

**Temporal quantitative profiling of sialyllactoses and sialic acids after oral
administration of sialyllactose to mini-pig with osteoarthritis**

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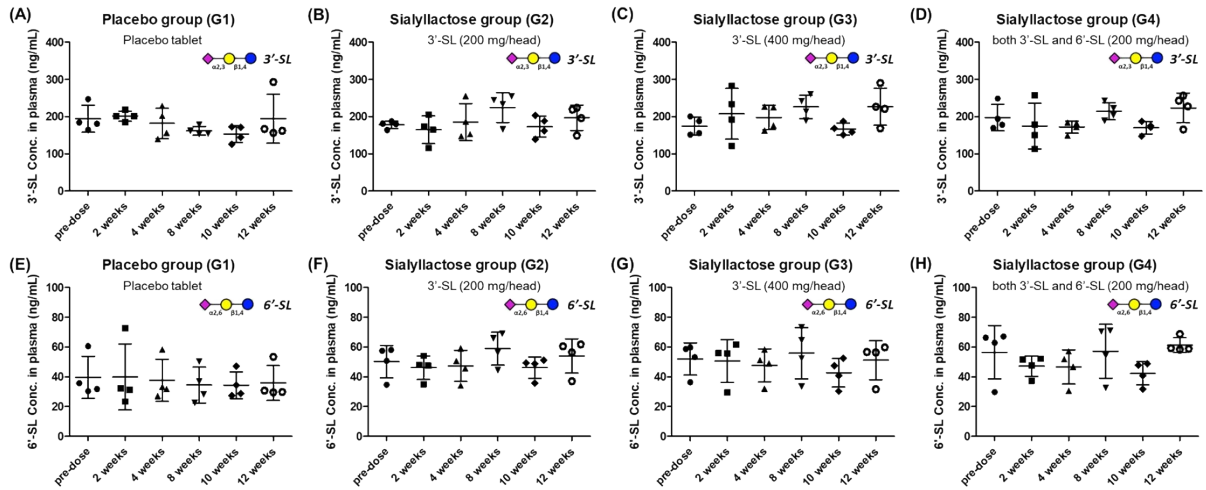


Figure S 1. Concentration-time profiles of 3'-SL and 6'-SL as a group. Concentrations (ng/mL) are shown in left panel for 3'-SL (A-D) and 6'-SL (E-H). Placebo group (Placebo tablet supplementation), SL group (SL tablet supplementation). Values are means \pm SDs, G1 (placebo), $n = 4$; G2 (3'-SL, 200 mg), $n = 4$; G3 (3'-SL, 400 mg), $n = 4$; G4 (3'- and 6'-SL, respectively 200 mg), $n = 4$. *Means differ, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

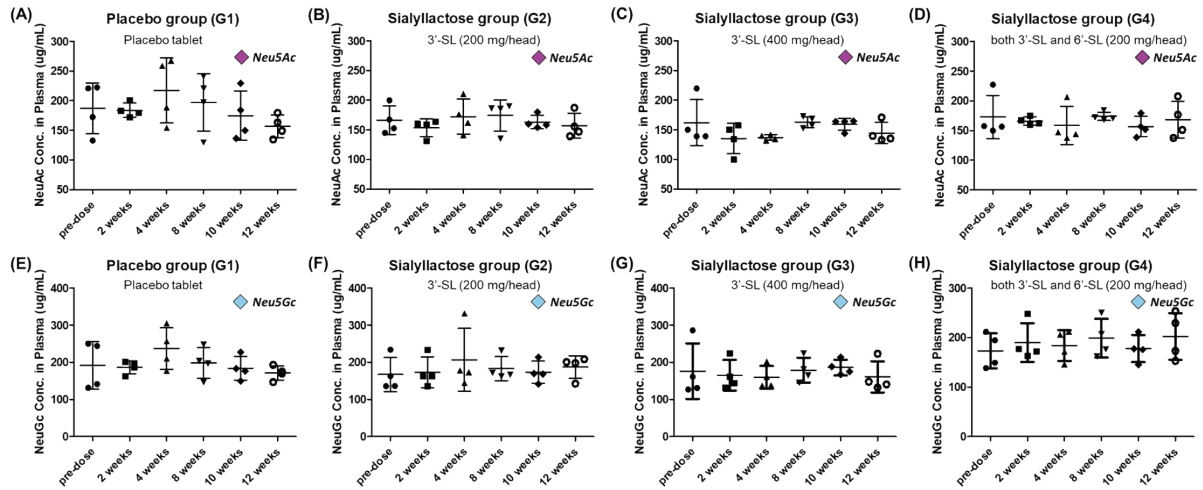


Figure S 2. Concentration-time profiles of Neu5Ac and Neu5Gc as a group. Concentrations ($\mu\text{g/mL}$) are shown in left panel for Neu5Ac (A-D) and Neu5Gc (E-H). Placebo group (Placebo tablet supplementation), SL group (SL tablet supplementation). Values are means \pm SDs, G1 (placebo), $n = 4$; G2 (3'-SL, 200 mg), $n = 4$; G3 (3'-SL, 400 mg), $n = 4$; G4 (3'-and 6'-SL, respectively 200 mg), $n = 4$. *Means differ, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table S 1. The primers for real-time PCR.

Genes	Sequence (5' to 3')
GAPDH	F AGTGAACGGATTTGGCCGC
	R GTGGAGGTCAATGAAGGGGTC
MMP1	F CAAGGTATTGGAGGGGATGC
	R TTCATGAGCAGCCACACGAT
MMP12	F GCTGACGATAGAAACAACGCC
	R TGTCCGGTCACATTTAGCCC

Results for method validation of 3'-SL and 6'-SL in mini-pig plasma

Matrix effect

The matrix effect was evaluated by the IS-normalized MF in samples spiked with low- and high-quality control samples using six individual samples (**Table S2**). In 3'-SL, the low concentration average was 1.14 with a CV of 4.64 %, and the high concentration average was 1.01 with a CV of 14.49 %. In 6'-SL, the IS-normalized MF average of low concentration was 1.14 with a CV of 6.07 %, and the high concentration was 1.19 with a CV of 13.84 %. Values satisfied the acceptance criteria.

Recovery

Recovery was assessed at three concentrations of QC samples in plasma (**Table S2**). The recovery percentages were compared peak area between the spiked sample in solution extracted only blank plasma and extracted sample already spiked plasma. The recovery rate of 3'-SL was observed from 62.27 % to 71.87 %, and 6'-SL was from 51.00 % to 62.33 %. Regarding CV, it was found to be within ± 15 % of the acceptable standard for 3'-SL, 6'-SL, and IS.

Linearity

The calibration curve, ranging from 40 to 2000 ng/mL, was obtained excellent linearity 0.9953 and 0.9959 for 3'-SL and 6'-SL, respectively (**Table S3**). The calibration accuracy range of the standard samples met the acceptance criteria within ± 15 % of the nominal value (± 20 % for LLOQ).

Selectivity

Selectivity was evaluated with LLOQ samples using real plasma of six different origins (**Table S4**). The actual plasma samples were 6 of 16 mini-pig plasma before SL-fed. None of the six plasma samples that spiked the LLOQ concentration had interfering peaks with the analyte. The CV (%) of the sixth repetition analysis was found to be within the acceptable standard (± 20 % for LLOQ) of 10.23 % – 16.20 % in all analyses of the lowest quantification limit

concentration.

Accuracy and precision

Accuracy and precision were evaluated at the lowest limit, low, medium, and high concentration levels (40; 160; 800 and 1600 ng/mL, respectively). In the **Table S5**, the intra-day results ranged from 92.09 to 103.06 % at the 3'-SL and 86.26 to 104.05 % at the 6'-SL. Inter-day results ranged from 90.52 to 107.65 % and 90.05 to 114.93 % for 3'-SL and 6'-SL, respectively. The CV (%) did not exceed the acceptable limits for accuracy and precision both inter- and intra-day.

Stability

Stability was evaluated in low and high QC samples of standard stock solutions of 3'-SL and 6'-SL (**Table S6**). Benchtop and standard stock solution stabilities were compared after leaving the sample at room temperature for 4 h. The benchtop stability of QC samples of 3'-SL and 6'-SL were observed with an accuracy of 95.72 to 109.41 % for 3'-SL, and 111.59 to 112.27 % for 6'-SL. The freeze/thaw stability was also measured within acceptable standards with an accuracy ranged from 92.91 to 104.81 % and from 105.44 to 105.79 % for both 3'-SL and 6'-SL. Standard stock solutions were compared with peak area values before and after standing at room temperature. The stability was confirmed at 102.28 % and 101.18 % for 3'-SL and 6'-SL, respectively.

Table S 2. Method validation data for 3'-SL and 6'-SL: matrix effect and recovery.

	3'-SL			6'-SL		
Nominal Concentration (ng/mL)	160	800	1600	160	800	1600
Matrix effect						
Mean	1.14	-	1.01	1.14	-	1.19
CV (%)	4.64	-	14.49	6.07	-	13.84
Recovery						
Mean (%)	62.27	68.00	71.87	51.00	57.48	62.33
CV (%)	2.55	2.44	0.96	1.09	3.74	1.81

Table S 3. Method validation data for 3'-SL and 6'-SL: linearity (calibration curve).

3'-SL						
Nominal Concentration (ng/mL)	40.00	100.00	200.00	400.00	1000.00	2000.00
Mean estimated conc. (ng/mL)	36.84	102.19	216.43	440.63	907.50	2036.67
CV (%)	8.19	2.35	3.62	0.46	1.26	0.76
RE (%)	-7.91	2.19	8.22	10.16	-9.25	1.83
6'-SL						
Nominal Concentration (ng/mL)	40	100	200	400	1000	2000
Mean estimated conc. (ng/mL)	34.50	107.03	219.67	438.83	899.87	2040.00
CV (%)	9.94	5.28	3.60	1.47	1.72	1.11
RE (%)	-13.74	7.03	9.83	9.71	-10.01	2.00

Table S 4. Method validation data for 3'-SL and 6'-SL: selectivity.

	3'-SL	6'-SL
Nominal Concentration (ng/mL)	40	40
Minipig 1	118.48	112.86
Minipig 2	115.81	87.83
Minipig 3	88.02	91.44
Minipig 4	88.49	90.72
Minipig 5	94.79	91.54
Minipig 6	80.25	87.47
Mean estimated conc. (ng/mL)	97.64	93.64
SD	15.82	9.58
CV (%)	16.20	10.23

Table S 5. Method validation data for 3'-SL and 6'-SL: accuracy and precision.

	3'-SL				6'-SL			
Nominal Concentration (ng/mL)	40	160	800	1600	40	160	800	1600
Intra-day								
Mean estimated conc. (ng/mL)	36.84	164.90	801.97	1477.00	34.50	163.57	832.37	1550.67
Accuracy (%)	92.09	103.06	100.25	92.31	86.26	102.23	104.05	96.92
CV (%)	8.19	2.19	1.06	1.98	9.94	0.92	1.60	1.51
Inter-day								
Mean estimated conc. (ng/mL)	36.21	172.24	841.38	1534.60	36.02	183.38	919.43	1710.20
Accuracy (%)	90.52	107.65	105.17	95.91	90.05	114.61	114.93	106.89
CV (%)	6.36	7.66	6.53	5.41	8.88	14.81	13.60	12.85

Table S 6. Method validation data for 3'-SL and 6'-SL: stability.

	3'-SL		6'-SL	
Nominal concentration (ng/mL)	160	1600	160	1600
Benchtop stability (room temp. for 4 h)				
Mean estimated conc. (ng/mL)	175.05	1531.50	180.36	1785.38
CV (%)	1.66	6.42	2.54	3.45
Freeze/thaw stability (three cycles)				
Mean estimated conc. (ng/mL)	167.69	1486.56	168.70	1692.60
CV (%)	7.57	5.49	2.38	7.80
Stock solution stability (room temp. for 4 h)				
	before	after	before	after
Mean (area)	1954326.46	1998879.52	3126451.62	3163437.04
CV (%)	1.35	0.76	3.01	2.55