Establishment and implementation of a determination method of nusinersen by peptide adsorption-controlled-LC-MS/MS in rat samples obtained by a microsampling technique

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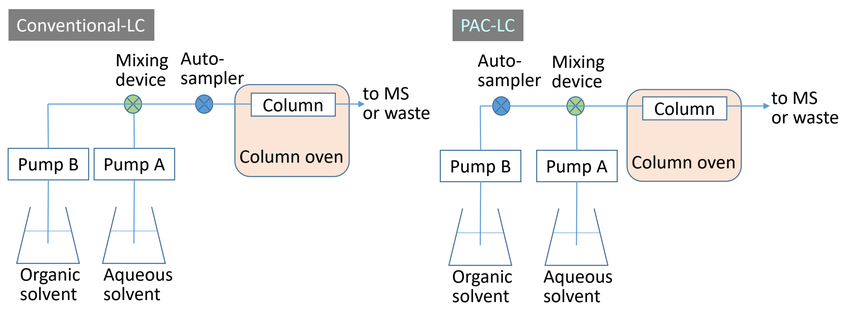
Introduction

Microsampling contributes to 3Rs (Replacement, Reduction and Refinement) of animals and it also enables to evaluate the relationship directly between the safety data and drug exposure in the same animal. Previously, much sampling blood volume was required for the determination of drugs in toxicokinetics analysis, but recent progress in sensitivity of analytical instruments contributed to the spread of sample reducing techniques. We focused on oligonucleotide therapeutics because relatively less number of microsampling applications are reported in the field. In order to determine nusinersen[1)] concentration in rat plasma obtained by microsampling, a determination method was developed using peptide adsorption-controlled-liquid chromatography (PAC-LC)[2)] combined with a mass spectrometer. Then, we measured the nusinersen concentration in rat plasma actually obtained by microsampling.

[1)] : antisense oligonucleotides for spinal muscular atrophy treatment
[2)]: Goda R., Sudo K., Biomed. Chromatogr., 21, 1005-1015 (2007)

About PAC-LC

PAC-LC is a LC system in which the connection of the liquid line is changed as shown in the illustration below, and enables the complete retention of analytes on the column while preventing adsorption during handling. In the system, after the sample is loaded into the line for mobile phase B, the analytes can be mixed with mobile phase A to recover their ability to retain in the column, and continue the retention until the organic solvent content of the eluent reaches a critical point. Due to this sample injection mechanism, the injectable sample volume of PAC-LC is theoretically unlimited.



Modified citation from Goda R., Pharmacia, Vol 56, No.6 (2020)

Sample preparation method

About dilution in microsampling :

For easy handling (operating or storage) or to obtain enough sample volume to re-analysis, the dilution is useful for microsampling. To reduce the amount of animal plasma used, we diluted plasma with L-consera®, which is based on human serum, as an alternative matrix.

50 µL of rat sample (the diluted plasma*)
↓ Add lysis buffer (Clarity®OTX** kit reagent)
Mixture
↓ Load to Clarity®OTX 25 mg plate
↓ Wash with 50 mM NaH_2PO_4 (pH5.5)
↓ Wash with 500 mM NaH_2PO_4 (pH5.5)/water/ACN (10/40/50, v/v/v)
↓ Elute with 400mM NH_4HCO_3/ACN (50/50, v/v)
Eluent
↓ Add Tris-EDTA buffer and mix
↓ Inject (150 µL) to LC-MS/MS
↓ Dry under N_2 stream
Residue
↓ Reconstitute with 50 µL of TE buffer/methanol (70:30, v/v)
↓ Filter
Filtrate
↓ Inject (7 µL) to LC-MS/MS

Due to the advantages of PAC-LC, the injection volume could be increased.

Time reduction of about 3 hours of the sample preparation method

*: Rat plasma (Anticoagulant: EDTA-2K) / L-Consera® (NISSUI PHARMACEUTICAL CO.,LTD., Tokyo, Japan) (1:9,v/v). L-consera® was added to plasma at test facility.
**: Phenomenex, Torrance, CA

LC-MS/MS Condition

PAC-LC	Shimadzu XR system (Shimadzu, Kyoto, Japan)			
Column	CAPCELL PAK INERT C18 MGⅢ, 3µm, 2×50mm (OSAKA SODA CO., LTD., Osaka, Japan)			
Column temp.	50°C			
Mobile phase (pump A)	Water/TEA/HFIP/acetylacetone (1000:2:30:0.05, v/v/v/v)			
Mobile phase (pump B)	Methanol/water/TEA/HFIP/acetylacetone (500:500:2:30:0.05, v/v/v/v/v)			
Run time	12.5 min			
Remark	The mobile phase was used with N_2 gas bubbling. Patent pending: WO2021/172380			
MS	**Triple TOF®5600 and Analyst®TF (ver. 1.6) (SCIEX, Framingham, MA)**			
Scan type	Product ion scan			
Polarity	Negative			
Ion spray voltage Floating	-4500 V			
TEM	600°C			
Monitoring ions	Analyte	Precursor (*m/z*)	DP	CE
	Nusinersen	889.7	-130	-95
	I.S.	881.7	-130	-90
XIC	94.940±0.015 Da			
Remark	Nusinersen-OMe, bearing the identical base sequence as nusinersen but with 2'-O-methyl (2-OMe) modifications in place of 2-O-methoxyethyl (2'-MOE) modifications, was used as an internal standard (I.S.).			

Validation Study

Result:

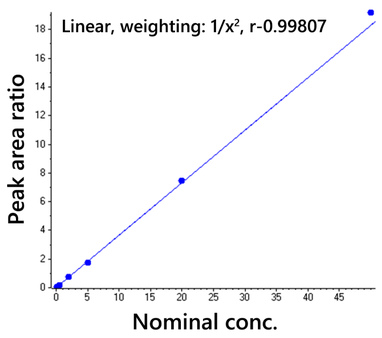
Validation tests of a highly-sensitivity determination method using PAC-LC-MS/MS were conducted. Good linearity, coefficient of variation (CV) and relative error (RE) were observed over the concentration range of 0.1 to 50 ng/mL in the diluted plasma. Nusinersen in the diluted plasma was stable under various storage conditions. The results of selectivity of 6 lots plasma and dilution integrity up to 1000-fold were also good (data not shown). About 70% area of LLOQ was detected in the blank matrix measured immediately after injection of the ULOQ. The carry-over peak could be washed away by once additional injection of blank sample. There is probably room for improvements in the current method, in order to mitigate sample carryover.

Equipment and Software:
Triple TOF®5600, Analyst®TF (ver. 1.6), PeakView™ (ver. 1.2), and MultiQuant™ (ver. 2.1.1) (SCIEX)

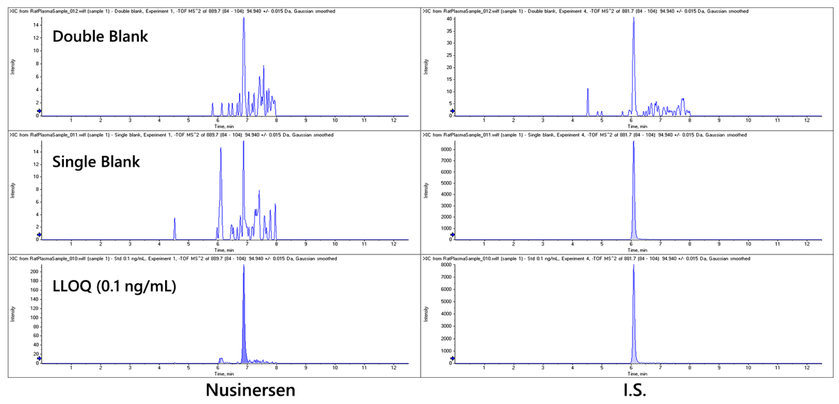
Linearity

Nominal concentration* (ng/mL)	Back calculated concentration* (ng/mL)	RE (%)
0.100	0.0979	-2.1
0.200	0.215	7.5
0.500	0.458	-8.4
2.00	2.01	0.5
5.00	4.79	-4.2
20.0	20.3	1.5
50.0	52.4	4.8

*: Concentration in the diluted plasma



Typical chromatograms



Between-run

Nominal concentration* (ng/mL)	Low QC 0.300	Medium QC 4.00	High QC 40.0
Day 1 Determined concentration* (ng/mL)	0.317	4.03	46.2
	0.318	3.95	41.9
	0.294	3.96	43.9
	0.345	4.51	42.4
	0.322	4.49	44.1
Day 2 Determined concentration* (ng/mL)	0.327	3.78	42.8
	0.277	4.13	40.0
	0.280	4.10	41.8
	0.295	4.09	40.2
	0.281	4.04	42.5
Day 3 Determined concentration* (ng/mL)	0.283	3.35	36.4
	0.248	3.54	35.7
	0.262	3.38	36.4
	0.278	3.34	34.7
	0.242	3.49	37.9
Mean* (ng/mL)	**0.291**	**3.88**	**40.5**
SD (ng/mL)	**0.030**	**0.39**	**3.5**
RE (%)	**-3.0**	**-3.0**	**1.3**
CV (%)	**10.3**	**10.1**	**8.6**

Stability in the diluted plasma

Nominal concentration* (ng/mL)	Storage condition	RE (%)
Low QC (0.300 ng/mL) and High QC (40.0 ng/mL) [each n=3]	Room temp. 24 h	6.0 and 9.8
	4°C 24 h	3.0 and 13.8
	Freeze and thaw 3 cycles	8.0 and 13.0
	-80°C 161 days	12.3 and 13.0
	Processed sample 10°C 72 h	2.7 and 6.0

Dilution integrity at test facility

Sample	Rat plasma volume (µL)	L-consera® volume (µL)	RE (%)	CV (%)
High volume n=5	18	162	-4.0	7.3
Low volume n=5	8	72	-11.5	8.5

Nominal plasma concentration: 200 ng/mL

In Vivo Study | Administration: Crl:CD(SD), intravenous, 10 mg/kg, single dosing, n=3

Microsampling

Sampling Method and Sampling Preparation:

Blood (50 µL) was collected without anesthesia from the subclavian vein using the FN syringe containing EDTA-2K solution and 27G needles. The collected blood was transferred to a polypropylene tube, and centrifuged (4°C, 10000×g, 3 minutes) to obtain plasma (>10 µL each). L-consera® was added to the obtained plasma volume so as to be diluted 10-fold at the test facility. The dilution operation was confirmed before this study started, and the dilution reproducibility of the two volumes both gave reasonable results. (The results are shown in validation study.)

Quantitative Analysis of Nusinersen

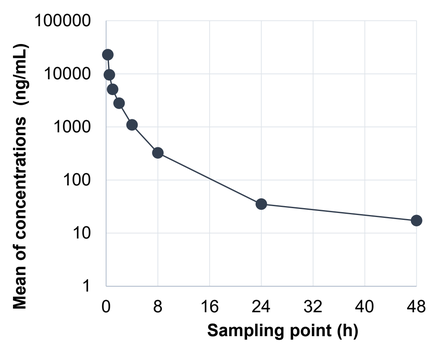
Result:

Concentrations of nusinersen in plasma tend to decrease until 48 hours after administration. The incurred sample reanalysis (ISR) results showed the assay variability※ of -12.7% to 7.2% (data not shown), confirming the measurement reproducibility of the actual sample.

※: Assay variability (%) =
[(Concentration obtained by ISR - Concentration in the original analysis)/Mean of the two] × 100.
(MHLW, "Guideline on Bioanalytical Method Validation in Pharmaceutical Development", July 11 (2013))

Animal No.	Plasma concentration (ng/mL)							
	0.25 h	0.5 h	1 h	2 h	4 h	8 h	24 h	48 h
1	23200 #1	9240 #2	4480 #2	2450 #3	870 #3	286 #3	38.2	19.6
2	23400 #1	9570 #2	5630 #2	2890 #3	1160 #3	330 #3	35.1	16.0
3	21700 #1	9800 #2	5130 #2	2990 #3	1240 #3	352 #3	32.0	15.9
Mean	22800	9540	5080	2780	1090	323	35.1	17.2
SD	900	280	580	290	190	34	3.1	2.1

Some samples were further diluted by L-consera® prior to the sample preparation (#1: 1000-fold dilution, #2: 100-fold dilution, #3: 10-fold dilution)



Conclusions

- **We developed highly-sensitivity determination method of nusinersen using PAC-LC-MS/MS.**
- **The PAC-LC-MS/MS method has the advantages of reducing time for sample preparation and increasing injection volume. In addition, improvements are considered to be required to reduce carryover.**
- **We could properly determine the plasma concentration of nusinersen in the samples obtained by microsampling.**