validation, intracellular injections, and excipients, among many other areas. The aim of this white paper was to evaluate the possibilities offered by all Daicel chromatography columns in these challenging separations of oligonucleotides. With their versatility and orthogonality, Daicel columns could bring new recognition mechanisms.

SCOPE OF THE STUDY

Samples:

The aim of our study was to evaluate how selective/ innovative Daicel columns could be regarding short chain oligonucleotides. Without access to representative

therapeutic oligonucleotides, tests started from a 6-mer DNA oligonucleotide with the following sequence: CGTACT (named "basic 6 mer"). Corresponding 7- and 8-mers, as well as isomers with sequence modification of this 6-mer oligonucleotide, were included to compose a representative pool for the current challenges, namely having as much selectivity as possible for compounds having a high structural similarity (Table 1).

All nucleotides were linked with a standard phosphodiester bond. These products were all purchased from Sigma-Aldrich as desalted forms.

Compound I.D.	Sequence
Basic 6-mer	CGTACT
"n+1" 7-mer	CGTACT**G**
"n+2" 8-mer	CGTACT**GA**
5' end modified 6-mer	**GC**TACT
3' end modified 6-mer	CGTA**TC**
Mid modified 6-mer	CG**AT**CT

Table 1: Oligonucleotides Used for Separation Study

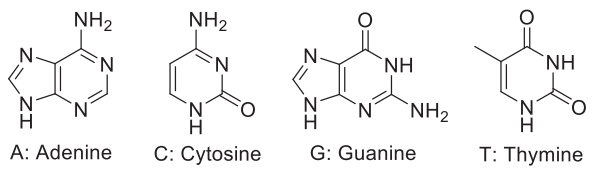

Figure 1: The Four Nucleotide Bases

Columns:

Within the Daicel portfolio, the following sets of complementary columns were considered. This included achiral as well as chiral polysaccharide and ion exchange columns. The selection was not exhaustive, but aimed to explore the recognition patterns of different column types for the set of selected oligonucleotides.

DCpak® PTZ and DCpak® P4VP are achiral polymeric columns.
Current main application range of DCpak® PTZ is HILIC-like mode for very polar molecules.

DCpak® P4VP was initially developed for SFC application, but was found to have encouraging recognition mechanisms in liquid chromatography. Advantageously, it is a versatile column for low to high polarity compounds.

CHIRALPAK® IB N, CHIRALPAK® IC and CHIRALPAK® IG are immobilized polysaccharide-based columns.
IB N and IC are cellulose-based chiral selectors and have already proven specific recognition pattern for larger molecules. CHIRALPAK® IG is an amylose-based chiral selector. It was added to the successful series of selectors with substituents on the carbamate in meta-positions.

CHIRALPAK® QD-AX and CHIRALPAK® QN-AX are weak-anion exchangers based on Quinidine and Quinine.
These columns are specifically designed for the separation of chiral acids.

CHIRALPAK® ZWIX(+) and ZWIX(-) are zwitterionic exchangers.
These columns incorporate both anion- and cation-exchange functional groups and work particularly well for underivatized amino-acids, small peptides, and other more challenging compounds.

For these tests, 150mm Length x 4.6mm Internal Diameter (ID) columns, packed with a 5 µm particle size, were used as this is considered a universal column geometry. The exception is for the zwitterionic columns which were 4.0mm ID and were packed with a 3 µm particle size.

More detailed information on the columns can be found at Explore our Chiral Selectors Portfolio | Daicel Chiral Technologies.

Mobile phase:

Investigations were focused on one buffer type, namely triethylammonium acetate (TEAA), which is a commonly used ion-pairing agent in the oligonucleotides field. It offers the following advantages:

- Volatility and Compatibility with MS-detection
- Cost-effectiveness
- Limited toxicity
- Sufficient solubility of the oligonucleotides when mixed with acetonitrile (ACN).

The following parameters were investigated: salt concentration (from 50 to 150mM) and pH-value (from 6.5 to 7.5).

Gradient conditions were applied with

A being buffer solution/ACN 95/5 (v/v)
B being buffer solution/ACN 5/95 (v/v)

Example of mobile phase preparation for a 100mM buffer solution: Add 5.7mL of glacial acetic acid in 980mL of pure water. Mix well. Add slowly ca 13.9mL of Triethylamine up to desired pH. Adjust final volume to 1L with pure water. Filter through a 0.45µm membrane.

The following time program was applied: 0-5min at 100%A, 5-20min at 100%B, 20-25min at 100%B, re-equilibration to 100% A for 5min.

Reagent references:
Purified water from an Elix® Advantage 15 system from Millipore
Acetonitrile: Carlo Erba for HPLC: Ref 412412000
Glacial acetic acid: Sigma Aldrich Ref. 45726 purity >99.5%
Triethylamine: Sigma Aldrich Ref. 471283

Chromatographic conditions:

The tests were run on an Agilent 1100 chromatographic system equipped with binary pump, autosampler, thermostatic oven and Diode Array Detector.

Flow rate:	1.0mL/min
Temperature:	25-40°C
Detection:	all the chromatograms reported in this document were at 260nm.

Samples were dissolved in pure water at 1mM. 1µL-injections were run.

MAIN RESULTS

Among the different conditions tested, complementary recognition patterns were observed with particularly good results obtained with the zwitterionic columns.

The evaluated mobile phase and the corresponding gradient profile were fully suitable to properly elute the pool of evaluated oligonucleotides within reasonable retention times. Buffer concentration of at least 100mM was needed to reach sufficient selectivity between the different entities. Overall working at 30°C or 40°C improved the recognition mechanism compared to room temperature.

Two **polymeric achiral columns** DCpak® PTZ and DCpak® P4VP were both explored.

- DCpak® PTZ did not yield any encouraging separations for the oligonucleotides tested. Because of the unique HILIC nature of the column, the ion-pair agent likely prevented any suitable recognition mechanism. DCpak® P4VP showed more promise, however was still limited in the number of conditions tested that yielded sharp and symmetric peaks (Figure 2). The most suitable conditions were those combining a 150mM buffer and a pH of 7.5. At lower pH values, quite strong peak distortion of the short eluting peaks was observed. On this column, the selectivity was significantly influenced by temperature.

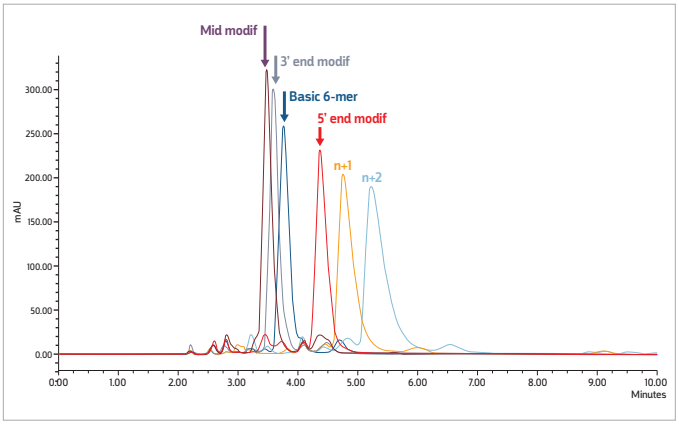

Figure 2: Overlaid chromatograms of the 6 oligonucleotides on DCpak® P4VP - gradient conditions with a 150mM TEAA at pH=7.5 – 1mL/min - 30°C

- All three **polysaccharide-based selectors** resulted in very similar retention times (ca 10min). On these columns, no selectivity was attained between the 6-mers with the 3'end & mid sequence modifications. CHIRALPAK® IB N showed the most encouraging selectivity profiles (Figure 3), especially for the separation between the reference 6-mer and the 7- and 8-mers (n+1 and n+2).

Mobile phase pH of 6.5 or 7.5 were the most encouraging. Recognition was significantly improved as the concentration of the ion-pair agent was increased. Moreover, separations were also significantly improved at 40°C compared to 35°C.

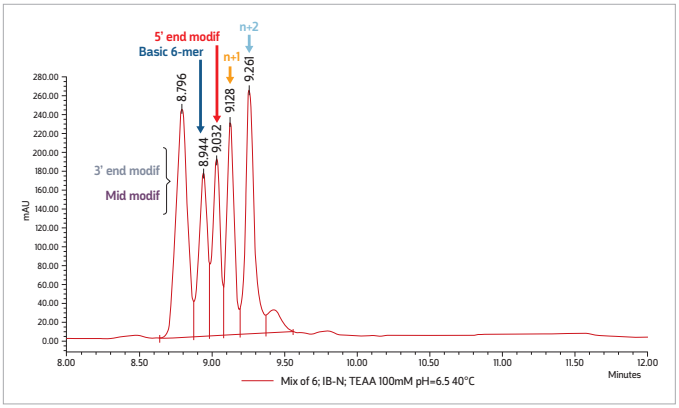

Figure 3: Injection of a manual mixture of the 6 oligonucleotides on CHIRALPAK® IB N – gradient conditions with a 100mM TEAA at pH=6.5 – 1mL/min - 40°C

- The CHIRALPAK® QD- and QN-AX **anion exchangers** behaved very similarly (Figure 4). As expected, due to the ionic mechanism, pH had a strong effect. No separation was achieved at pH of 6.5, while recognition was significantly improved at higher pH values. Specifically, a pH of 7.5 resulted in considerably sharper peaks compared to a pH of 7.

Like the polysaccharides, no selectivity was attained between the 6-mers with 3'end & mid sequence modifications.

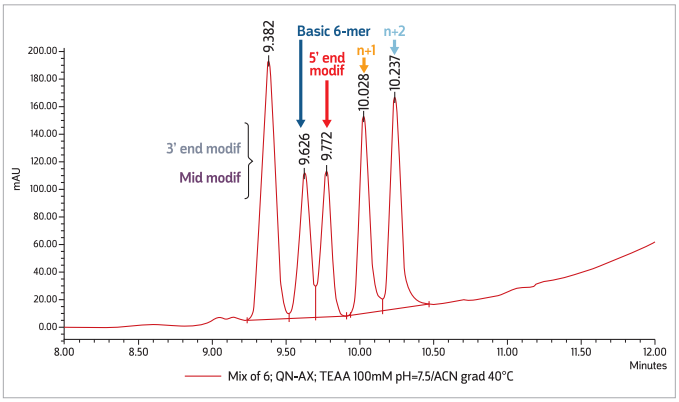

Figure 4: Injection of a manual mixture of the 6 oligonucleotides on CHIRALPAK® QN-AX – gradient conditions with a 100mM TEAA at pH=7.5 – 1mL/min - 40°C

- Contrary to the anion exchangers, both versions of the **zwitterionic phases** behaved differently. Specifically, CHIRALPAK® ZWIX(-) offered significantly higher selectivity than the CHIRALPAK® ZWIX(+).

Overall, pH had a limited impact on the recognition mechanism, unlikely for the other columns, where retention times tended to increase with an increase in pH. As shown below, apart from the separation of the 6 oligonucleotides, the resolution of a minor component is significantly improved at a pH of 7.5 compared to a pH of 6.5 (Figure 5).

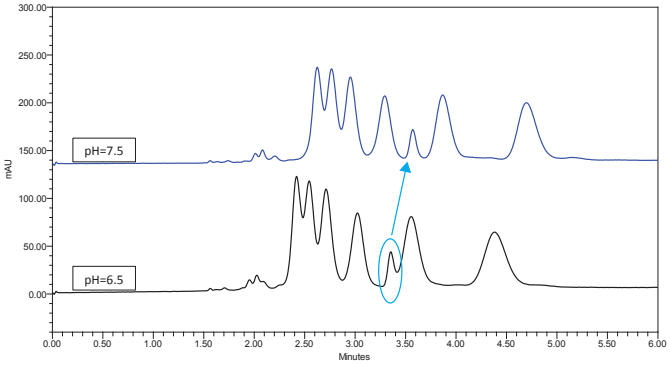

Figure 5: Injection of a manual mixture of the 6 oligonucleotides on CHIRALPAK® ZWIX(-) – gradient conditions with a 100mM TEAA at pH=6.5 (black) and at pH=7.5 (blue) – 1 mL/min - 40°C

CHIRALPAK® ZWIX(-) offered the best separation in the shortest run time. In less than 7 minutes, the 6 selected oligonucleotides were resolved. This separation can be compared to a reference separation attained on a non-chiral, C18 column under similar conditions (Figure 6). The elution order is the same, but CHIRALPAK® ZWIX(-) offers a significantly enhanced separation of the 6-mers (Figure 7).

Figure 6: Overlaid chromatograms of the 6 oligonucleotides on XBridge Shield® C18 (150*4.6mm, 3µm) – gradient conditions with a 100mM TEAA at pH=7.5 – 1 mL/min – 60°C

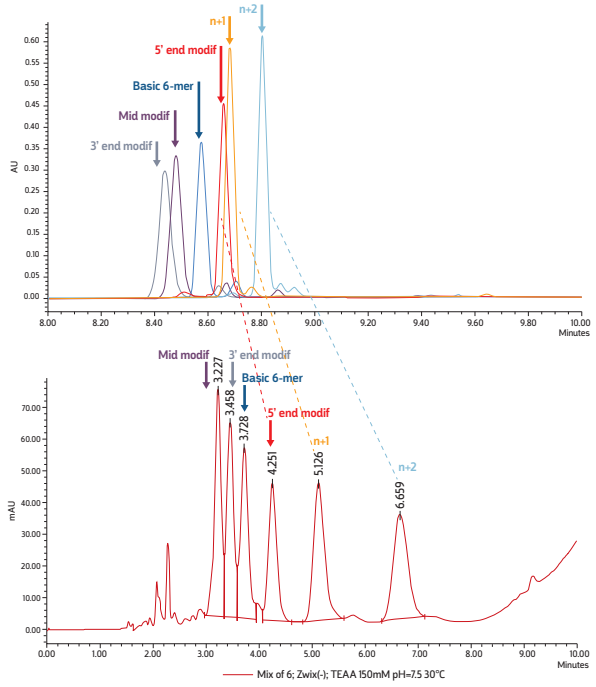

Figure 7: Injection of a manual mixture of the 6 oligonucleotides on CHIRALPAK® ZWIX(-) – gradient conditions with a 150mM TEAA at pH=7.5 – 1 mL/min – 30°C

CONCLUSION

The purpose of this preliminary investigation was to evaluate the ability of several column types to elute and differentiate short chain oligonucleotides with small sequence modifications. Columns bearing chiral and achiral selectors functionalized onto a silica support were included in the study, in the search of complementary and/or orthogonal elution patterns.

Very encouraging results were attained for the chosen pool of 6-, 7- and 8-mers on Daicel chiral columns. The use of TEAA ion-pair agent together with ACN in gradient mode resulted in sharp peaks within a short run time, as well as in unique recognition mechanisms. Trends regarding pH-value, buffer concentration and temperature were also established for the different types of columns.

The zwitterionic column, CHIRALPAK® ZWIX(-), offered the highest selectivity. Compared to a well-established C18 column, the selectivity for the 6-mers with slight base position modifications was significantly improved.

These first sets of results open up a very promising investigation path to apply and further improve the existing separation conditions; this would include use of smaller particle sizes, hyphenation with MS-detection, combinations with other columns in a 2D-configuration, and preparative applicability.

Although the experimental model was based on synthetic small oligonucleotide standards, the differences observed could be positive hints to include chiral and/or polymeric non-chiral selectors into the column toolbox, helping to understand the “fingerprints” of different oligonucleotide samples.

Chiral analysis has become of tremendous importance for small molecule drug safety since the thalidomide disaster in the early 1960s. For oligonucleotides, it has been established that the activity of some therapeutic phosphorothioate oligonucleotides is directly linked to the stereochemistry. That is the stereochemistry resulting from the addition of a sulphur on the phosphate internucleoside linkage, whose objective is to improve the resistance of the drug to nuclease degradation [4]. In the near future, the stereochemistry of oligonucleotides will very likely continue to grow in its importance. Having applications like those developed in this white paper involving chiral columns may bring the added information needed to evaluate the stereochemistry of these next generation oligonucleotides.

References

[1] Karin E. Lundin, Olof Gissberg and C.I. Edvard Smith, *Oligonucleotide Therapies: the Past and the Present,* Human Gene Therapy (2015), 26(8): 475-485

[2] A. Cary McGinnis, Buyun Chen, Michael G. Bartlett, *Chromatographic methods for the determination of therapeutic oligonucleotides,* Journal of Chromatography B (2012), 883-884: 76-94

[3] Feiyang Li, Michael Lämmerhofer, *Impurity profiling of siRNA by two-dimensional liquid chromatography-mass spectrometry with quinine carbamate anion-exchanger and ion-pair reversed-phase chromatography,* Journal of Chromatography A (2021), Vol. 1643

[4] Naoki Iwamoto et all, *Control of phosphorothioate stereochemistry substantially increases the efficacy of antisense oligonucleotides,* Nature Biotechnology (2017), Vol. 35, No.9