Quantitative determination of PMOs and PPMOs in mouse and monkey tissues by UPLC-HRMS

Authors: John Chen[1], Jianbo Zhang[2], Ran An[1], Jian Shi[1], Chengjie Ji[1], John Hadcock[2]

[1]NovaBioAssays LLC; [2]Sarepta Therapeutics, Inc

NovaBioAssays

Innovative Mass Spectrometric Solutions

SAREPTA THERAPEUTICS

Introduction

Phosphorodiamidate Morpholino oligomers (PMO) are synthetic oligonucleotides whose bases attached to a backbone of morpholine rings linked through phosphorodiamidate groups. Conjugating cell-penetrating peptide (CPPs) to PMO as PPMO could improve drug efficiency by increasing drug delivery to their intracellular targets.

To determine drug exposure and clearance for PMOs and PPMOs, UPLC-HRMS methods have been developed and implemented to quantify PMOs and PPMOs in multiple tissue samples from mouse and monkey studies.

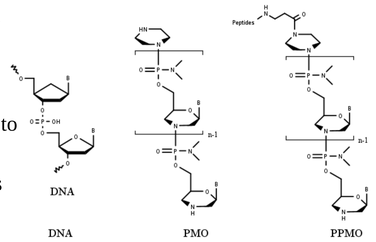
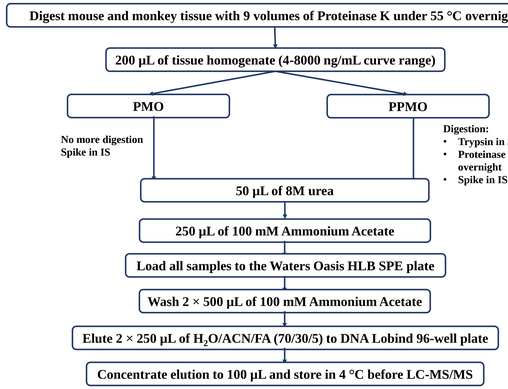


Figure 1. Structures of DNA, PMO and PPMO

Methodology

1. Tissue Sample Preparation before UPLC-HRMS Analysis



2. UPLC-HRMS method

Column Type	Thermo DNApac RP, 4 µm, 2x50 mm
Column Temp.	70 °C
Flow Rate	0.4 mL/min
Injection Volume	20 µL
Mobile Phase	A=2% Formic Acid in water
	B=2% Formic Acid in acetonitrile
LC Gradient	15-35%B in 3 minutes
Mass Spectrometer	Thermo Scientific Q Exactive Plus
Ionization Mode	Electrospray Ionization, Positive ion mode
Source Temp.	350 °C
Monitoring Mode	PRM, resolution 70000
IT	250 ms
AGC	$2e^5$
Inclusion List	Analyte:

1. PMO: 2 Q1 ions of different charges > 3 Q3 isotopes
2. PPMO: 2 Q1 ions of different charges > 3 Q3 isotopes
3. Internal Standard: 1 Q1 ion > 3 Q3 isotopes

Results and Discussion

1. Method Development

Optimization of UPLC-HRMS Conditions and Sample Extraction

HLB SPE plate was used to clean up tissue samples and provided the highest recovery and best precision. To achieve the best selectivity and sensitivity of analytes, the most two intense precursor ions and their common highest product ion under optimized CE were applied in high resolution PRM method, combining with the optimized LC condition , IS and matrix did not have interference to PMO and PPMO analytes (Figure 2 and 3), and the sensitivity was improved five to ten times than previous assays.

PPMO Stability Issues

CPPs in PPMO structure can be degraded to multiple metabolites during sample processing. After Trypsin/Proteinase–K digestion, PPMO was converted to one single end product of one AA linked PMO, and the end product concentration represented the total concentration of PPMO in tissues.

2. Assay Selectivity and Sensitivity

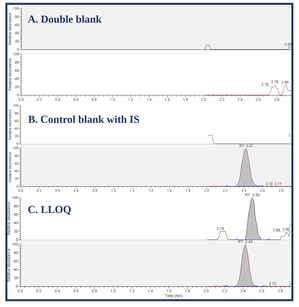


Figure 2. Chromatograms of double blank (A), control blank (B) and LLOQ=4 ng/mL in tissue homogenate or 40 ng/g in tissue (C) in PMO assay

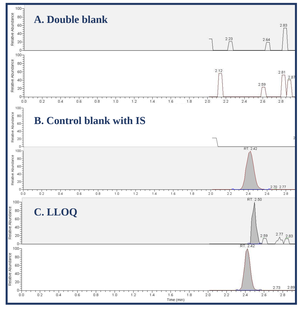


Figure 3. Chromatograms of double blank (A), control blank (B) and LLOQ=4 ng/mL in tissue homogenate or 40 ng/g in tissue (C) in PPMO assay

3. Standard Curve and Regression

Table 1. Back-Calculated Concentrations of Calibration Standards for PMO

Stats	Assay Number	4.00	8.00	16.0	32.0	160	800	3200	6400	8000
	1	3.30*	8.14	17.9	29.3	216**	765	3072	6314	7927
		4.04	10.7**	19.6*	30.3	183	684	2921	6304	7490
	2	4.20	6.65*	15.8	30.8	184*	739	3205	6894	9649*
		3.57	8.45	15.1	37.1*	185	627*	2989	5444	7787
	3	4.09	8.01	15.9	33.0	178	734	3542	7200	8292
		4.37	7.99	16.4	28.4	190*	749	2610*	6368	7506
Mean		3.93	7.85	16.8	31.5	184	716	3056	6420	8108
%CV		10.4	8.82	9.92	10.0	2.33	7.17	10.1	9.38	10.0
%Theoretical		98.2	98.1	105	98.4	115	89.5	95.5	100	101
n		6	5	6	6	5	6	6	6	6

* > ±15% deviation from theoretical
** Deactivated as a statistical outlier

Table 2. Back-Calculated Concentrations of Calibration Standards for PMO-Gly

Stats	Assay Number	4.00	8.00	16.0	32.0	160	800	3200	6400	8000
	1	3.66	8.81	11.7**	33.7	157	798	3868*	6992	8162
		4.71*	6.75*	17.0	27.4	158	895	3408	6198	6564*
	2	4.31	7.84	13.3*	30.4	156	906	3372	5741	6091*
		7.17**	8.44	13.0*	31.4	160	828	3849*	7611*	8423
	3	4.31	8.13	13.3*	27.9	153	906	3584	5955	5501**
		4.08	7.19	16.4	30.6	169	858	3525	6672	7033
Mean		4.22	7.86	14.6	30.2	159	865	3601	6528	7255
%CV		9.16	9.86	13.1	7.62	3.53	5.22	5.93	10.8	13.9
%Theoretical		105	98.2	91.3	94.5	99.4	108	113	102	90.7
n		6	6	5	6	6	6	6	6	5

* > ±15% deviation from theoretical
** Deactivated as a statistical outlier

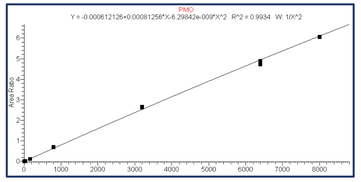


Figure 4. Representative standard calibration curve (4-8000 ng/mL) and regression in PMO assay.

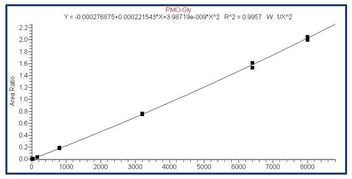


Figure 5. Representative standard calibration curve (4-8000 ng/mL) and regression in PPMO assay.

4. Inter Assay Accuracy and Precision

Table 3. Quality Control Samples for Inter Assay Accuracy and Precision for PMO

Stats	Assay Number	Low QC 20.0 ng/mL	Low-Medium QC 80.0 ng/mL	Medium QC 1200 ng/mL	High QC 4800 ng/mL
	1	19.0	79.7	1182	5103
		17.9	70.2	1231	4830
Mean		18.8	74.9	1206	4967
%Theoretical		94.1	93.7	101	103
	2	20.8	88.7	1394*	5077
		23.5*	83.6	1285	4544
Mean		22.1	86.2	1339	4810
%Theoretical		111	108	112	100
	3	20.1	75.9	1135	5278
		16.3*	70.4	1233	5088
Mean		18.2	73.2	1184	5183
%Theoretical		91.1	91.4	98.7	108
Mean		19.7	78.1	1243	4986
%CV		12.5	9.46	7.20	5.21
%Theoretical		98.7	97.6	104	104
n		6	6	6	6

* > ±15% deviation from theoretical

Table 4. Quality Control Samples for Inter Assay Accuracy and Precision for PPMO

Stats	Assay Number	Low QC 20.0 ng/mL	Low-Medium QC 80.0 ng/mL	Medium QC 1200 ng/mL	High QC 4800 ng/mL
	1	21.1	75.9	1227	5740*
		23.0	78.8	1218	5158
Mean		22.1	77.4	1222	5449
%Theoretical		110	96.7	102	114
	2	22.0	80.1	1306	4588
		20.3	93.9*	1289	5832*
Mean		21.2	87.0	1298	5210
%Theoretical		106	109	108	109
	3	17.7	81.3	1298	5142
		20.9	87.4	1462*	4936
Mean		19.3	84.4	1380	5039
%Theoretical		96.4	105	115	105
Mean		20.8	82.9	1300	5232
%CV		8.65	7.95	6.73	9.10
%Theoretical		104	104	108	109
n		6	6	6	6

* > ±15% deviation from theoretical

Conclusions

- High throughput SPE extraction and UPLC-HRMS methods have been successfully developed for PMO and PPMO analysis in various mouse and monkey tissue samples. Five to ten times better sensitivity was achieved comparing to the previously reported tissue assay for PPMO quantitation using UPLC-HRMS.
- Developed assays have been successfully implemented for PK/TK studies to support Sarepta PMOs and PPMOs drug development programs.

Reference

(1) Hong M. Moulton and Jon D. Moulton, Morpholinos and their peptide conjugates: Therapeutic promise and challenge for Duchenne muscular dystrophy, Biochimica et Biophysica Acta 1798 (2010) 2296–2303
(2) Z. Zhang, and et. Al., Quantification of Peptide Phosphorodiamidate Morpholino Oligomers in Plasma for Multiple Species Using LC-MS/MS, APA 2019
(3) M. Meng, and et. al., Quantitative Determination of AVI-7100, a PMO in Human Plasma Using LC-MS/MS, AAPS 2015, W4252

Disclosures: J. Chen, R. An, J. Shi, and C. Ji have nothing to disclose. J. Zhang and J. Hadcock are employees of Sarepta Therapeutics, Inc. and may own stock in the company. Products are investigational only.

Funding Statement: This study was sponsored by Sarepta Therapeutics, Inc.