Validation of HILIC-HRMS Method for Oligonucleotide Analysis

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Abstract

Synthetic oligonucleotide therapeutics (ONTs) are regulatorily challenging due to their molecular complexity and the presence of structurally similar product-related impurities. This study aims to validate a hydrophilic interaction liquid chromatography (HILIC) – high resolution mass spectrometry (HRMS) method recently developed in the Office of Testing and Research (OTR) lab for oligonucleotide analysis with a focus on low-level impurities. Method precision, sensitivity, calibration curve range, linearity, and accuracy were validated following regulatory guidances. The developed method is demonstrated to be sensitive and accurate for analysis of complex oligonucleotides and impurities.

Introduction

Synthetic oligonucleotide therapeutics (ONTs) are an emerging class of drugs that shows a great potential to target previously considered undruggable diseases. It may modulate gene expression by interacting with mRNA at molecular level (**Figure 1**). Solid phase synthesis of oligonucleotides includes multiple synthetic cycles, each consisting of multiple steps (**Figure 2**). Failure in any step in the synthetic process as well as degradation of final products during storage may contribute to formation of impurities that may potentially impact their physiochemical properties, efficacy and toxicity. ONTs pose unique regulatory challenges, which is largely attributed to the molecular complexity present in both intended full-length product (FLP) and product-related impurities. Recently, we developed a HILIC–HRMS method for oligonucleotide analysis. In this study, this method was validated following regulatory guidances: Guidance for Industry *M10 Bioanalytical Method Validation and Study Sample Analysis* (ICH 2022), and Guidance for Industry *Q2(R1) Validation of Analytical Procedures: Text and Methodology* (FDA 2021, ICH Q2(R1) 2005).

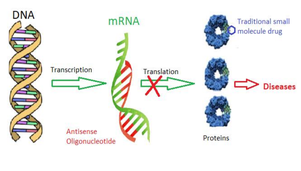


Fig 1. ONT as modulator of gene expression

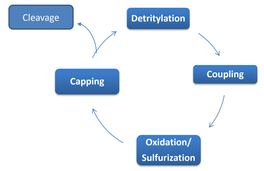
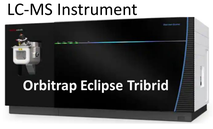


Fig 2. Synthetic cycles of phosphorothioate ONTs

Materials and Methods

- LC-MS Instrument



- HILIC column: Shodex HILICpak VN-50 2D, 2.0x150 mm, 5 um, 100 Å)
- Mobile phases A (MPA) and B (MPB): 70/30 (v/v) and 30/70 (v/v) water/acetonitrile with 20 mM NH4Ac, respectively. pH adjusted to 5.5. Gradient: 0-1 min 10%, 1-10 min 10-25%, and 10-12 min 25% MPA.
- LC-MS data were processed by Biopharma Finder (BPF, Thermo Scientific).
- FLP with the same nucleotide sequence and modifications as nusinersen and representative common impurities tested listed in **Table 1**.

Table 1. Custom synthesized oligonucleotide sequences

Sample type	Sample name	Sequence
FLP (18-mer)	FLP	5′-TCACTTTCATAATGCTGG-3′
PO impurity (18-mer)	PO	5′-TCACTTTCATAATGCTGG-3′ where one phosphorothioate (PS) linkage was replaced with phosphodiester (PO) linkage
n-1 impurity (17-mer)	n-A	5′-TCCTTTCATAATGCTGG-3′
n+1 impurity (19-mer)	n+A	5′-TCAACTTTCATAATGCTGG-3′
n-2 impurity (16-mer)	n-2	5′-ACTTTCATAATGCTGG-3′

Results and Discussion

1. Calibration curve range and linearity

- The calibration curve was generated for each of the tested oligonucleotide sequences, specifically focusing on low concentration levels ranging from 0.005 to 5 pmol/µL, which is equivalent to column loads from 0.01 to 10 pmol at a fixed injection volume of 2 µL. Two repeated runs were performed on different days.
- The calibration curves showed excellent linearity with $R^2 > 0.99$ for all 5 tested compounds for both runs. The results from Run 1 are shown in **Figure 3**.

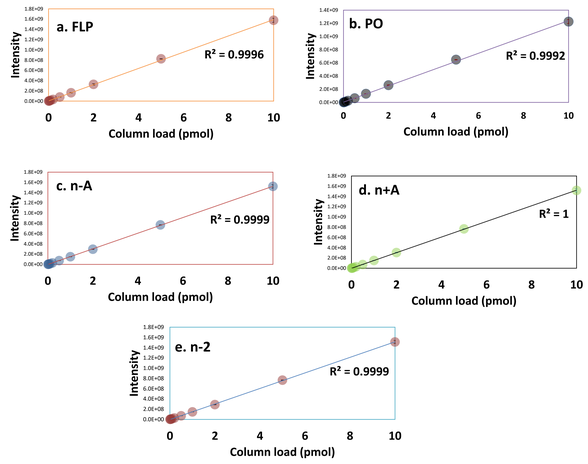


Fig 3. Calibration curves. (a) FLP, (b) PO, (c) n-A, (d) n+A, and (e) n-2

2. Precision

- Precision was evaluated for each concentration level (or column load) tested by coefficient of variation (% CV). At each concentration level, 6 repetitive injections in one analytical run or 12 injections from two separate runs were performed to evaluate the precision for within run or in-between runs, respectively.
- Except for the lowest column load level, all levels showed a % CV < 6% for within-run for both runs and < 8% for in-between runs (maximal % CV values were in bold in **Table 2** using FLP data as an example).
- At the lowest column load level (0.01 pmol), % CVs within 15% were observed for either within run or in- between runs (**Table 3,** numbers in bold indicate the largest % CV among all the runs).

Table 2. Intensities and % CV of LC-MS data acquired for different column loads on two separate runs processed by BPF deconvolution for FLP as an example

Column Load (pmol)	1st run		2nd run		In-between runs	
	Avg. intensity	% CV	Avg. intensity	% CV	Avg. intensity	% CV
0.01	8.34E+05	6.41	9.26E+05	9.80	8.80E+05	9.76
0.02	2.20E+06	**4.78**	2.49E+06	2.83	2.35E+06	**7.48**
0.05	7.20E+06	1.22	8.07E+06	4.14	7.64E+06	6.70
0.1	1.54E+07	0.59	1.67E+07	0.95	1.61E+07	4.19
0.2	3.13E+07	2.03	3.33E+07	**4.62**	3.23E+07	4.73
0.5	8.02E+07	2.04	8.46E+07	3.30	8.24E+07	3.81
1	1.62E+08	3.41	1.79E+08	0.34	1.71E+08	5.42
2	3.26E+08	4.27	3.51E+08	2.37	3.39E+08	4.99
5	8.25E+08	0.48	9.02E+08	1.07	8.63E+08	4.72
10	1.58E+09	2.63	1.63E+09	1.47	1.60E+09	2.63

2. Precision – cont.

Table 3. Intensities and % CV of LC-MS data acquired for the lowest column load tested (0.01 pmol) on two separate runs for all 5 compounds

	1st run		2nd run		In-between runs	
	Avg. intensity	% CV	Avg. intensity	% CV	Avg. intensity	% CV
FLP	8.34E+05	6.41	9.26E+05	9.80	8.80E+05	9.76
PO	5.47E+05	10.81	6.33E+05	**14.19**	5.90E+05	14.42
n-A	7.28E+05	8.43	8.24E+05	4.48	7.76E+05	8.98
n+A	4.64E+05	**11.95**	5.79E+05	7.01	5.22E+05	**14.50**
n-2	8.99E+05	3.33	9.83E+05	3.65	9.41E+05	5.733

3. Accuracy

- To evaluate the accuracy, linear regression equations were first obtained from calibration curves (**Figure 3**) and summarized in **Table 4**. Grand average value from two separate runs for all tested compounds (FLP and 4 representative impurities) showed a RSD within 10% for slope and within 20% for intercept, indicating a species-independent linear relationship of peak intensity *vs* column load for the tested 16-mer to 19-mer sequences.
- The regression equation of each run was then used to back calculate column loads and compare to the true column loads for % Recovery at different concentration levels for two separate runs. FLP data in **Table 5** (numbers in bold indicate the % Recovery maximally deviated) as a representative example showed % Recovery within ± 6%.
- % Recovery deviated from the nominal concentrations were within ± 15% for all tested compounds within the entire range of 0.01-10 pmol (% Recovery maximally deviated listed in **Table 6**).

Table 4. Linear regression equations* for all 5 compounds

		FLP	PO	n-A	n+A	n-2	Grand Ave.
Slope	1st run	1.62E+08	1.28E+08	1.49E+08	1.50E+08	1.47E+08	(1.52±0.13) E+08 **RSD: 8.44%**
	2nd run	1.73E+08	1.38E+08	1.60E+08	1.59E+08	1.57E+08	
	% Difference between runs	6.57%	7.52%	7.12%	5.83%	6.58%	
Intercept	1st run	-8.60E+05	-7.98E+05	-9.15E+05	-1.20E+06	-7.45E+05	(-8.86±1.67) E+05 **RSD: 18.9%**
	2nd run	-8.22E+05	-7.68E+05	-8.85E+05	-1.16E+06	-7.11E+05	
	% Difference between runs	4.52%	3.83%	3.33%	3.39%	4.67%	

*: **Log values** of calibration curve data were used to generate regression equations.

Table 5. % Recovery at different column loads on two separate runs using FLP as an example

Column Load (pmol)	Back calculated column load (pmol)					
	1st run			2nd run		
	Average	% CV	Recovery (%)	Average	% CV	Recovery (%)
0.01	0.011	3.16	104.62	0.010	5.19	100.88
0.02	0.019	3.44	**94.50**	0.019	2.13	95.65
0.05	0.050	1.09	99.60	0.051	3.76	102.67
0.1	0.101	0.56	100.75	0.101	0.91	101.21
0.2	0.199	1.98	99.35	0.197	4.51	98.43
0.5	0.501	2.02	100.20	0.493	3.27	98.55
1	1.009	3.40	100.90	1.036	0.34	103.57
2	2.021	4.26	101.06	2.030	2.37	101.50
5	5.100	0.48	102.00	5.207	1.06	104.13
10	9.747	2.63	97.47	9.403	1.47	**94.03**

3. Accuracy – cont.

Table 6. % Recovery maximally deviated from the nominal concentrations observed for all 5 compounds on two separate runs

	1st run		2nd run	
	Column Load (pmol)	% Recovery	Column Load (pmol)	% Recovery
FLP	0.02	94.50	10	94.03
PO	0.02	93.43	10	93.61
n-A	0.01	109.95	0.01	107.08
n+A	0.01	111.03	0.01	109.09
n-2	0.01	112.14	0.01	107.81

4. LLOQ

- LLOQs are determined based on linearity, precision and accuracy by meeting criteria including % CV within 20%, and accuracy (% Recovery) within 20% deviated from nominal concentration.
- Based on of calibration curve linearity (**Fig. 3**), precision (**Tables 2** and **3**), and accuracy (**Tables 5 and 6**), a LLOQ at a column load of 0.01 pmol was determined for all 5 tested compounds including FLP and 4 common impurities.

5. Specificity

- Specificity was evaluated by comparing peak area of a specific compound at LLOQ level with that in a blank sample.
- By extracted ion chromatograms (EICs) of 5 tested compounds, no detectable peaks at expected retention times were observed for blank samples, which confirms the method specificity.

Conclusions

- A HILIC-HRMS method developed for quantifying oligonucleotides was validated for calibration curve range, linearity, precision, accuracy, sensitivity, and specificity following regulatory guidances.
- The developed method is sensitive and accurate for the analysis of oligonucleotides and common product-related impurities including structurally closely related impurities.
- Future studies will address potential matrix effects from drug product formulation and the impact of high-level FLP and other coelutes.

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