Ion-pair-free RPLC-MS of synthetic oligonucleotides

Impurity analysis of siRNA with a polybutylene terephthalate-based column

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In this study, we developed an ion-pair-free reversed-phase chromatography method using DCPak PBT. The ion-pair-free RP provides sufficient retention and adequate selectivity for oligonucleotides to allow direct coupling with ESI-MS.

As of today, reversed-phase liquid chromatography using ion-pair (IP) reagents still plays a dominant role in the separation of synthetic oligonucleotides. However, the use of ion-pair reagents leads to a contamination of the ionization source of mass spectrometers (MS) and significantly suppresses ionization and thus the sensitivity of the method. In this work, we demonstrate RPLC devoid of ion-pair reagents in the mobile phase using a polybutylene terephthalate-based column. With methanol as organic modifier it succeeded in generating orthogonal selectivities compared to ion-pair RPLC and HILIC Amide for the shortmer impurities of synthesized Patisiran siRNA antisense and sense single strands. The separated impurities could subsequently be detected and characterized directly by ESI-TOF-MS without contaminating the system with ion-pair reagents.

Synthetic oligonucleotides such as ASO and siRNA are gaining importance as new therapeutic modalities because they can treat a wide range of different diseases by regulating gene expression [1-3]. Due to the high polarity resulting from sugarphosphate backbones, oligonucleotides often do not exhibit sufficient retention and selectivity on C18 stationary phases in conventional reversed-phase liquid chromatography (RPLC) and therefore require alkylamines as ion pairing (IP) reagents in the mobile phase [4,5]. To minimize ion suppression effects during the ESI-MS detection that usually result from the employed triethylammonium acetate, Apffel et al. suggested combining hexafluoroisopropanol (HFIP) with triethylamine (TEA) as mobile phase additives to increase MS sensitivity because less TEA is required in presence of HFIP [6–8]. This is an approach that has long been popular in widespread use. Apart from this, however, the use of alkylamines causes contamination of the ion source, which can be particularly problematic when MS instruments are not used exclusively for oligonucleotide analyses. For this reason, alternative separation methods are highly desirable [9,10]. Recently, we already have succeeded in generating retention as well as selectivity for RNA oligonucleotides using a cholesterol-based separation material without any addition of ion-pair reagents [11]. In our search for other stationary phases with similar retention potential, we came across a polybutylene terephthalate-based column DCpak PBT (from Daicel, supplied by Chiral Technologies Europe, Illkirch, France), which has a mixed phase character due to the various functional groups ranging from hydrophobic domains (butylene residue), over π-π-interactions (aromatic phthalate group), to dipole-dipole interactions and hydrogen bonding (ester moiety) (Fig. 1A). This circumstance allows for mixed hydrophobic, aromatic and hydrophilic interactions. Initially designed for supercritical fluid chromatography (SFC), DCpak PBT also found its way into the separation of polar compounds [12,13]. Its unique structure prompted us to investigate DCpak PBT for its selectivity for oligonucleotides. For this purpose, the oligonucleotide strands of the small interfering RNA (siRNA) therapeutic patisiran (Fig. 1B) were used as model test compounds. Patisiran (trade name Onpattro), approved by the US Food and Drug Administration (FDA) in 2018, is an siRNA therapeutic for the treatment of polyneuropathy in patients with hereditary transthyretin-mediated amyloidosis. To increase its stability to enzymatic degradation in the human body and improve bioavailability, several nucleotides of the RNA strands are modified by methylation in 2' position of the ribose residues (see Fig. 1B). Individual sense (passenger) and antisense (guide) strands (Fig. 1B) were purchased as desalted raw products without further purification; the test compounds used herein do not correspond to the actual final pharmaceutical product quality but deliberately contain a large number of impurities which allows for assessment of the selectivity of the column for structurally closely related oligonucleotides. When the DCpak PBT column was employed with common reversed-phase conditions with a linear gradient of methanol using 20 mM ammonium formate as buffer salt, a large number of impurities were resolved from each other for both single strands. These conditions are ESI-MS friendly and allowed the direct coupling and characterization of

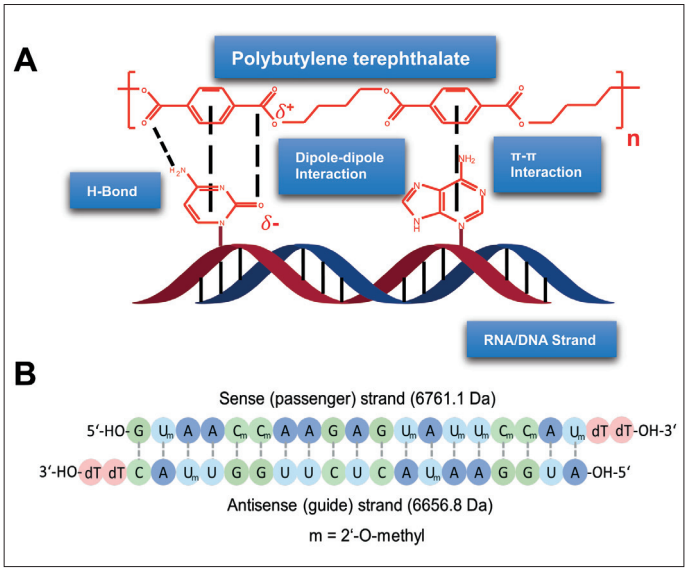

Fig. 1. A: The chemical structure of the selector of DCpak PBT. The tentative mechanism for interaction with DNA/RNA is schematically illustrated. B: The structure (base sequence) of the two single strands of patisiran.

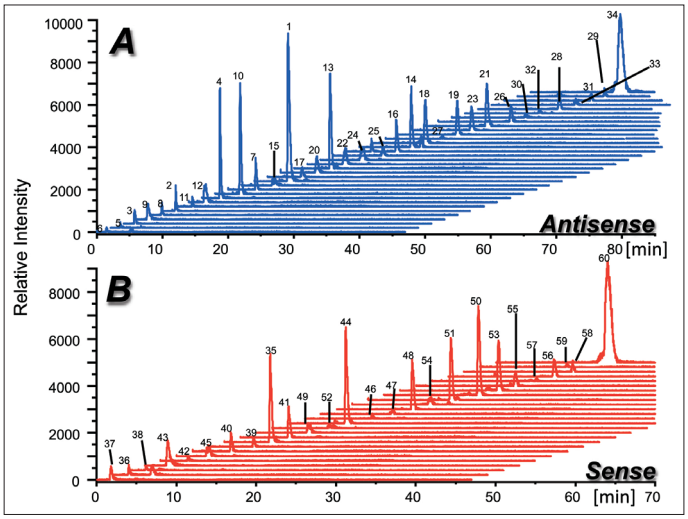

Fig. 2. Extracted ion chromatograms (EIC) of detected impurity species of (A) antisense and (B) sense strands. Please refer to Tables 1 and 2 for numbering. LC-MS conditions: Column dimension: DaicelPak PBT, 150x3.0 mm, 3 µm. Mobile phase A: water, 20 mM ammonium formate, pH 6.3. Mobile phase B: Methanol/water 9:1 (v/v), 20 mM ammonium formate, pH 6.3 (adjusted with aqueous fraction only). Gradient: 10%-45% MPB in 28 min. Column temperature: 40 °C. Flow rate: 0.6 mL/min. MS instrumentation: SCIEX TripleTOF 5600+ QTOF mass spectrometer with Duospray source. ESI mode, negative. The MS parameters for nebulizer gas, heater gas, curtain gas, source temperature, ion spray voltage, declustering potential and collision energy were set as follows: 90 psi, 90 psi, 35 psi, 550 °C, -4500 V, -200 V, -10 V for TOF-MS and -35 V for MS/MS experiments. Mass (m/z) range of MS1 experiments were from 100 to 2000. MS2 data was generated by information dependent acquisition (IDA) mode (with one MS full scan experiment followed by four MS2 experiments with an accumulation time of 200 ms each). The precursor isolation was set from 200 to 1250 m/z.

the detected peaks by time-of-flight mass spectrometry (TOF-MS). Figure 2 shows the staggered extracted ion chromatograms for both single strands (2A: antisense, 2B: sense). A total of 60 different oligonucleotide species were detected in both samples (see Tables 1 and 2). During the synthesis of oligonucleotides a number of impurities can arise [14]. Due to coupling errors, impurities are formed that are missing one or more nucleotides (e.g., N-1, N-2 shortmers, etc.). In these shortmers, nucleotides may be missing at both the 3' and 5' ends, depending on the mechanism of formation [14,15]. In addition, synthetic oligonucleotides may be contaminated by structurally closely related synthesis by-products. In general, four different structural

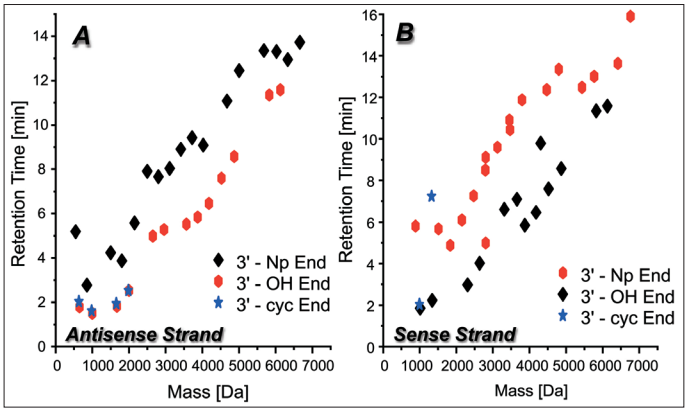

Fig. 3. Correlation between deconvoluted mass of detected impurities for patisiran and retention time on DCPak PBT. Black trace, square: species with free hydroxy at the 3' end; red trace, circle: species with phosphate at the 3' end; blue trace, triangle: cyclic 2',3' phosphate (phosphodiester) at the 3' end. A: patisiran antisense strand; B: patisiran sense strand.

variables can be distinguished in oligonucleotides: i) oligonucleotide length (due to the different number of deletions), ii) base sequence (due to 5' and 3' nucleotide deletions; coupling error products with missing nucleotides at both the 3' and 5' ends were present), iii) 2' modifications (hydroxyl, methoxy, and hydrogen for 2nt overhang), and iv) 3' end modifications. At the 3'-end, the oligonucleotides have either a hydroxyl end (OH), a phosphate end (Np), or a 2',3'-cyclic phosphodiester end (cyc) [16]. Basically, the detected impurities can be divided into 3 subgroups according to these structural features: (i) a 3'->5' impurity series with nucleotide deletions at the 5' end and a free 3'-OH (3'-OH end), (ii) a 5'->3' impurity series with nucleotide deletions at the 3' end and a phosphorylated 3' end (3'-Np end), and iii) a corresponding 5'->3' impurity series in which the 3'-Np end is converted to a 2',3'-cyclic phosphodiester bond (3'-cyc end). Within each series, retention increases with each additional nucleotide, but not to the same extent for different nucleotides as expected based on the difference in base hydrophobicity (C<G<A<T/U) [17]. However, there are some negative retention shifts with additional nucleotide, e.g., from 2-mer to 3-mer of the 3-OH end series (change from deoxynucleotide sequence to ribonucleotide series), from 3-mer to 4-mer (U to mU), from 6-mer to 7-mer (U to G), 10-mer to 11-mer (C to U), 15-mer to 16-mer (U to G). They cannot be explained by the hydrophobicity of the bases alone. The stronger retention for the 5'-2-mer-OH (5'-dTdT-OH-3') compared to the 5'-3-mer-OH (5'-CdTdT-OH-3') can be explained by the deoxy-ribonucleotides, in which a hydrogen atom instead of a hydroxyl group in the 2'-position leads to a lower polarity and thus to potentially stronger hydrophobic interactions with the stationary phase surface. Interestingly, these negative retention shifts occur mainly when the sequence is extended by either U or C (vide infra) (Fig. 3, Table 1, 2).

Conclusion

In this study, we developed an ion-pair-free reversed-phase chromatography method using DCPak PBT, which was originally designed for supercritical fluid chromatography. The ion-pair-free RP provides sufficient retention and adequate selectivity for oligonucleotides (as documented for the antisense and sense strands of patisiran) to allow direct coupling with ESI-MS. The silica-coated polybutylene terephthalate selector with its variety of different interaction sites (hydrophobic moieties, aromatic systems, and dipole/H-bond interaction sites) interacts with the oligonucleotides without the need for an ion-pairing reagent. In general, a large number of impurities could be separated and characterized by ESI-TOF-MS using the optimized method. PBT-based ion-pair-free RPLC appears to be a suitable method for oligonucleotide analysis without contaminating MS ion sources, and is therefore suitable for LC-ESI-MS instruments that are not dedicated exclusively to oligonucleotide analysis and therefore should not come into contact with ion-pair reagents.

Table 1 Summary of detected oligonucleotide species in the raw product of the antisense strand.

Name	Sequence	Deconvoluted mass	Peak No. Fig. 2A	t_R RP
5' 2mer - OH	5' dTdT 3' - OH	546.129	1	5.19
3' 2mer - cyc	5' AU 3' - cyc	635.071	2	2.03
3' 2mer - Np	5' AU 3' - Np	653.082	3	1.79
5' 3mer - OH	5' CdTdT 3' - OH	851.170	4	2.78
3' 3mer - cyc	5' AUG 3' - cyc	980.118	5	1.61
3' 3mer - Np	5' AUG 3' - Np	998.135	6	1.51
5' 5mer - OH	5' mUACdTdT 3' - OH	1501.257	7	4.24
3' 5mer - cyc	5' AUGGA 3' - cyc	1654.209	8	1.94
3' 5mer - Np	5' AUGGA 3' - Np	1672.221	9	1.82
5' 6mer - OH	5' UmUACdTdT 3' - OH	1806.282	10	3.87
3' 6mer - cyc	5' AUGGAA 3' - cyc	1983.260	11	2.53
3' 6mer - Np	5' AUGGAA 3' - Np	2001.264	12	2.53
5' 7mer - OH	5' GUmUACdTdT 3' - OH	2151.330	13	5.58
5' 8mer - OH	5' GGUmUACdTdT 3' - OH	2496.368	14	7.91
3' 8mer - Np	5' AUGGAAmUA 3' - Np	2650.364	15	4.99
5' 9mer - OH	5' UGGUmUACdTdT 3' - OH	2802.396	16	7.66
3' 9mer - Np	5' AUGGAAmUAC 3' - Np	2955.407	17	5.28
5' 10mer - OH	5' UUGGUmUACdTdT 3' - OH	3108.418	18	8.04
5' 11mer - OH	5' CUUGGUmUACdTdT 3' - OH	3413.459	19	8.90
3' 11mer - Np	5' AUGGAAmUACUC 3' - Np	3566.466	20	5.52
5' 12mer - OH	5' UCUUGGUmUACdTdT 3'-OH	3719.470	21	9.42
3' 12mer - Np	5' AUGGAAmUACUCU 3' - Np	3873.496	22	5.85
5' 13mer - OH	5' CUCUUGGUmUACdTdT 3' - OH	4024.528	23	9.08
3' 13mer - Np	5' AUGGAAmUACUCUU 3' - Np	4178.519	24	6.46
3' 14mer - Np	5' AUGGAAmUACUCUUG 3' - Np	4523.570	25	7.60
5' 15mer - OH	5' mUACUCUUGGUmUACdTdT 3' - OH	4673.609	26	11.08
3' 15mer - Np	5' AUGGAAmUACUCUUGG 3' - Np	4868.606	27	8.57
5' 16mer - OH	5' AmUACUCUUGGUmUACdTdT 3' - OH	5002.661	28	12.45
5' 18mer – OH	5' GAAmUACUCUUGGUmUACdTdT 3' - OH	5676.783	29	13.35
3' 18mer - Np	5' AUGGAAmUACUCUUGGUmUA 3' - Np	5823.723	30	11.35
5' 19mer - OH, N-2	5' GGAAmUACUCUUGGUmUACdTdT 3' - OH	6021.828	31	13.31
3' 19mer - Np	5' AUGGAAmUACUCUUGGUmUAC 3' - Np	6128.748	32	11.58
5' 20 mer - OH, N-1	5' UGGAAmUACUCUUGGUmUACdTdT 3' - OH	6327.814	33	12.95
Antisense	5' AUGGAAmUACUCUUGGUmUACdTdT 3' - OH	6656.866	34	13.73

Table 2 Summary of detected oligonucleotide species in the raw product of the sense strand.

Name	Sequence	Deconvoluted mass	Peak No. Fig. 2B	t_R RP
5' 3mer_A-OH	5' mUdTdT 3' – OH	866.170	35	5.80
3' 3mer - cyc	5' GmUA 3' - cyc	994.133	36	2.07
3' 3mer -Np	5' GmUA 3' - Np	1012.130	37	1.85
3' 4mer- Np	5'GmUAA 3' - Np	1341.186	38	2.23
5' 5mer- OH	5' mCAmUdTdT 3' - OH	1514.268	39	5.67
5' 6mer - OH	5' mCmCAmUdTdT 3' - OH	1833.322	40	4.88
5' 7mer - OH	5' mUmCmCAmUdTdT 3' - OH	2153.353	41	6.09
5' 7mer - Np	5' mUmCmCAmUdTdT 3' - Np	2233.336	42	3.53
3' 7mer – Np	5' GmUAAmCmCA 3' - Np	2308.347	43	2.98
5' 8mer - OH	5' mUmUmCmCAmUdTdT 3' - OH	2473.396	44	7.26
3' 8mer – Np	5' GmUAAmCmCAA 3' - Np	2637.394	45	4.02
5' 9mer – OH (A->mU)	5' mUmUmUmCmCAmUdTdT 3' - OH	2793.442	46	8.50
5' 9mer - OH	5' AmUmUmCmCAmUdTdT 3' - OH	2802.449	47	9.12
5' 10mer - OH	5' mUAmUmUmCmCAmUdTdT 3' - OH	3122.492	48	9.60
3' 10mer – Np	5' GmUAAmCmCAAGA 3' - Np	3311.495	49	6.61
5' 11mer – OH (G->A)	5' AmUAmUmUmCmCAmUdTdT 3' - OH	3451.544	50	10.90
5' 11mer – OH	5' GmUAmUmUmCmCAmUdTdT 3' - OH	3467.530	51	10.44
3' 11mer – Np	5' GmUAAmCmCAAGAG 3' - Np	3656.547	52	7.10
5' 12mer – OH	5' AGmUAmUmUmCmCAmUdTdT 3' - OH	3796.590	53	11.88
3' 13mer - Np	new, 5' GmUAAmCmCAAGAGmUA 3' - Np	4305.673	54	9.79
5' 14mer – OH	5' AGAGmUAmUmUmCmCAmUdTdT 3' - OH	4470.688	55	12.37
5' 15mer – OH	5' AAGAGmUAmUmUmCmCAmUdTdT 3' - OH	4799.718	56	13.01
5' 17mer – OH	5' mCmCAAGAGmUAmUmUmCmCAmUdTdT 3' - OH	5437.841	57	12.48
5' 18mer – OH	5' AmCmCAAGAGmUAmUmUmCmCAmUdTdT 3' - OH	5766.896	58	13.35
5' 20mer - OH (N-1)	5' mUAAmCmCAAGAGmUAmUmUmCmCAmUdTdT 3' - OH	6416.030	59	13.63
Sense, Patisiran	5' GmUAAmCmCAAGAGmUAmUmUmCmCAmUdTdT 3' - OH	6761.087	60	15.90

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