Analytical Method Development of DNA/RNA heteroduplex oligonucleotide (HDO) in Rat Brain using LC-MS/MS

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Introduction

In drug development, evaluation of 'drug metabolism and disposition' is important, and quantitative analysis of drug in biological samples is essential. LC-MS/MS method has been widely used for quantitative analysis of oligonucleotides in biological samples because of the high quantification capability and versatility, and the ability to directly identify the metabolites. Recently, DNA/RNA heteroduplex oligonucleotide (HDO) has been reported to be delivered to the target organs without DDS [Nishida K. et al. Nat Commun. (2015)].

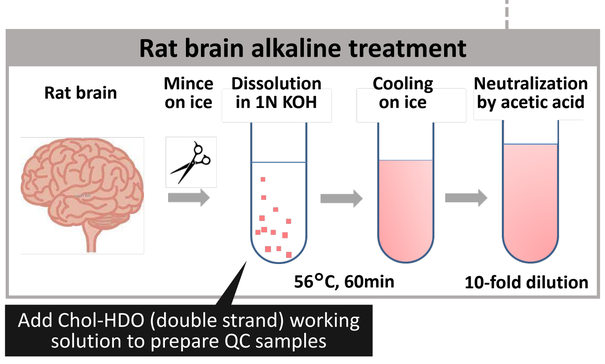
Therefore, we developed an analytical method for quantification of cholesterol binding HDO (Chol-HDO*1), which can be delivered to CNS [Nagata T. et al. Nat Biotechnol. (2021)], in rat brain by LC-MS/MS.

*1: Structure of Chol-HDO

cRNA: 3'-G^A^U^CAAGUGACUU^A^C^G-Chol-5'
ASO: 5'-mC^T^A^g^t^t^c^a^c^t^g^a^a^T^G^mC-3'
N: RNA, n: DNA, N: 2'-OMe, N: LNA, ^: PS, Chol: TEG-cholesterol

Pre-treatment

50 µL of rat brain lysate
↓ Add ASO of Chol-HDO (single strand) working solution to calibration standards
↓ Add I.S. working solution
↓ Add 0.025% ammonium
Mixture
↓ Add phenol/chloroform/isoamyl alcohol (25:24:1)
Mixture
↓ Centrifuge
Supernatant
↓ Add acetic acid/water (1:25, v/v)
↓ Add MeOH
Mixture
↓ Inject (2 µL) to LC-MS/MS

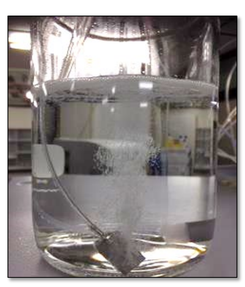


LC-MS/MS Condition

LC	Nexera X2 system (Shimadzu, Kyoto, Japan)
Column	X Bridge™ BEH C18 2.5µm, 2.1 × 50mm (Waters, Milford, MA)
Column temp.	60°C
Mobile phase A*2	Water/methanol/TEA/HFIP/acetylacetone (900:100:2:40:0.05, v/v/v/v/v)
Mobile phase B*2	Methanol/water/TEA/HFIP/acetylacetone (900:100:2:40:0.05, v/v/v/v/v)
Run time	14.0 min

MS	QTRAP®6500+ and Analyst®(ver. 1.7) (SCIEX, Framingham, MA)		
Ionization mode	Turbo ion spray		
Scan type	MRM		
Polarity	Negative		
Ion spray voltage	-3000 V		
TEM	450°C		
Monitoring ions	Analyte	Q1 (*m/z*)	Q3 (*m/z*)
	ASO of Chol-HDO	755.2	94.9
	I.S. (Nusinersen-OMe*3)	881.7	94.9

*2: Under shading and N_2 bubbling (Patent pending: WO2021/172380)



*3: Structure of Nusinersen-OMe

5'-T^mC^A^mC^T^T^T^mC^A^T^A^A^T^G^mC^T^G^G-3'
N: RNA, N: 2'-OMe, ^: PS

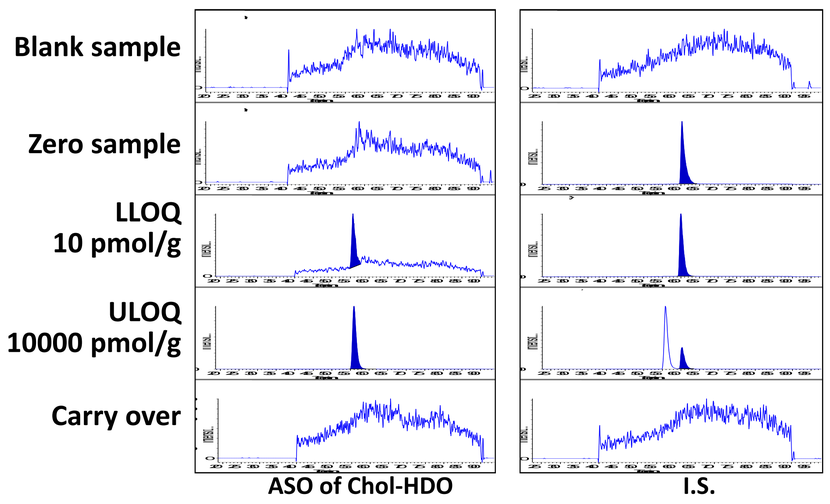
Nusinersen which its chemical modification of 2'-MOE is changed to 2'-OMe is used as I.S. for this analytical method.

Result

Good linearity, precision and accuracy were observed over the concentration range of 10 to 10000 pmol/g in rat brain. S/N ratio of LLOQ peak was enough and carry over peak was not observed. Good reinjection reproducibility was observed up to 48 hours. ASO of Chol-HDO during alkaline treatment was stable. Recovery of pre-treatment was 86.7 % or more.

Items	Contents	Results
Carry over	n=1	No interfering peak
Calibration curve	10-10000 pmol/g (10 calibration standards), n=1 each	Accuracy: 91.0-106.0%, r: 0.9983
Within-run accuracy and precision	3 concentrations, n=3 each	Accuracy: 88.0-93.0% Relative standard deviation: 0.6-3.8%
Reinjection reproducibility	Calibration standards, n=1 each, 4°C, 24 and 48 hours	Accuracy: 89.5-105.3%, r≥0.9986
Stability during alkaline treatment	1 concentration, n=3	Residual ratio: 90.2%
Recovery of pre-treatment	ASO of Chol-HDO and I.S., 1 concentration, n=3 each	Recovery: 90.6% (ASO of Chol-HDO) Recovery: 86.7% (I.S.)

Typical chromatograms



Within-run accuracy and precision

Nominal conc. (pmol/g)	Observed conc. (pmol/g)	Mean (pmol/g)	Accuracy (%)	Relative standard deviation (%)
QC-L 20.0	17.5 18.7 18.8	18.3	91.5	3.8
QC-M 400	352 350 354	352	88.0	0.6
QC-H 8000	7400 7370 7550	7440	93.0	1.3

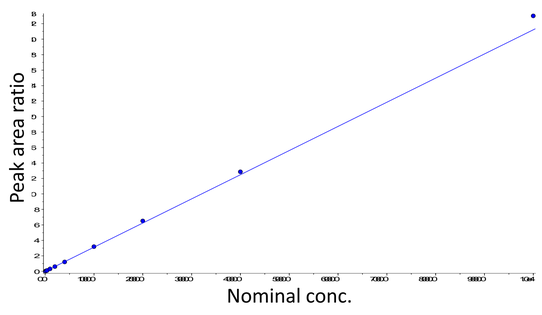
Stability during alkaline treatment

Nominal conc. (pmol/g)	Sample name	Peak area ratio	Mean	Residual ratio (%)
400	Standard samples	0.993 1.04 1.02	1.02	-
	Test samples	0.909 0.935 0.916	0.920	90.2

Calibration curve

Type Weight Slope Intercept r	Linear 1/X² 0.00312 -0.00456 0.9983	
Nominal conc. (pmol/g)	Back calculated conc. (pmol/g)	Accuracy (%)
10.0	10.6	106.0
20.0	18.6	93.0
40.0	36.4	91.0
100	100	100.0
200	195	97.5
400	388	97.0
1000	1020	102.0
2000	2090	104.5
4000	4120	103.0
10000	10600	106.0

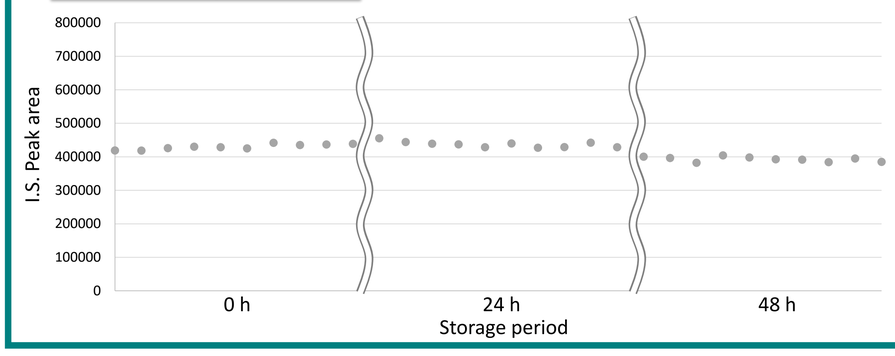
Plot of Calibration curve



Reinjection reproducibility

Storage period	Initial		24 hours		48 hours	
Calibration curve						
Type Weight Slope Intercept r	Linear 1/X² 0.00286 -0.00323 0.9996		Linear 1/X² 0.00294 -0.00427 0.9992		Linear 1/X² 0.00282 -0.00194 0.9986	
Nominal conc. (pmol/g)	Back calculated conc. (pmol/g)	Accuracy (%)	Back calculated conc. (pmol/g)	Accuracy (%)	Back calculated conc. (pmol/g)	Accuracy (%)
10.0	10.1	101.0	9.87	98.7	10.5	105.0
20.0	19.2	96.0	20.3	101.3	17.9	89.5
40.0	41.3	103.3	42.1	105.3	41.8	104.5
100	98.9	98.9	95.6	95.6	96.7	96.7
200	197	98.5	197	98.5	195	97.5
400	390	97.5	377	94.3	397	99.3
1000	996	99.6	1010	101.0	994	99.4
2000	2000	100.0	2050	102.5	2040	102.0
4000	4120	103.0	3960	99.0	4120	103.0
10000	10200	102.0	10400	104.0	10400	104.0

Plot of I.S. peak area



Conclusion

- **Good linearity, precision and accuracy were observed over the concentration range of 10 to 10000 pmol/g in rat brain.**
- **We have developed an LC-MS/MS method for analysis of Chol-HDO in rat brain.**

We have no financial relationships to disclose for this presentation.