Validation of HILIC-HRMS Method for Quantitative Oligonucleotide Analysis

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Introduction

Synthetic oligonucleotide therapeutics (ONTs) are an emerging class of drugs that shows a great potential to target previously considered undruggable diseases by modulating gene expression through interacting with mRNA at molecular level (**Figure 1**). Solid phase synthesis of ONTs consists of repetitive synthetic cycles, each including multiple steps (**Figure 2**). Failure in any steps during synthesis as well as degradation of final products during storage may contribute to formation of impurities that may potentially impact their physiochemical properties, efficacy and safety. 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 (J Mass Spectrom 2022; 57(4):e4819). 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).

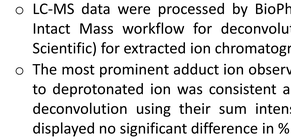

Fig 1. ONT as modulator of gene expression

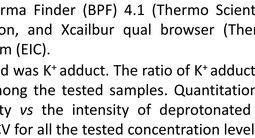

Fig 2. Synthetic cycles of phosphorothioated ONTs

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.
- FLP with the same nucleotide sequence and modifications as nusinersen and representative common impurities (**Table 1**) were custom synthesized by GenScript at HPLC grade (purity ≥ 90%) and used as is.

Table 1. Custom synthesized oligonucleotide sequences

Sample type	Sample name	Sequence
FLP (18-mer)	**FLP**	5'-TCACTTTCATAATGCTGG-3'
PO impurity (18-mer)	**PO**	5'-TCACTTTCAT***A**ATGCTGG-3' where 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'

Data Processing

- LC-MS data were processed by BioPharma Finder (BPF) 4.1 (Thermo Scientific) Intact Mass workflow for deconvolution, and Xcailbur qual browser (Thermo Scientific) for extracted ion chromatogram (EIC).
- The most prominent adduct ion observed was K^+ adduct. The ratio of K^+ adduct ion to deprotonated ion was consistent among the tested samples. Quantitation by deconvolution using their sum intensity *vs* the intensity of deprotonated ion displayed no significant difference in % CV for all the tested concentration levels.
- Quantitation by EIC with an extraction window covering top 8 isotopic peaks *vs* by deconvolution displayed no significant difference in % CV for most of the tested concentration levels, with a slightly better % CV for the low concentration end.
- Intensity of deprotonated ion by deconvolution was used in quantitation below.

Results and Discussion

1. Calibration curve range and linearity

- Equal molar mixtures of 5 tested oligonucleotide samples at varied concentration levels ranging from 0.005 to 5 pmol/µL (i.e., equivalent to column loads from 0.01 to 10 pmol at a fixed injection volume of 2 µL) were used to generate the calibration curves. Two repeated runs were performed on different days.
- The calibration curves showed excellent linearity with $R^2 > 0.999$ for all 5 tested compounds for both runs. The results from Run 1 are shown in **Figure 3**.

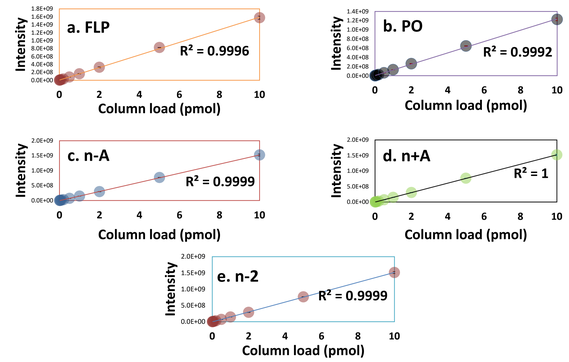

Fig 3. Calibration curves. (a) FLP, (b) PO, (c) n-A, (d) n+A, and (e) n-2

2. Precision

- Precision was evaluated for each tested column load 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 (**Table 2** using FLP data as an example, the maximal % CV values not counting the lowest column load were in bold).
- At the lowest column load level (0.01 pmol), % CVs within 15% were observed for either within run or in- between runs (**Table 3,** the largest % CV among all the runs were in bold).

Table 2. Intensities and % CV of LC-MS data acquired at different column loads on two separate runs for FLP

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

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 % CV 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 as a representative example in **Table 5** (the % Recovery maximally deviated were in bold) showed % Recovery deviation was 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 Avg.
Slope	1st run	1.62E+08	1.28E+08	1.49E+08	1.50E+08	1.47E+08	(1.52±0.13) E+08
	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%	**% CV: 8.44%**
Intercept	1st run	-8.60E+05	-7.98E+05	-9.15E+05	-1.20E+06	-7.45E+05	(-8.86±1.67) E+05
	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%	**% CV: 18.9%**

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

Table 5. % Recovery at different column loads on two separate runs for FLP

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

Compounds	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 calibration curve linearity (**Figure 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 when examined with equal molar mixtures.

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.
- A model molecule representing therapeutic oligonucleotides (*i.e.*, an 18-mer RNA with modifications of nusinersen) was used in the study. The developed method is sensitive and accurate for the analysis of the full-length product and its common product-related impurities including structurally closely related impurities.
- Future studies will address the impact of coexisting high-level full-length product and other coelutes, and the potential matrix effects from excipients in drug product formulation.

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