Oligonucleotides

Capillary flow ion pair reversed-phase separation for very sensitive oligonucleotide LC-HRMS analysis and characterisation

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Abstract

Here we present a workflow with a DNAPac RP 300 µm capillary column, the low flow Vanquish Neo UHPLC system coupled to the Orbitrap Exploris 480 MS for a full and sensitive ion-pair reverse-phase LC-HRMS workflow utilizing low capillary flowrates suitable for characterisation of oligonucleotides from smaller ASO RNA to larger therapeutic mRNA[1-2], while greatly decreasing the eluent and sample consumption compared to standard microflow flowrates currently utilized for most oligonucleotide LC-MS methods.

Introduction

The emergence of mRNA vaccines has resulted in a growing demand for expanding the analytical toolbox for all types of oligonucleotide therapeutics. Ion pair reverse-phase (IPRP) liquid chromatography is a powerful technique for analysis of oligonucleotides.

However, current IPRP methods employs 200–500 µl/min flow rates which require a significant consumption of sample, organic solvent, and ion pair reagent. For some applications like mRNA sequencing a relatively high amount of mRNA is required at 20–50 µg[1], and a need for more sensitive methods is required as a small IVT production produces only 100 µg mRNA.

We have therefore developed a IPRP capillary flow rate workflow to significantly increase the sensitivity of the LC-HRMS characterisation of oligonucleotides while greatly reducing eluent consumption.

Materials and methods

Capillary flow ion-pair reversed-phase (IPRP) separations were performed with the new monodisperse pH stable low flow Thermo Scientific™ DNAPac™ RP column (300 µm, 50 mm).
Low flow 2–5 µl/min gradients were applied using a Thermo Scientific™ Vanquish™ Neo UHPLC system, coupled to a Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer equipped with an Thermo Scientific™ EASY-Spray™ low-flow ion source.

Samples:
10mer: GAGCGGCTGT
20mer: GAGCGGCTGTGAGCGGCTGT
30mer: GAGCGGCTGTGAGCGGCTGTGAGCGGCTGT
40mer: GAGCGGCTGTGAGCGGCTGTGAGCGGCTGTGAGCGGCTGT
50mer: GAGCGGCTGTGAGCGGCTGTGAGCGGCTGTGAGCGGCTGTGAGCGG CTGTGAGCGGCTGT
55mer: GAGCGGCTGTGAGCGGCTGTGAGCGGCTGTGAGCGGCTGTGAGCG GCTGTGAGCGGCTGTGAGCG
Oligonucleotide duplex (sense and antisense): 6.2k Da and 6.0k Da.

The eluent systems and UHPLC cleaning procedure were optimized to eliminate amine and metal adducts, while maintaining high chromatographic resolution and very sensitive mass spectrometric response. See **Table 1** and **Table 2** for HRMS and UHPLC conditions, respectively.
Data was acquired using either Thermo Scientific™ Xcalibur™ Software or Thermo Scientific™ Chromeleon™ CDS Software v. 7.3.2 MUb and analysed in either Chromeleon or Thermo Scientific™ BioPharma Finder™ software v. 5.2.

Table 1. Optimized HRMS Conditions for minimizing in-source produced impurities to obtain clean data for efficient impurity screening.

Full MS – Orbitrap Exploris 480 Mass Spectrometer	
Spray Voltage: Negative Ion 1.4 kV	Scan Range: 570–3,000
Ion Transfer Tube Temp.: 250 ˚C	Microscans: 2
In-Source CID: Off	Resolution: 120,000
RF Lens: 50%	Max. Injection Time: Auto

Table 2. Eluent gradient conditions used with the Vanquish Neo UHPLC System for capillary flow ion pair RP separation of oligonucleotides.

Time (min)	Flow (µl/min)	% Eluent B	Curve	Temp. (˚C)
0.0	4.00	5.0	5	50
0.5	4.00	8.0	5	50
4	4.00	12.0	5	50
5	4.00	90.0	5	50
6	4.00	90.0	5	50
Buffer A - 20 mM triethylamine (TEA) and 60 mM HFIP in water Buffer B - 20 mM triethylamine (TEA) and 60 mM HFIP in methanol				

Results

In recent years, a lot of work has been done in developing and improving methods for characterisation of oligonucleotides therapeutics. However, most IPRP LC-MS methods utilize relatively high flowrates, which results in a relatively high sample consumption for some applications such as mRNA sequencing.
Here, we apply a new 300 µm ID monodisperse DNAPac RP column for IPRP LC-HRMS that together with the Vanquish Neo UHPLC provide efficient chromatography for oligonucleotides ranging in size. The low-flow IPRP workflow provided excellent and robust chromatography, with sensitive MS data, while greatly decreasing sample amount and with 100x less eluent usage compared to current microflow workflows.

Baseline separation and robust chromatography of an oligonucleotide mix of 10, 20, 30, 40, 50, and 55mer was obtained with a short 4 min gradient at 4 µl/min, see **Figure 1.**

Using an EASY-Spray Source and the Orbitrap Exploris 480 MS, clean mass spectra was obtained for all oligonucleotides with no significant presence of amine or metal ions due to the fully inert flow path of the Vanquish Neo UHPLC and DNAPac RP column, see **Figure 2**. Using the soft source and efficient ion transmission of the Orbitrap mass spectrometers, enables optimization of the source conditions to not produce any in-source induced artifacts, such as base loss, while still minimizing the amount of ion pair adducts, thus making data analysis and quantitation simple. As increasingly harsh source conditions can be shown to fragment oligonucleotides during the analysis[2]. The optimized source settings for minimal ion pair adduct formation and in-source fragmentation are shown in **Table 1.** However, due to low flow rates, source activation and temperature could be kept at a minimum without getting any significant amine adducts without inducing any source fragmentation.

Figure 1. Efficient separation of oligonucleotide mixture ranging from 10mer to 55mer by capillary flow ion-par chromatography coupled to HRMS.

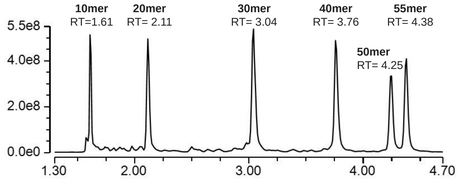
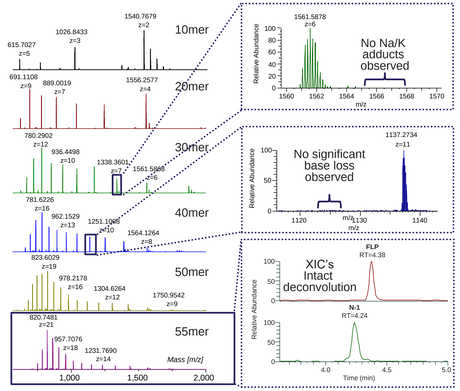


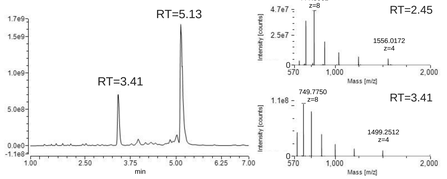
Figure 2. Clean mass spectra observed with no observable metal adducts or base loss, enabling efficient deconvolution, identification, and impurity screening at capillary flow LC-HRMS.



Metal adducts such as K and Na have become accepted as unavoidable in LC-MS oligonucleotide analysis. However, by using novel and simple cleaning techniques for the fully inert and stainless steel free UHPLC system, high-quality reagents and eluents, as well as avoiding the use of any silicate glass in sample preparation and for eluent containers, results in no detectable metal adducts[1]. We show here, that metal adduct free mass spectra can also be obtained on the low-flow Vanquish Neo UHPLC system by using the above-mentioned procedures, see **Figure 2**.

Intact deconvolution of the data with isotopic sliding windows additionally provides retention time information for all identified components from the XIC traces generated by the deconvolution, see **Figure 2**, which further verifies the authenticity of the impurity identification. As N-1 impurities are expected to elute before the full-length product (FLP), N+1 impurities are expected to elute after the FLP, and any in-source produced artifacts will elute with the same retention time as the FLP.

Figure 3. Efficient separation of DNA duplex containing of sense and antisense oligonucleotide by capillary flow LC-HRMS with clean mass spectra.



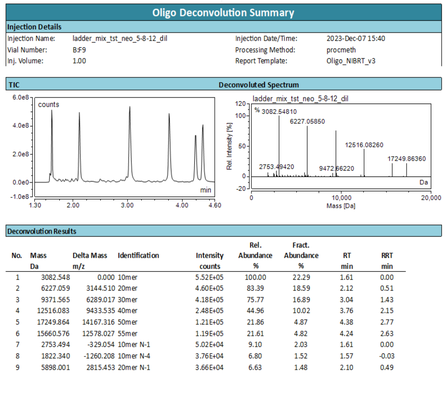
An oligonucleotide duplex containing corresponding sense and antisense oligonucleotides were additionally analysed by capillary flow IPRP LC-HRMS, see **Figure 3**. Which shows efficient separation of the sense and antisense oligonucleotides as well as impurities from both on a short 7 min gradient. Very clean MS data was also obtained as well with no observed adduct formation or in-source produced impurities. This further highlights the usability of method for analysing different oligonucleotides.

The BioPharma Finder (BPF) software has been used with added sequence information for the oligonucleotide to quickly identify impurities present in the sample by intact deconvolution. If the impurity delta mass is known assignment of identity for each impurity will be performed as well.

The compliant-ready Chromeleon software was used for deconvolution of the sample. The software can additionally control both the UHPLC and HRMS as well as acquire the data. This enables a full workflow for sequence creation, data acquisition, data analysis, and reporting in one compliant workflow. The deconvolution engine is the same in both BPF and Chromeleon which enables an efficient transition from discovery to identification and quantitation of impurity monitoring.

A report has additionally been developed to Chromeleon that can identify impurities from intact deconvolution delta mass directly in Chromeleon. The current database contains 200 impurities, but it can easily be scaled to use. Chromeleon 7.3.2 utilizes a new feature where either the target mass or target chemical formula can be added to the sequence for each injection and matches that to the components detected experimentally with intact deconvolution. Adding the chemical formula further enables the use of different isotope tables for deconvolution, which improves deconvolution results for modified oligonucleotides.

Figure 4. Shows a report template example from Chromeleon CDS that reports mass, delta mass, identity, RRT, XIC, etc. for each component or impurity found by isotopic mass sliding window intact deconvolution. Which enables efficient data analysis in an automated workflow.



Oligo Deconvolution Summary

Injection Details

Injection Name:	ladder_mix_tst_neo_5-8-12_dil	Injection Date/Time:	2023-Dec-07 15:40
Vial Number:	B:F9	Processing Method:	procmeth
Inj. Volume:	1.00	Report Template:	Oligo_NBRT_v3

TIC — counts, min

Deconvoluted Spectrum — ladder_mix_tst_neo_5-8-12_dil; 3082.54810, 6227.05860, 12516.08260, 17249.86360, 2753.49420, 9472.86220; Rel. Intensity [%]; Mass [Da]

Deconvolution Results

No.	Mass Da	Delta Mass m/z	Identification	Intensity counts	Rel. Abundance %	Fract. Abundance %	RT min	RRT min
1	3082.548	0.000	10mer	5.52E+05	100.00	22.29	1.61	0.00
2	6227.059	3144.510	20mer	4.60E+05	83.39	18.59	2.12	0.51
3	9371.565	6289.017	30mer	4.18E+05	75.77	16.89	3.04	1.43
4	12516.083	9433.535	40mer	2.48E+05	44.96	10.02	3.76	2.15
5	17249.864	14167.316	50mer	1.21E+05	21.86	4.87	4.38	2.77
6	15660.576	12578.027	55mer	1.19E+05	21.61	4.82	4.24	2.63
7	2753.494	-329.054	10mer N-1	5.02E+04	9.10	2.03	1.61	0.00
8	1822.340	-1260.208	10mer N-4	3.76E+04	6.80	1.52	1.57	-0.03
9	5898.001	2815.453	20mer N-1	3.66E+04	6.63	1.48	2.10	0.49

Summary

The capillary flow UHPLC-HRMS workflow for IP-RP analysis of oligonucleotides is easy to use which also enable the workflows useability in a more routine biopharmaceutical setting. Additionally, 100x less eluent and sample usage is obtainable compared to standard micro flow eluent workflows. Using the same workflow larger oligonucleotides, including mRNA sequencing can be analysed with more sensitivity than standard workflows. Small IVT production of mRNA produces only 100 ug of material so there is not that much sample available for sequence analysis.

The Vanquish Neo UHPLC system provides efficient chromatographic gradients from low nl/min to 100 µl/min without any stainless-steel parts in the flow-path, which is essential for oligonucleotide analysis. This opens the possibility of decreasing sample and eluent consumption for IPRP LC-HRMS analysis of oligonucleotides. Applications on biological samples and small IVT reactions are now realistic.

Conclusions

Using an IPRP eluent system with the new monodisperse capillary flow DNAPac RP column and the Vanquish Neo UHPLC system coupled to an Orbitrap Exploris MS it was possible to obtain:

- Efficient chromatographic separation of oligonucleotides ranging 10–55mer and an oligonucleotide duplex with ion pair reverse-phase (IPRP) capillary flow coupled to HRMS
- Very sensitive and clean mass spectrometric data with no metal adducts or source induced base loss artefacts with at least a 100-fold increase in sensitivity to standard micro flow with a 2.1 mm column
- The efficient and sensitive method will drastically improve the usability of LC-MS for analysis of biological samples with limited sample amounts, and workflows that normally require larger sample amounts, e.g., mRNA sequencing by LC-MS/MS[1]. And uses 100x less eluent compared to current microflow techniques
- Efficient and automated data processing and reporting in Chromeleon CDS enables high throughput workflows with automated identification of impurities.

References

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