Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

Evaluation of micropillar array columns for chromatographic separation of phosphorothioated oligonucleotides and their diastereomers

Lieve Dillen[1] | Tiny Deschrijver[2] | Kurt Van Mol[3] | Jeff Op de Beeck[3] | Paul Jacobs[3] | Filip Cuyckens[1]

[1] Drug Metabolism and Pharmacokinetics, Janssen R&D, Beerse, Belgium

[2] Chemical Process R&D, Process Analytical Research, Janssen R&D, Beerse, Belgium

[3] PharmaFluidics, Ghent, Belgium

Correspondence
Lieve Dillen, Drug Metabolism and Pharmacokinetics, Janssen R&D, Beerse, Belgium.
Email: ldillen@its.jnj.com

Abstract

Chromatographic analysis of therapeutic oligonucleotides is challenging due to the presence of closely related impurities, degradants or metabolites and due to the presence of phosphorothioate bonds, which introduce chiral centers. In the present study, ion pair reversed phase chromatography of oligonucleotides on micropillar array columns was investigated. Two commonly used mobile phase conditions were included. With 16.3 mM triethylamine and 400 mM hexafluoroisopropanol, the separation of 16-mer oligonucleotides differing in the number and positions of phosphorothioate linkages as well as some *n*-1 and *n*-2 truncations demonstrated complete suppression of diastereoselectivity. Although the positional phosphorothioate isomers evaluated could not be resolved, an increase in phosphorothioate bonds resulted in more retention. A therapeutic 19-mer RNA sequence with 2′-fluor and 2′-*O*-methyl modifications showed partial separation of some very close impurities. When using 15 mM triethyl ammonium acetate in the mobile phase, diastereomer selectivity was clearly observed for all analytes. The best result was obtained for the 19-mer RNA therapeutic mimic with four phosphorothioate bonds, since all 16 theoretical diastereomers were clearly observed under the conditions tested. A limited benchmark exercise demonstrated the improved capability of the new micropillar array columns. Therefore, these columns can be positioned as a valuable alternative when challenging oligonucleotide separations are expected.

1 | INTRODUCTION

Oligonucleotides have recently been included in portfolios of many pharmaceutical companies because of their potential as therapeutic molecules. Both antisense oligonucleotides and small interference (si)RNA have gained a lot of interest.[1,2] Suppression of the translation of a target gene/protein is realized by complementarity to the messenger RNA of that target. Different mechanisms of action can be recognized depending on the design of the oligonucleotide constructs and the site of action.[3] Therapeutic oligonucleotides are chemically modified to improve their drugability. Typical examples of these modifications are the incorporation of phosphorothioate (PS) linkages in the backbone to improve stability or modifications at the 2′ position in the ribose to increase affinity toward the target mRNA with reduced innate immune stimulation. With other modifications, such as 5′ or 3′ extensions with *N*-acetyl galactosamine or lipids, targeted delivery to organs has been realized.[4,5]

In support of development programs, analytical tools are essential to investigate impurity profiles, degradation pathways, and to study the exposure after administration. The identification and separation of closely related oligonucleotide structures/impurities requires development of analytical methods with increased resolution and selectivity.[6]

Additional diastereoselective methods for oligo analysis have also gained interest.[7,8] The presence of PS linkages creates chiral centers and produces diastereomers with Rp and Sp configuration that have different pharmacological and physicochemical properties. Depending on the number of PS linkages in an oligonucleotide structure, the complexity increases as 2^n (n = # internal PS linkages) isomers are formed.

Ion pair reversed phase chromatography (IP-RP) is the preferred chromatographic method since oligonucleotides are large polar, acidic molecules with limited retention in reversed phase chromatography. Therefore, alkylamines have been used as ion pairing reagents to retain the oligonucleotides on reversed phase columns.[9] Initially, triethylammonium acetate (TEAA) was mainly applied, providing excellent separation at high concentration (>100 mM).[10] TEAA, especially the nonvolatile acetate counter ion, is, however, detrimental for MS sensitivity. Reducing the TEAA levels allows better compatibility with MS but compromises the separation performance. Other counter ions (bicarbonate, formate, bromide, chloride) have been evaluated but it was only when a combination of triethylamine as ion pairing and hexafluoroisopropanol (HFIP) as acidic modifier was introduced by Apffel et al.[11] that a major leap toward sensitive LC-MS analysis was realized. However, when diastereomer separation is considered, smaller (less hydrophobic) alkylamines in combination with acetate have shown most performant in IP-RP.[8] With respect to the choice of stationary phases, standard silica or ethylene bridged hybrid silica, anion-exchange as well as polymer-based monolithic columns have been described.[12–20] Capillary monoliths (200 µm internal diameter) have been introduced to reduce void volumes which exist between packed particles and to abolish diffusion mass transfer in the pores of porous stationary phases.[21,22]

Ion pair reversed phase chromatography combined with UV detection and electrospray ionization (ESI) mass spectrometry is the most powerful method for oligonucleotide analysis. The detection orthogonality allows identification and quantification even when structurally closely related impurities coelute. MS analysis of these structures is complex due to the relatively large molecular weight resulting in several multiply charged ions for each analyte.[23,24] Structurally related impurities or metabolites can represent a mixture of analytes since some modifications (defluorination, deamination) can often occur at different positions in the structure. Low resolution mass spectrometry analysis with a limited m/z range, routinely used during development, can be prone to interference of other impurity related charged species and adducts. Chromatography with an extended resolving power for the closely related oligonucleotide impurities, could simplify the analysis.

In this study, the potential of micropillar array columns for oligonucleotide analysis was evaluated with respect to its resolving power for closely related oligo impurities, degradants, or metabolites and for diastereomers.

Micropillar array columns are produced by micromachining technology, using lithography to transfer the designed separation bed to silicon wafers which is formed by sophisticated Deep Reactive Ion Etching. Consequently, the intercolumn reproducibility is exceptionally high. The perfectly ordered array of pillars forms the backbone of the stationary phase. For the columns tested in this work, the pillar diameter is 5 µm and the pillar height is 18 µm. The high order and very reproducible spacing between the pillars improve LC resolution because peak dispersion due to heterogeneous flow paths in the separation bed is eliminated. The pillars are formed out of one silicon substrate by etching, with the top of the pillars bonded to the covering glass wafer. No potentially moving parts are involved, and no frits are required, which improves column robustness. Finally, as the pillars are freestanding and accurately positioned at an inter pillar distance of 2.5 µm, the permeability of the columns is very well controlled, enabling long columns which still can be operated at moderate pressures, and consequently increasing the separation power despite the longer diffusional distances. A lower back pressure also leads to less wear and tear in the system, preventing leakage at the fittings as experienced with the benchmarking columns. Furthermore, elevated temperatures to reduce the mobile phase viscosity are not needed. Micropillar array columns up to 200 cm long are commercially available. A 50 cm micropillar array column consists of five lanes with a width of 315 µm, concatenated by 180° turn structures (additional details are provided in Supporting information Figure S1). The pillars and the inner surface of the microchannels is made superficially porous and the resulting surface is grafted with a monofunctional C_{18} silane and end capped with hexamethyldisilazane. Micropillar array columns have been successfully applied in proteomics,[25–29] phosphoproteomics,[30] and lipidomics,[31] with remarkable capabilities with respect to repeatability and reproducibility.[32] The current study aimed to investigate the potential of this innovative type of columns for separation of oligonucleotide therapeutics.

The obtained results were benchmarked to two commonly used particle-based nanoLC columns.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All solvents, including water, acetonitrile, and methanol, were HPLC grade and obtained from Biosolve BV (Valkenswaard, The Netherlands). Triethylamine and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were obtained from Merck KGaA (Darmstadt, Germany) as well as TEAA buffer, premade as a 1 M solution in water. 1× Tris-EDTA (TE) buffer pH 8.0 was purchased from Integrated DNA Technologies (Leuven, Belgium)

HFIP needs to be handled with extra caution since direct contact is harmful to skin, lungs, and eyes.

2.2 | Oligonucleotide samples

A comprehensive list of all oligonucleotides discussed in this manuscript is provided in Table 1 and 2. They were custom made

by Integrated DNA Technologies (Leuven) except for sequence **13** that was in-house synthesized. No further purification was performed for **13** since UHPLC UV-MS analysis showed acceptable purity (UV purity 84 area%). Oligonucleotides **1** to **12** were purchased as "standard desalting" but structures **14** to **20** were additionally purified by ion exchange chromatography by the vendor.

The compounds were dissolved in water (1 µg/µL) and further diluted in water to a concentration of 250 ng/µL. To determine peak capacity for 200 and 50 cm micropillar array columns, a reference standard mixture ranging from 5 to 30 thymidine-oligonucleotides was prepared in-house. These oligonucleotides were used at a final concentration of 75 ng/µL each dissolved in 1× TE buffer pH 8.0 purchased from IDT Technologies.

2.3 | Liquid chromatography instrumentation, columns, and conditions

All experiments were performed on a Thermo Fisher Scientific Ultimate 3000 RSLC nano HPLC System with Pro Flow technology (Thermo Fisher Scientific, Belgium), configured with a binary pump, a diode-array UV detector, a column thermostat, and autosampler unit. An external nanoliter injection valve (C4N-4004-.004EUHA) from VICI (VICI AG International, Schenkon, Switzerland) allowed for 4 nL injections. The temperature of the column thermostat was set to 35°C for all experiments, unless otherwise stated.

A PharmaFluidics 50 cm µPAC C_{18} and 200 cm µPAC C_{18} column were compared to a Waters nanoEase M/Z Peptide BEH C_{18} column (130 Å, 1.7 µm, 75 µm × 150 mm) and a ThermoFisher Scientific Acclaim PepMap 100 C_{18} column (100 Å, 2 µm, 75 µm × 150 mm). Thermo Scientific Dionex Chromeleon Chromatography Data System software (version 7.2.7) was used for data acquisition.

2.4 | Preparation of buffers

Two mobile phase conditions were included. Preparation was done by weight measurement to increase buffer composition consistency during the complete testing.

The first one consisted of a TEAA buffer (0.015 M, pH 7) which was prepared by diluting the 1 M stock to a 15 mM working solution by adding 1.5 g 1 M stock to a total of 100 mL water. Mobile phase A consisted of 15 mM TEAA with a confirmed pH of approximately 7 without pH adjustment. Mobile phase B consisted of 40% acetonitrile with 15 mM TEAA concentration. Both solutions were mixed on a magnetic stirrer for 15 min.

Similarly, the second setup uses a TEA-HFIP buffer which was prepared by titrating the acidic HFIP solution with TEA. To prepare 0.1 L mobile phase A with 16.3 mM TEA—400 mM HFIP buffer pH 7.9 in water, 0.227 mL TEA was added to 4.21 mL HFIP, and mixed on a magnetic stirrer to ensure full dissolution. Gilar et al.[19] report that only higher concentrations of TEA can be dissolved in 400 mM HFIP if methanol is added. The same amounts of HFIP and TEA were used in 40% methanol/water to prepare mobile phase B.

3 | RESULTS AND DISCUSSION

Ion pair reversed phase chromatography of oligonucleotides is challenging due to the presence of structural closely related impurities/degradants but also due to their structural characteristics (large acidic negative charged molecules). Although the mechanisms of chromatographic retention and selectivity are not completely understood, it is known that the nature and concentration of the ion pairing reagent, the gradient, and the counter ion as well as the nucleotide sequence and length, all critically contribute to the separation.[13–18] Also, the modifications present in the molecules have an impact on the retention behaviour.[33] The incorporation of a PS linkage in an oligonucleotide creates a stereogenic phosphorus atom introducing diastereomers. This further complicates the interpretation of the chromatograms since improving chromatographic resolving power to separate PS oligonucleotide impurities or metabolites often results in (partial) separation of the diastereomers as well.

The present investigation aimed for evaluation of the separation power of micropillar array columns for oligonucleotides and their diastereomers. For this study, two mobile phase solvent systems were selected based on results published in literature: 15 mM TEAA and 16.3 mM TEA/400 mM HFIP. The selection of TEAA was driven by a recent study by Enmark et al.[8] providing some insight in factors influencing the separation of the diastereomers and has positioned TEAA as the most promising ion pairing reagent for diastereomer selectivity in line with some earlier findings. Next to this TEAA method, 400 mM HFIP with 16.3 mM TEA was applied as it has shown improved efficiency for separation of ladder oligonucleotide sequences.[18,19]

Several oligonucleotides were synthesized, and details are given in Table 1 and 2. Group 1 (Table 1, IX **1-7**+**12**) includes 16 mer DNA oligonucleotides with different numbers of PS linkages at both 5′ and 3′ ends, as well as for one sequence (IX **5)** the concomitant 3′ and 5′ *n*-1 and *n*-2 shortmers (IX **8-11**) without any other modification. A second group of oligonucleotides are examples of known process impurities and degradants based on a specific parent sequence (IX **13)** as shown in Table 2. The parent sequence, also referred to as full length product (FLP) has 19 RNA nucleotides, a mixed PO/PS backbone and contains common modifications in therapeutic oligonucleotides at the 2′ ribose position: 2′-*O*-methylation and 2′-fluorination as shown in Supporting information Figure S2. The related impurities include *n*-1, *n*-2, *n*-4 shortmers (IX **16-18)**, PS (IX **14)**, PO (IX **15**), and arabinosyl (IX **19-20**) modifications. The arabinosyl impurity is formed by defluorination of 2′-fluorinated pyrimidine nucleotide followed by water addition. It has only a molecular weight difference of 2 Da and is challenging to resolve both by UV and MS.[34] Two analogues were synthesized to assess possible influence of the arabinosyl position. The PS impurity represents the opposite modification of PO impurity: a phosphate diester is replaced by a PS diester instead of the well-known PS diester by phosphate diester modification.

Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

TABLE 1 Oligonucleotide sequences with their phosphorothioate linkages indicated and the theoretical number of diastereomers calculated

IX	Nucleotide sequence 5′-3′	# PS bonds	Shortname (5′PS+3′PS)	# diastereomers
1	a-t-c-t-a-t-a-c-a-a-g-c-t-g-t-c	0	Full PO	0
2	as-t-c-t-a-t-a-c-a-a-g-c-t-g-t-c	1	1+0	2
3	a-t-c-t-a-t-a-c-a-a-g-c-t-g-ts-c	1	0+1	2
4	as-t-c-t-a-t-a-c-a-a-g-c-t-g-ts-c	2	1+1	4
5	**as-ts-c-t-a-t-a-c-a-a-g-c-t-g-ts-c**	3	2+1	8
6	as-t-c-t-a-t-a-c-a-a-g-c-t-gs-ts-c	3	1+2	8
7	as-ts-c-t-a-t-a-c-a-a-g-c-t-gs-ts-c	4	2+2	16
8	ts-c-t-a-t-a-c-a-a-g-c-t-g-ts-c	2	2+1 5′*n*-1	4
9	c-t-a-t-a-c-a-a-g-c-t-g-ts-c	1	2+1 5′*n*-2	2
10	as-ts-c-t-a-t-a-c-a-a-g-c-t-g-t	2	2+1 3′*n*-1	4
11	as-ts-c-t-a-t-a-c-a-a-g-c-t-g	2	2+1 3′*n*-2	4
12	as-ts-cs-ts-as-ts-as-cs-as-as-gs-cs-ts-gs-ts-c	15	Full PS	32 768

n, deoxynucleotide.
s or PS, phosphorothioate. PO, phosphodiester.
Shortname for the truncated structures (IX8-11) mentions the # of PS bonds at 5′ and 3′ ends of the parent oligonucleotide in combination with the # of nucleotide losses at either 5′ or 3′ end.

TABLE 2 Test oligonucleotide sequence containing the most common modifications and some related impurities

IX	Nucleotide sequence 5′-3′	# PS bonds	#Diastereomers	Comment
13	As-Afs-Cs-Af-U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	4	16	Parent sequence = FLP
14	As-Afs-Cs-Af-U-Uf-G**s**-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	5	32	1 PO → PS
15	A-Afs-Cs-Af-U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	3	8	1 PS → PO
16	Afs-Cs-Af-U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	3	8	5′*n*-1
17	Cs-Af-U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	2	4	5′*n*-2
18	U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	1	2	5′*n*-4
19	As-Afs-Cs-Af-U-AraU-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	4	16	2′F → 2′OH; arabinosyl1
20	As-Afs-Cs-Af-U-Uf-G-A-G-AraC-G-Af-U-Af-U-Cf-C-As-C	4	16	2′F → 2′OH; arabinosyl2

N = 2′OMe, *Nf* = 2′F, s or FLP, full length product; PS, phosphorothioate; PO, phosphodiester.

3.1 | Chromatographic separation of closely related oligonucleotide sequences

3.1.1 | Separation of PS-modified DNA 16 mer oligonucleotides

In this study, the separation of different oligonucleotides containing 16 bases was considered. Figure 1 shows partial separation of seven oligonucleotides that have identical nucleotide sequences only differing by the number or position of the PS linkages (Table 1, IX **1-7**). The method with 16.3 mM TEA and 400 mM HFIP as mobile phase enabled the separation of oligonucleotides with a different number of PS linkages on a 50 cm micropillar array column applying a 30 min gradient at a flow rate of 1.5 µL/min. The compound containing only phosphodiester linkages (PO, IX **1**) elutes first. Increased retention times were observed with increasing number of PS bonds as expected. The structure with 15 PS bonds (PS, IX **12**) is most retained on this column in line with the increased hydrophobicity introduced with the PS linkages. No diastereomer separation was visible and the positional PS isomers almost completely overlap (e.g., in case of the presence of three PS linkages, independent of whether two are located at the 5′ end and one at the 3′ end (2 + 1, IX **5**) or vice versa (1 + 2, IX **6**). The chromatographic details are specified in the figures.

3.1.2 | Separation of 16 mer truncated metabolites/degradants

The application of micropillar array columns toward the selectivity of truncated metabolites/degradants of sequence **5** was evaluated. Therefore, shortmers of the 16 mer with two 5′ PS linkages and one at the 3′ end (Table 1, sequences **8-11**) were synthesized. Four shortmers with the loss of 1 and 2 nucleotides from both ends were considered. The chromatograms obtained on the 50 cm micropillar array column using the 16.3 mM TEA/400 mM HFIP solvent resulted in adequate separation of the shortmers from the reference sequence **5** applying

Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

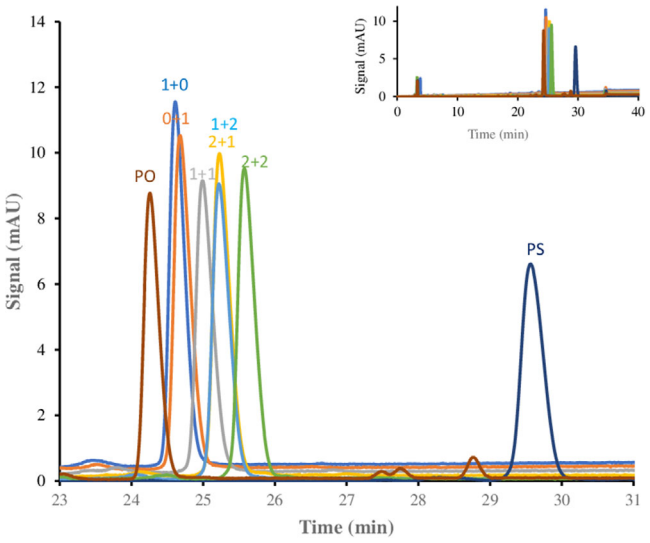

FIGURE 1 Chromatographic separation of eight 16-mer oligonucleotides with different # and position of PS bonds (IX 1-7, 12 in Table 1) on a 50 cm micropillar array column. The chromatographic peaks are labeled with the # of PS bonds at the 5′ and 3′ ends respectively and reflect the short names in Table 1. Insert shows the full chromatogram. Chromatographic conditions: mobile phase (MP) A: 16.3 mM TEA and 400 mM HFIP, MPB: 16.3 mM TEA and 400 mM HFIP in 40% methanol/water 30 min gradient from 5 to 60% MPB flow rate: 1500 nL/min 4 nL injection volume (conc. 1 µg/µL)

a 60 min gradient at 250 nL/min (Figure 2A). The combined loss of a nucleobase and a PS linkage induces a relative larger shift in retention time compared to the loss of a nucleobase with a PO linkage, although it is a combined effect of the hydrophobicity of the linkage and nucleobase itself. Cytosine is the most hydrophilic nucleobase and upon cleavage of the 3′ C (IX **10**; 3′ *n*-1) a relatively more hydrophobic nucleotide (*Rt* = 42.5 min) is left compared to the loss of the 5′ adenosine (IX **8**; 5′ *n*-1) (*Rt* = 41.2 min). The extra loss of the 3′ T (the most hydrophobic nucleobase but now with no loss of PS linkage) results in a *Rt* of 40.9 min (IX **11**; 3′ *n*-2) and partially coelutes with 5′ *n*-1 (IX **8**) (see inset Figure 2A). Only with detectors, such as mass spectrometers, that allow distinction of the two analytes based on mass, the identification/confirmation of the coeluting analytes can be achieved.

3.1.3 | Separation of 2′-F and 2′-*O*-Me phosphorothioate-modified RNA sequences

Figure 3 shows the separation obtained for parent sequence **13** and derived sequences **14-20** on a 50 cm micropillar array column with the TEA/HFIP solvent system applying a 60 min gradient at a flow rate of 250 nL/min. Shortmers *n*-4 (IX **18**; *Rt* 46.5 min), *n*-2 (IX **17**; *Rt* 48.8 min), and *n*-1(IX **16**; *Rt* 50.7 min) were (almost for *n*-1) completely resolved from the parent sequence **13** (*Rt* 51.4 min). The PO structure (IX **15**; *Rt* 51.4 min) was not separated from the parent sequence and all other analytes, arabinosyl 1 and 2 (IX **19** and **20**; *Rt* 50.7 and 50.5 min) and PS (IX **14**; *Rt* 51.7 min) showed partial coelution with the parent sequence. A 200 cm column did not provide complete separation of these structures neither (data not shown). Coupling with a MS spectrometer could provide orthogonality for the (partial) coeluting analytes but remains challenging. Additional method development with different ion pair reagents, including optimization of the IP concentration in combination with optimal selection of the counterions and organic modifiers,

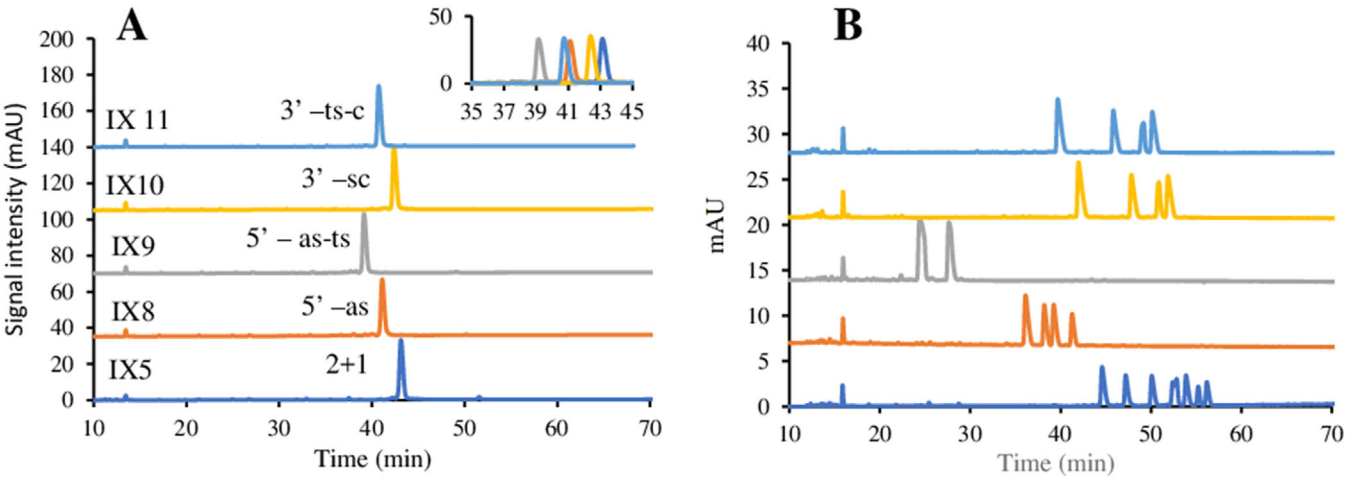

FIGURE 2 Chromatographic separation of a 16-mer oligonucleotide with three PS bonds and its 5′ and 3′ truncated metabolites (IX 5, 8-11 in Table 1) on a 50 cm micropillar array column. (A) Chromatographic separation with TEA and HFIP with the insert showing a zoomed overlay. (B) Diastereomeric separation obtained using the TEAA as solvent system. The losses of nucleotide(s) combined with loss of PS bonds at the 5′ and 3′ ends, respectively, are indicated. Chromatographic conditions (2A): mobile phase (MP) A: 16.3 mM TEA and 400 mM HFIP MP B: 16.3 mM TEA and 400 mM HFIP in 40% methanol/water 60 min gradient from 20 to 99% MPB flow rate: 250 nL/min 4 nL injection volume (conc. 1 µg/µL). Chromatographic conditions (2B): mobile phase (MP) A: 15 mM TEAA, MPB: 15 mM TEAA in 40% acetonitrile 60 min gradient from 20 to 30% MPB flow rate: 250 nL/min 4 nL injection volume (conc. 1 µg/µL)

Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

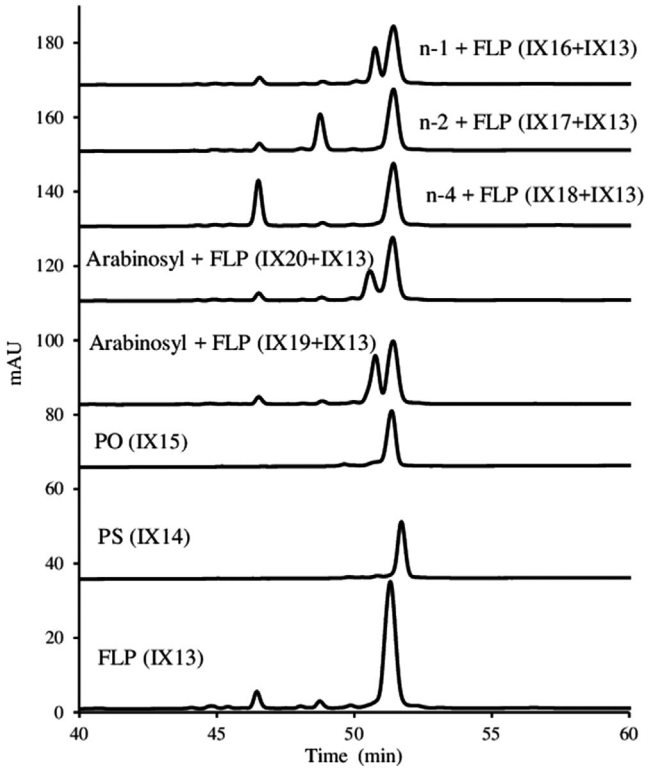

FIGURE 3 Chromatographic separation of full-length product (FLP) (IX 13) and related impurities (IX 14-20) on a 50 cm micropillar array column. To improve correct (visual) interpretation some impurities were injected combined with the FLP. PO, phosphodiester impurity; PS, additional PS impurity. Chromatographic conditions: mobile phase (MP) A: 16.3 mM TEA and 400 mM HFIP; MPB: 16.3 mM TEA and 400 mM HFIP in 40% methanol/water 60 min gradient from 20 to 99% MPB flow rate: 250 nL/min 4 nL injection volume

may further provide improved selectivity but is beyond the scope of this research.

3.2 | Chromatographic separation of oligonucleotide diastereomers

3.2.1 | Diastereoselective separation of PS-modified DNA 16 mer oligonucleotides

With 15 mM TEAA as ion pairing reagent and applying a short 10 min gradient and 1.5 µL/min flow rate, partial diastereomer separation on a 50 cm micropillar array column was obtained for the 16 mer oligonucleotides with different number and position of terminal PS linkages (IX **2-7** Table 1) (Figure 4). For the terminal 5′ and 3′ analytes with one PS linkage, the 3′ positioned PS (IX **3**) shows improved resolution of its diastereomers (baseline separated), which can be rationalized by presence of cytosine as end nucleobase compared to adenosine at the 5′ end (IX **2**). A pentamer of cytosine has been demonstrated to have twice the diastereomer selectivity of a thymine pentamer (C > G > A > T).[8] Similar differences in resolution can be noticed for the diastereomers of the molecule with two 5′ PS linkages (as-ts-c) and one 3′ (ts-c) PS linkage (IX **5**; the theoretical eight diastereomers observed) compared to one with one 5′ (as-t) PS linkage and two 3′ (gs-ts-c) PS linkages (IX **6**; only 6 of the 8 diastereomers separated). Increasing the number of linkages further increases the complexity of the diastereomer separation as more diastereomers are present and the selectivity becomes insufficient. Obviously, the 32 768 theoretical diastereomers of the full PS oligonucleotide (IX **12**) could not be separated. The analyte with two PS linkages at either 5′ and 3′ ends (IX **7**), resulting in 16 diastereomers (but only nine observed) was also analyzed on a 50 cm micropillar array column with a 60 min gradient which resulted in slightly enhanced selectivity. On a 200 cm column, improved resolution was only demonstrated when extending the gradient time (data not shown). The results in Figure 4 were obtained with a relatively short 10 min gradient and for sequence 5 (two 5′ PS bonds and one 3′ PS bond) reanalysis with a 60 min gradient showed substantial improvement in the diastereomer selectivity (Figure 2B).

3.2.2 | Diastereoselective separation of 16 mer truncated metabolites/degradants

Investigation and identification of metabolites becomes more complicated when diastereomer separation is present unless selectivity is unprecedented and allows clear distinction between the truncated structures. The shortmers of the oligonucleotide sequence with three PS linkages (sequence 5 in Table 1—shortmers **8-11**) were analyzed on a 50 cm micropillar array column, now with TEAA as mobile phase. Figure 2B reveals complete diastereomer selectivity for the four shortmers tested as well as for compound **5** under the applied 60 min gradient conditions. For the 5′ shortmers, diastereomers of the *n*-1 and *n*-2 (IX **8** and **9**, respectively) are also resolved from the diastereomers of compound **5**. However, the 3′ shortmer diastereomers (IX **10**; 3′*n*-1 and IX **11**; 3′ *n*-2) partially overlap with the diastereomers of the compound **5** and with the 5′*n*-1 (IX **8**) diastereomers. In biological samples when these analytes are simultaneously present, improved chromatographic performance combined with a mass-specific detector can facilitate identification and differentiation.

3.2.3 | Diastereoselective separation of 2′-F and 2′-*O*-Me phosphorothioate-modified related sequences

The oligonucleotide sequence **13** was synthesized as tool compound, closely resembling therapeutic RNA oligonucleotides, and injected on a 50 cm micropillar array column using the 15 mM TEAA solvent. The diastereoselectivity observed (Figure 5) was astonishing as all 16 diastereomers were well resolved applying a 72 min gradient. A slight increase in gradient time (from 60 to 72 min) was required to allow elution of the last pair of diastereomers at 77 min within the elution window and keeping percent mobile phase B increase per minute identical. Remarkably the diastereomers appear in clusters, probably reflecting Rp and Sp pairs.

Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

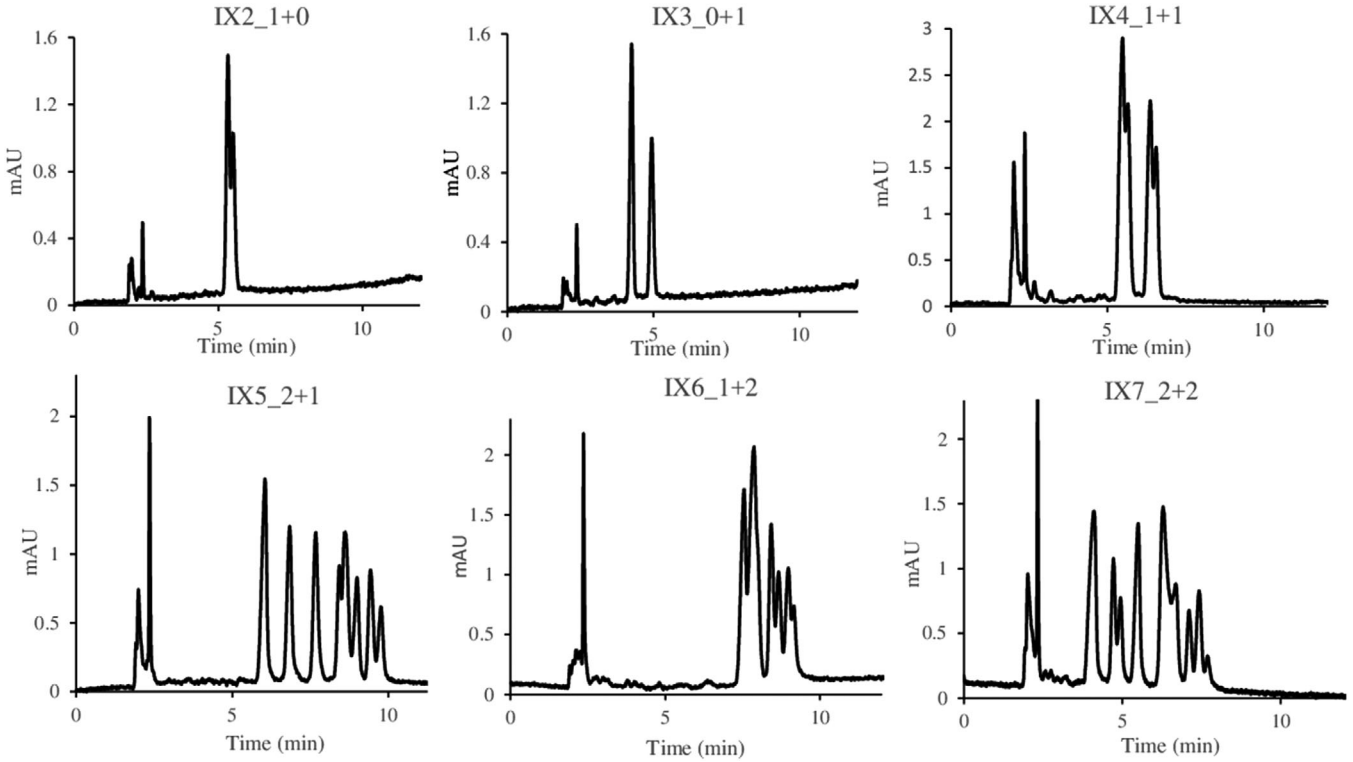

FIGURE 4 Chromatographic separation of six 16-mer oligonucleotides with different # and position of PS bonds (IX 2-7 Table 1) on a 50 cm micropillar array column. The numbers indicate the PS bonds at either the 5′ and 3′ ends of the analytes (1 + 0 indicates 1 PS at 5′ end and no PS bonds at 3′ end and refers to the short name mentioned in Table 1). Chromatographic conditions: mobile phase (MP) A: 15 mM TEAA; MPB: 15 mM TEAA in 40% acetonitrile 10 min gradient from 22.5 to 27.5%, MPB 9-11% acetonitrile (except for the 2 + 2 analyte from 25 to 35% to allow elution within the gradient window), flow rate: 1500 nL/min 4 nL injection volume (conc. 0.25 μg/μL for 0 + 1 and 1 + 0 analyte and 1 μg/μL for all other analytes)

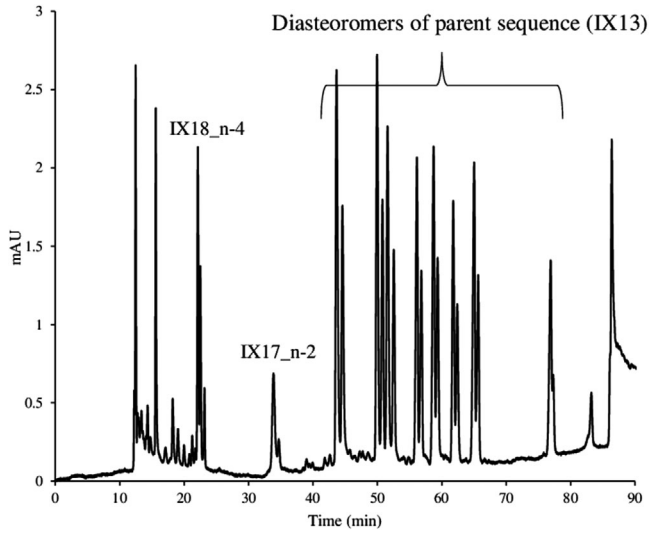

FIGURE 5 Chromatographic separation of the diastereomers of the full-length product (FLP) (IX 13) and related impurities (*n*-2, *n*-4; IX 17 and 18) on a 50 cm micropillar array column. Chromatographic conditions: mobile phase (MP) A: 15 mM TEAA; MPB: 15 mM TEAA in 40% acetonitrile 72 min gradient from 28 to 40% MPB 11-16% acetonitrile, flow rate: 250 nL/min 4 nL injection volume

With five phosphodiester linkages present in the PS sequence (IX **14**), limited diastereoselectivity was observed on a 50 cm micropillar array column (12/32 detected, results not shown). Unexpectedly, for compound **15** with one PS converted to PO at the 5′ end, only four out of eight diastereomers were seen. This result also conflicts with our data obtained for the 16 mer compound with three PS bonds (IX **5**, Figure 2B). The length, sequence, and position of the PS bond seem critical for diastereoselective separation. Partial diastereoselectivity was also observed for *n*-1 sequence (IX **16**) and arabinosyl analogues (IX 19-20): four out of eight for analyte **16,** six out of eight diastereomers for analytes **19-20** on 50 cm micropillar array columns were discriminated. Two more diastereomers were observed when switching to a 200 cm column for analyte **20**. However, since these analytes were purified after synthesis via anion exchange chromatography, some diastereomers could have been removed during the purification process.[34] The chromatograms of the *n*-2 (IX **17**) and *n*-4 (IX **18**) analytes clearly reflect the presence of the expected two and four diastereomers, respectively (not shown).

The TEAA mobile phase also seems powerful for diastereomer separation of these 2′-F and 2′-O-Me-modified RNA sequences. A more in-depth investigation could be done to define the influence of 2′-F, 2′-O-Me nucleosides combined with PS numbers and positions.

Full Article
doi.org/10.1002/ansa.202000175
Chemistry Europe
European Chemical Societies Publishing

3.3 | Performance of micropillar array columns

3.3.1 | Micropillar array columns (50 versus 200 cm)

Supporting information Figures S3 and S4 compile the results obtained on 50 versus 200 cm micropillar array columns with the two solvent systems. It can be noticed that retention times are reduced on the 50 cm column with minimal compromise on the selectivity. Overall, the peak symmetry and resolution slightly improve on the 200 cm column but at the expense of extended run times. As column volume increases, the effect of the injection procedure on the peak shape decreases, resulting in improved peak shapes for longer columns.

The performance of both micropillar array columns was also evaluated with the TEA/HFIP ion pairing system over a range of flow rates, and this for the internal reference standard mixture of T5 to T30 oligonucleotides. Full Width Half Maximum-based peak capacity was calculated according to Neue[35] where the elution window ($t_E = t_n - t_1$; t_n referring to the retention of the last eluting peak and t_1 of the first one) rather than the total gradient time (t_G) was used to give a more accurate representation of the true separation power that can be achieved. When plotting the peak capacity as a function of t_E/t_0 (Supporting information Figure S5A), it becomes clear that the maximum peak capacity, that can be achieved for the different columns, increases with the square root of the number of theoretical plates (N). At a t_E/t_0 value of 40 and at the optimal flow rate of 250 nL/min, peak capacity values of respectively 700 and 350 were obtained for the 200 and 50 cm column. A different and more practical perspective is obtained by plotting the peak capacity values as function of the retention time obtained for the last eluting analyte (t_n) (Supporting information Figure S5B). The use of longer columns does result in increased peak capacity but at the cost of extended run times. For an optimal balance between separation, resolution and sample throughput, the 50 cm column is the preferred choice for the applications under evaluation in the current study. The benefit of matching the flow rate to the sample complexity (and thus gradient length) has been illustrated in Supporting information Figure S5C. For short analyses ($t_G \leq 30$ min), better performance will be achieved at higher flow rates, whereas separation at the optimal flow rate (250 nL/min) will produce better performance for more complex separations at extended gradient lengths ($t_G \geq 60$ min). Therefore, the current study focusses on using either a short ($t_G = 10$ min) method at 1500 nL/min or a longer method ($t_G \geq 60$ min) at 250 nL/min.

3.3.2 | Benchmarking

In a limited benchmarking exercise, the diastereomer separation of oligonucleotide analytes on the 50 cm micropillar array column was compared with the results obtained on two standard packed-bed C_{18} nanoLC columns (15 cm Acclaim Pepmap and nanoEase columns). The selection of the length of these standard nanoLC columns was driven by the backpressure that, with longer columns, was exceeding the 400-bar upper limit of the nanovolume sample injector used. A selection of results is presented in Figure 6 and clearly shows that the micropillar array columns outperform the current golden standard nanoLC columns when evaluating diastereomer selectivity of oligonucleotides. This improved performance and peak shape was attributed to the particular nature of the microarray columns with perfect ordered pillars virtually eliminating column peak dispersion.

The present study was initiated to evaluate the performance of micropillar array columns for chromatographic separation of oligonucleotides. Oligonucleotide separation is predominantly carried out using ion pairing reversed phase chromatography. Many ion pairing reagents in combination with different counter ions have extensively been discussed in literature over the last years with emphasis on the chromatographic resolution and mass spectrometric sensitivity. The current study has focused on the potential and chromatographic performance of a new type of nanoLC columns based on micropillar array technology. Therefore, two mobile phase solvent systems were

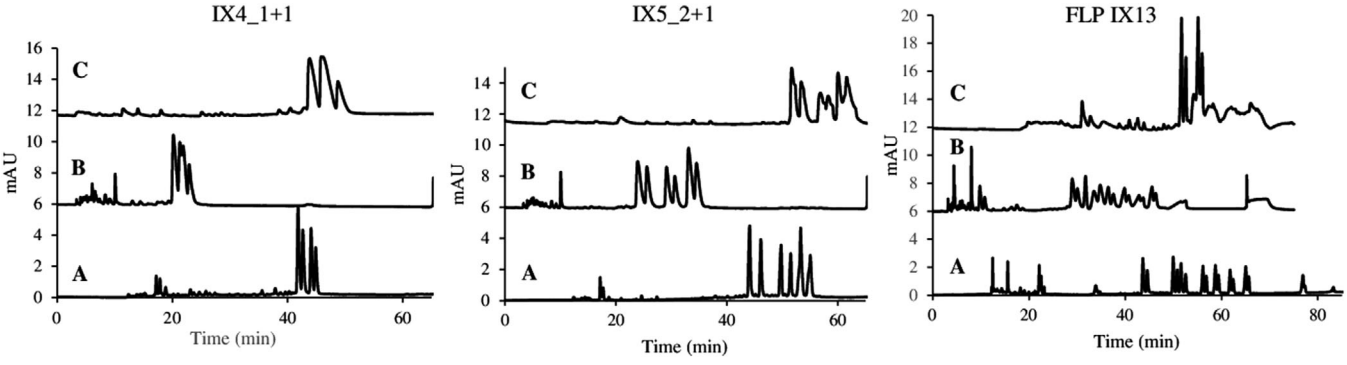

FIGURE 6 Chromatograms obtained with the conditions specified below for 16 mer oligonucleotides with 2 and 3 PS bonds (1+1 (IX 4) and 2+1 (IX 5)) and for the full-length product (FLP) (IX 13) on three different NanoLC columns. A: 50 cm micropillar array column; B: Pepmap 75 µm m × 15 cm; C: NanoEase 75 µm × 15 cm. Chromatographic conditions: 50 cm micropillar column mobile phase (MP) A: 15 mM TEAA; MPB: 15 mM TEAA in 40% acetonitrile 60 min gradient from 28 to 38%, MPB flow rate: 250 nL/min 4 nL injection volume Pepmap 75 µm × 15 cm mobile phase (MP) A: 15 mM TEAA, MPB: 15 mM TEAA in 40% acetonitrile 60 min gradient from 25 to 35% MPB (FLP from 33 to 43% MP-B); flow rate: 250 nL/min; NanoEase 75 µm × 15 cm mobile phase (MP) A: 15 mM TEAA, MPB: 15 mM TEAA in 40% acetonitrile 60 min gradient from 15 to 25% MPB (FLP from 27 to 37% MP-B), flow rate: 250 nL/min

considered, one aimed to facilitate diastereomer selectivity evaluation (TEAA) and the choice of the second one (TEA/HFIP), including hexafluoro isopropanol, as buffering acid was made based on literature showing high efficiency of oligonucleotide separation without diastereomer selectivity.[10,19]

The TEAA solvent was included since it is widely applied, despite its well-known incompatibility with MS. However, in a recent publication, Enmark et al.[8] have also identified this ion pair reagent to give the highest diastereomer selectivity for PS containing oligonucleotides at lower concentrations.

Although understanding of the exact mechanisms governing suppression or enhancement of the diastereomer selectivity is limited, some general findings are consistent in literature (and in the current study). Independent of the choice of the RP column, TEAA solvent shows propensity for enhanced diastereomer selectivity especially at lower concentrations. Under these conditions, ion-pair formation in the eluent is favored and hydrophobic elution will dominate. Increasing IP concentration or changing to more hydrophobic ion pair reagents (hexylamine or tributylamine with acetate) results in reduced (or no) diastereomer selectivity in line with the theory that these conditions drive the ion pair reagent toward the stationary phase inducing more ionic interactions. A similar hypothesis has been proposed for TEA/HFIP eluents. Fountain et al.[18] showed enhanced peak capacity with 400 mM HFIP in combination with 16.3 mM TEA, which is the maximum solubility of TEA in 400 mM HFIP. This limited solubility of the ion pairing can contribute to an enhancement of the ion pairing efficiency, driving more TEA+ ions on the reversed phase sorbent. Again, this situation favors ionic interactions and suppresses diastereomer separation. In this investigation a common ion pairing reagent (TEA) was used in two mobile phase systems. The type and concentration of counterion (acetate vs HFIP) was responsible for the diastereomer resolution in line with the above arguments. Therefore, combining TEA and HFIP at lower HFIP concentrations IP could potentially also show diastereomer separation. To reiterate, the mechanism of ion pair chromatography in combination with a buffering acid for separation of oligonucleotides is not fully understood and explained but observed effects are also confirmed on this new type of columns. The micropillar array columns show great potential to enhance chromatographic capacity and resolution independent of the ion pair reagent and acid used. Both 50 and 200 cm micropillar array columns provide improved performance but in routine applications the 50-cm micropillar array column is more convenient.

4 | CONCLUSIONS

Compared to standard packed nanoLC columns, micropillar array columns demonstrated increased performance for the separation of oligonucleotides and their diastereomers. Using a 15 mM TEAA solvent system, PS diastereomers up to a maximum of four PS bonds could be separated. In contrast, under the investigated TEA/HFIP conditions diastereomer separation was completely absent but some of the closely related structures were chromatographically resolved. Since, the 200 cm column provided in general minimal or no improvement over the 50 cm micropillar array column, the latter is considered the best choice for oligonucleotide analyses due to its shorter equilibration and analysis times. As for any nanoLC column, working with micropillar array columns demands special attention to minimize extra-column dead volumes, in particular because the relative impact of extra column band broadening increases with improved resolution. Currently, also microflow micropillar array columns are commercialized to provide opportunity for shorter analysis time and better implementation with more routine HPLC instruments.

ACKNOWLEDGMENTS

We would like to thank Ronald de Vries and Thomas De Vijlder for interesting and fruitful discussions and Bart Noten and Luc Sips for their technical support.

CONFLICT OF INTEREST

L. Dillen, T. Deschrijver, and F. Cuyckens are employees of Janssen R&D and declare no conflict of interest.

K. Van Mol and J. Op de Beeck of PharmaFluidics.

P. Jacobs is COO and co-founder of PharmaFluidics.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Crooke S, Witztum J, Bennett F, Baker B. RNA-targeted therapeutics. *Cell Metab*. 2018;27:714-739.
2. Dowdy S, Levy M. RNA therapeutics (almost) comes of age: targeting, delivery and endosomal escape. *Nucleic Acid Ther*. 2018;28(3):107-108.
3. Crooke ST, Vickers T, Lima W, Wu H. Mechanisms of anti-sense drug action, an introduction. In: Crooke ST, ed. Antisense Drug Technology: Principles, Strategies and Applications. Boca Raton, FL: CRC Press; 2008:3-46.
4. Setten R, RossieJ HanS. The current state and future directions of RNAi-based therapeutics. *Nature Rev*. 2019;18:421-446.
5. Chernikov I, Vlassov V, Chernolovskaya E. Current development of siRNA bioconjugates: from research to the clinic. *Front. Pharmacol*. 2019;10:101-125.
6. Capaldi D, Teasdale A, Henry S, et al. Impurities in oligonucleotide drug substances and drug products. *Nucleic Acid Ther*. 2017;27(6):309-322.
7. Sakamuri S, Eltepu L, Liu D, et al. Impact of phosphorothioate chirality on double-stranded siRNAs: a systematic evaluation of stereopure siRNA designs. *Chembiochem*. 2020;21:1-6.
8. Enmark M, Rova M, Samuelson J, Ömskov E, Schweikart F, Fronstedt T. Investigation of factors influencing the separation of diastereomers of phosphorothioated oligonucleotides. *Anal. Bioanal. Chem*. 2019;411(15):3383-3394.
9. Chen B, Bartlett M. Evaluation of mobile phase composition for enhancing sensitivity of targeted quantification of oligonucleotides using ultra-high-performance liquid chromatography and mass spectrometry: application to phosphorothioate deoxyribonucleic acid. *J. Chromatogr. A*. 2013;1288:73-81.
10. Gilar M, Fountain K, Budman Y, Holyoke J, Davoudi H, Gebler J. Characterization of therapeutic oligonucleotides using liquid chromatography with on-line mass spectrometry detection. *Oligonucleotides* 2003;13(4):229-243.

11. Apffel A, Chakel J, Fischer S, Lichtenwalter K, Hancock W. New procedure for the use of high-performance liquid chromatography-electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides. *J. Chromatogr. A.* 1997;777(1):3-21.
12. Nuckowski L, Kaczmarkiewicz A, Studzinska S. Development of a SPE method for the extraction of phosphorothioate oligonucleotides from serum samples. *Bioanalysis* 2018;10(20):1667-1677.
13. Li N, El Zahar N, Saad J, van der Hage E, Bartlett M. Alkylamine ion-pairing reagents and the chromatographic separation of oligonucleotides. *J. Chromatogr. A.* 2018;1580:110-119.
14. Basiri B, Murph MM, Bartlett MG. Assessing the interplay between the physicochemical parameters of ion-pairing reagents and the analyte sequence on the electrospray desorption process for oligonucleotides. *J. Am. Soc. Mass Spectrom.* 2017;28:1647-1656.
15. Basiri B, van Hattum H, van Dongen WD, Murph M, Bartlett M. The role of fluorinated alcohols as mobile phase modifiers for LC-MS analysis of oligonucleotides. *J. Am. Soc. Mass Spectrom.* 2017;28(1):190-199.
16. Studzinska S, Rola R, Buszewski B. The impact of ion-pairing reagents on the selectivity and sensitivity in the analysis of modified oligonucleotides in serum samples by liquid chromatography coupled with tandem mass spectrometry. *J. Pharm. Biomed. Anal.* 2017;138:149-152.
17. Gong L, McCullag J. Comparing ion-pairing reagents and sample dissolution solvents for ion-pairing reversed-phase liquid chromatography/electrospray ionization mass spectrometry analysis of oligonucleotides. *Rapid Commun. Mass Spectrom.* 2014;28:339-350.
18. Fountain K, Gilar M, Gebler J. Analysis of native and chemically modified oligonucleotides by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 2003;17:646-653.
19. Gilar M. Analysis and purification of synthetic oligonucleotides by reversed-phase high-performance liquid chromatography with photodiode array and mass spectrometry detection. *Anal. Biochem.* 2001;298(2):196-206.
20. Studzinska S, Buszewski B. Analysis of microRNA and modified oligonucleotides with the use of ultra-high-performance liquid chromatography coupled with mass spectrometry. *J. Chromatogr. A.* 2018;1554:71-80.
21. Oberacher H, Huber CG. Capillary monoliths for the analysis of nucleic acids by high-performance liquid chromatography-electrospray ionization mass spectrometry. *Trends Anal. Chem.* 2002; 21:166-174.
22. Svec F, Huber CG. Monolithic materials: promises, challenges and achievements. *Anal. Chem.* 2006;78(7):2100-2107.
23. Elzahar N, Magdy N, El-Kosasy A, Bartlett M. Degradation product characterization of therapeutic oligonucleotides using liquid chromatography mass spectrometry. *Anal. Bioanal. Chem.* 2018;410(14):3375-3384.
24. Sutton JM, Guimaraes GJ, Annavarapu V, van Dongen WD, Bartlett MG. Current state of oligonucleotide characterization using liquid chromatography-mass spectrometry: insight into critical issues. *J. Am. Soc. Mass Spectrom.* 2020;31:1775-1782.
25. Tóth G, Panić-Janković T, Mitulović G. Pillar array columns for peptide separations in nanoscale reversed-phase chromatography. *J. Chromatogr. A.* 2019;1603:426-432.
26. Ihling C, Tänzler D, Hagemann S, et al. Mass spectrometric identification of SARS-VoV-2 proteins from gargle solution samples of COVID-19 patients. *J Proteome Res.* 2020;19:4389−4392.
27. Lam AT, Zhang XN, Courouble VV, et al. A bifunctional NAD^+ for profiling poly-ADP-ribosylation-dependent interacting proteins. *ACS Chem. Biol.* 2021;16:389-396.
28. Beyer T, Klose F, Kuret, et al. Tissue- and isoform-specific protein complex analysis with natively processed bait proteins. *J. Proteomics.* 2021;231:103947.
29. Nys G, Cobraiville G, Fillet M. Multidimensional performance assessment of micro pillar array column chromatography combined to ion mobility-mass spectrometry for proteome research. *Anal. Chim. Acta.* 2019;1086:1-13.
30. Reimann L, Schwäble AN, Fricke AL, et al. Phosphoroproteomics identifies dual-site phosphorylation in an extended basophilic motif regulating FILIP1-mediated degradation of filamin-C. *Nature Commun. Biol.* 2020;3:3253.
31. Sandra K, Vandenbussche J, t'Kindt R, et al. Evaluation of micro-pillar array columns (μPAC) combined with high resolution mass spectrometry for lipidomics. *LCGC Specl. Iss.* 2017;30(6):6-13.
32. Müller JB, Geyer PE, Colaço AR, et al. The proteome landscape of the kingdoms of life. *Nature.* 2020;582:592-596.
33. Calvitt J, Levn D, Shepperd B, Gruenloch CJ. Chemistry at the 2′ position of constituent nucleotides controls degradation pathways of highly modified oligonucleotide molecules. *Oligonucleotides.* 2010;20(5):239-251.
34. Thayer JR, Wu Y, Hansen E, Angelino MD, Raoa S. Separation of oligonucleotide phosphorothioate diastereoisomers by pellicular anion-exchange. *J. Chromatogr. A.* 2011;1218(6):802-808.
35. Neue UD. The theory of peak capacity in gradient elution. *J. Chromatogr. A.* 2005;1-2:153-161.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Dillen L, Deschrijver T, Van Mol K, Op de Beeck J, Jacobs P, Cuyckens F. Evaluation of micropillar array columns for chromatographic separation of phosphorothioated oligonucleotides and their diastereomers. *Anal Sci Adv*. 2021;2:354–363. https://doi.org/10.1002/ansa.202000175