LC-MS Method for Detection of 1st, 2nd and 3rd Generation Oligonucleotide Therapeutics in Liver and Kidney Homogenate Samples for Metabolism Studies

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ABSTRACT

Oligonucleotide-based therapeutics is an emerging and fast-growing area. Oligonucleotide-based drugs show a potential in the treatment of a wide variety of genetic disorders and infections and as use for vaccines. Understanding the metabolism of oligonucleotides and evaluation of safety of potential drug candidates with the use *in vitro* models including liver and kidney tissue homogenates is important for successful drug development process. However, this process is complex from sample preparation and chromatography prospective compared to small molecule analysis. The goal of the project is to optimize sample preparation and LC-MS methodology for 1st, 2nd and 3rd generation oligonucleotides including phosphorothioate, 2′-O-methyl and locked oligonucleotides, and phosphoramidate morpholino oligonucleotides. Protein Metrics software will be used for high- throughput oligonucleotide data analysis.

For an initial proof of concept study, we optimized liquid-liquid extraction with phenol/chloroform/isoamyl alcohol mixture (25:24:1 v/v/v) to reach high analyte recovery, good selectivity and minimal matrix effects. During LC-MS method development, different columns, mobile phase modifiers, and gradients were evaluated for suitable separation and intensity of parent oligonucleotides. Preliminary results show effective separation and sufficient intensity of phosphorothioate, 2′-O-methyl and locked oligonucleotides using an Agilent Poroshell 120 EC-C18 column and the mobile phase A containing 25 mM 1,1,1,3,3,3-hexafluro-2-propanol/ 8mM trimethylamine in water and mobile phase B containing methanol in negative electrospray ionization mode, while phosphoramidate morpholino oligonucleotide requires additional optimization to obtain high signal intensity.

The study will examine optimal conditions for biotransformation studies of a wide variety of modified oligonucleotide therapeutics.

OBJECTIVES

- Evaluate effects of mobile phase additives, gradients, ionization modes and LC-MS instrumentation in order to obtain suitable separation and intensity of parent oligonucleotides
- Optimize sample preparation for each oligonucleotide to reach high analyte recovery
- Perform in vitro incubations of oligonucleotides using liver and kidney homogenate in order to generate metabolites

METHOD

Table 1. Test oligonucleotides

#	Oligonucleotide sequence	Modification	Sequencing information
1	CAGATTGACGCCTTCAGAGT	Unmodified oligonucleotide	Control sequence
2	C*A*G*A*T*T*G*A*C*G*C*C*T*T*C*A*G*A*G*T	Phosphorothioate oligonucleotide (PS)	1st generation
3	mCmAmGmATTmGmAmCmGmCmCTTmCmAmGmAmGT	2′-O-methyl oligonucleotide (2′-O-ME)	2nd generation
4	+Q+A+G+A+T+T+G+A+Q+G+Q+Q+T+T+Q+A+G+A+G+T	Locked nucleic acids (LNA)	3rd generation
5	CAGATTGACGCCTTCAGAGT	Phosphorodiamidate morpholino oligonucleotide (PMO)	3rd generation

* - PO_3S phosphorothioate linkage, m - 2'-O-methylation of nucleosides, + - 2'-O,4'-C-methylene-bridge

In vitro incubation of oligonucleotides using liver or kidney homogenates for 0 and 38 hr

Unmodified oligo, PS, 2′-O-ME, LNA oligo sample preparation

- 100 µL of sample
- 35 µL of Proteinase K digestion solution
- Incubation at 55ºC and at 400 rpm for 3 hour

- Extraction with phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v)
- Collection of aqueous layer

- Evaporation of samples to approximately 200 µL
- Transfer to LC-MS vials

PMO oligo sample preparation

- 30 µL of sample
- 120 µL of 0.1% formic acid in H_2O/MeCN/MeOH (30:35:35; v/v/v)

- Collection of supernatant
- Transfer to LC-MS vials

METHOD (cont.)

Unmodified oligo, PS, 2′-O-ME and LNA LC-MS Conditions:

HPLC: Agilent 1290 Infinity II

Mass spectrometer: Agilent QTOF 6545 XT

Mode: Negative, 3200 m/z, 2 GHz resolution

Mass range: 600 -3200 m/z

Scan rate (spectra/sec): 2.00

Injection volume: 5 µL

Column: Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 50mm, 2.7µm)

Column temperature: 60ºC

Mobile phase A: 25 mM HFIP and 8 mM TEA in H_2O

Mobile phase B: Methanol

PMO LC-MS Conditions:

HPLC: Shimadzu Nexera X2

Mass spectrometer: LTQ Orbitrap XL

Resolution: 60K

Mass Range: 250-2000 m/z

Mode: Positive

Injection volume: 10 µL

Column: Agilent Polaris 3 C18-A (150 x 2.0 mm, 3µm)

Column temperature: 35ºC

Mobile phase A: 0.1% FA in H_2O

Mobile phase B: 0.1% FA in acetonitrile

RESULTS

Evaluation of different LC methods

Figure 1. LC methods

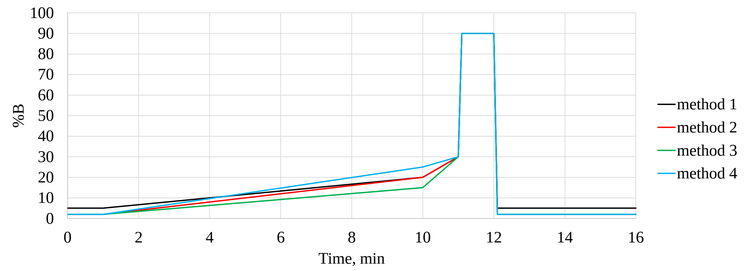


Figure 2. Extracted ion chromatogram of unmodified oligo in standard solution

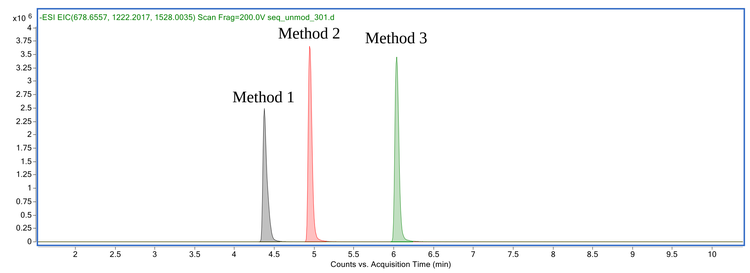
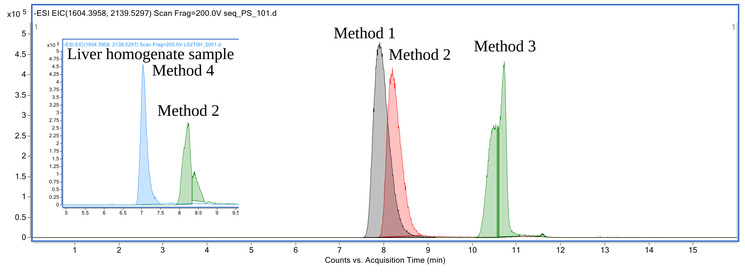


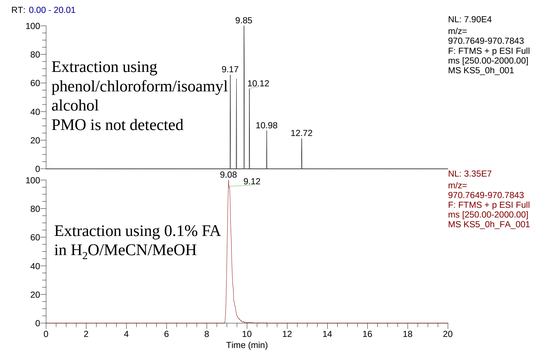
Figure 3. Extracted ion chromatogram of PS oligo in standard solution



Additional LC method modification was required in homogenate samples of PS oligo

RESULTS (cont.)

Comparison of different sample preparation methods



PMO oligo is not extracted with phenol/chloroform/ isoamyl alcohol sample preparation procedure

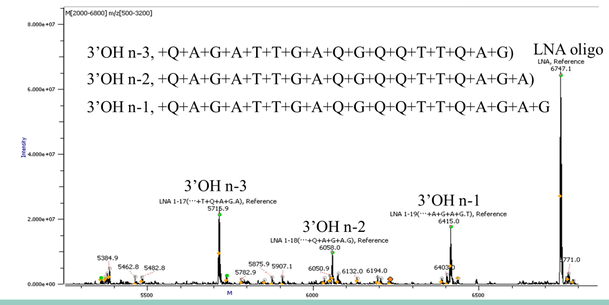
Results of *in vitro* incubation of oligonucleotides in kidney and liver homogenates

Table 2. Peak area and relative abundance of oligonucleotides **in kidney and liver homogenates**

Oligonucleotide	Time	MS Peak Area		Relative Abundance (%)[a]	
		Kidney homogenate	Liver homogenate	Kidney homogenate	Liver homogenate
Unmodified oligo	T = 0 hr	4.06E+07	3.03E+07	0.07%	0.00%
	T = 38 hr	2.76E+04	0.00E+00		
PS oligo	T = 0 hr	5.88E+06	1.79E+07	33.10%	40.5%
	T = 38 hr	1.95E+06	7.25E+06		
2′-O-ME oligo	T = 0 hr	2.28E+07	2.83E+07	0.00%	0.00%
	T = 38 hr	0.00E+00	0.00E+00		
LNA oligo	T = 0 hr	1.73E+07	3.00E+07	52.73%	42.8%
	T = 38 hr	9.14E+06	1.29E+07		
PMO oligo	T = 0 hr	5.15E+08	5.56E+08	106.05%	102.6%
	T = 38 hr	5.46E+08	5.71E+08		

[a] Relative Abundance = (peak area at 38 hr) ÷ (peak area at 0 hr) × 100

Figure 4 Metabolite identification results for LNA oligo



CONCLUSIONS

- LC-MS methods were developed for each oligonucleotide
- Later generation oligonucleotides appear to be more stable than earlier generations
- Time course points are critical for metabolism evaluation and metabolite identification

Future work

- Repeat *in vitro* incubation with additional time points for early generation oligos with a focus on the metabolite identification