SUPPORTING INFORMATION

Accurate Quantification of *N*-Glycolylneuraminic Acid in Therapeutic Proteins Using Supramolecular Mass Spectrometry

Hyun Hee L. Lee[†], Chae Eun Heo[†], Nari Seo[‡], Seung Gyu Yun[§], Hyun Joo An[‡,*], and Hugh I. Kim[†,*]

[†]Department of Chemistry, Korea University, Seoul 02841, Republic of Korea

[‡]Graduate School of Analytical Science & Technology, Chungnam National University, Daejon 34134, Republic of Korea

[§]Department of Laboratory Medicine, Korea University College of Medicine, Seoul 02841, Republic of Korea

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Isothermal Titration Calorimetry (ITC). The ITC experiments were performed using a VP-ITC calorimeter (Microcal, Norhampton, MA, USA) at 298 K. For the consistency of the pH of solutions, 0.01M HCl solution (pH 2.00) and 20 mM sodium phosphate buffer (pH 7.00) were utilized as buffer solutions. We confirmed that pH changes of final sample solutions do not occur. The reference cell was filled with buffer solutions, and the ITC cell was filled with 0.40 mM CB[7] solutions dissolved in the buffer solutions. The ITC syringe was filled with 10 mM sialic acid solution dissolved in the buffer solutions. The number of injections, initial equilibration time, and injection interval time were set as 40, 1000 s, and 300 s. The heats of dilution were subtracted for the analysis.

Analysis of Free Sialic Acids Using LC-ESI-MRM-MS. For investigating free sialic acids using ESI-MS, we utilized LCMS-8580 (Shimadzu, Japan), a triple-quadrupole mass spectrometer with multiple reaction monitoring (MRM), both in positive and negative ion modes. The source parameters were 300, 250, 400 ℃, corresponding to the interface, DL, and heating gas temperatures, respectively. The nebulizing, heating, and drying gases were set to 3, 10, and 10 L/min, respectively. For the separation in LC, an ACQUITY UPLC BEH C18 column (1.7 μm, 1.0 × 100 mm) was utilized at 30 ℃. The injection volume of the sample solution was 5 μl, and the solvent A (0.1% FA water) and B (0.1% FA ACN) were used at a flow rate of 0.1 mL/min. The gradients of the two solvents were set as follows: 0–0.4 min, 0% B; 0.4–2.8 min, linear increase from 0 to 60% B; 2.8–3.8 min, 60% B; 3.8–4.8 min, linear decrease from 60 to 0% B; and 4.8–6.0 min, 0% B.

Quantification of Sialic Acids Using Gas-phase Host-guest Chemistry. We performed a quantitative analysis of two sialic acids using the gas-phase host-guest chemistry with the addition of both sialic acid standards to the sample solutions. Since the linear relationship between the gas-phase complexation and concentration proportions in the solution corresponds only to the range of the concentration ratio from 10 to 90 %, the addition of a standard solution containing both Neu5Gc and Neu5Ac to the sample solution is required for quantifying sialic acid mixtures with a wide range of concentrations. For example, if we determine a single Neu5Gc solution using the gas-phase host-guest chemistry, the calculated quantifiable concentration range of Neu5Gc is from 0 to ~8 μM when adding both Neu5Gc and Neu5Ac standards of 1 μM into the sample solution. On the contrary, the quantifiable concentration range is 0.11 μM to 9 μM when only adding the Neu5Ac standard of 1 μM. Therefore, we added both sialic acid standard solutions to obtain absolute concentrations of Neu5Gc and Neu5Ac in the mixtures. The linear simultaneous equations for the quantification of binary mixtures of two sialic acids are as follows:

$$\begin{cases} \dfrac{I_A}{I_A + I_B} = m \times \left(\dfrac{c_A V_0 + c_{A,s1} V_{s1}}{V_0 (c_A + c_B) + V_{s1} (c_{A,s1} + c_{B,s1})} \times 100 \right) + b \\ \dfrac{I_A^{'}}{I_A^{'} + I_B^{'}} = m \times \left(\dfrac{c_A V_0 + c_{A,s2} V_{s2}}{V_0 (c_A + c_B) + V_{s2} (c_{A,s2} + c_{B,s2})} \times 100 \right) + b \end{cases} \quad \text{(S1)}$$

where I_A and I_B represent the total intensities of CB[7]-A and CB[7]-B complex ions after addition of the standard solution, respectively. V_0, V_s, $c_{A,s}$, and $c_{B,s}$ represent the initial volume, the volume of the standard solution, and concentrations of standards A and B, respectively. Before performing the ESI-MS experiments for sialic acids with unknown concentrations, we confirmed the slope (m) and y-intercept (b) by measuring the ESI-MS spectra of mixtures of standard sialic acids. For the confirmation, we used a total sialic acid concentration of the standards of 10 μM and concentration proportions of 10, 50, and 90%. The c_A and c_B are

obtained by solving Equation (S1) as all values except the two concentrations in the equation are known. As for Equation (S1), the linear simultaneous equations for the single sialic acid solutions are as follows:

$$\begin{cases} \dfrac{I_A}{I_A + I_B} = m \times \left(\dfrac{c_{A,s}}{c_{A,s} + c_{B,s}} \times 100 \right) + b \\ \dfrac{I_A^{'}}{I_A^{'} + I_B^{'}} = m \times \left(\dfrac{c_A V_0 + c_{A,s} V_s}{c_A V_0 + V_s \left(c_{A,s} + c_{B,s} \right)} \times 100 \right) + b \end{cases} \quad \text{(S2)}$$

For the quantification of the binary mixture in the present study, 1–10 μl of sialic acid standard solution with 50 μM of Neu5Gc and Neu5Ac was added to 50 μl of the sample solutions. In case of the single sialic acid solution, 1–10 μl of the standard solution was added to 100 μl of the sample solutions.

Calculation of Binding Energies of CB[7]-Sialic Acid complex ions. The binding energies (BE) of the final structures of complex ions were obtained following equation (S3):

$$\mathrm{BE} = E_{complex} - E_{CB[7]} - E_{protonated\ Sia} - E_{NH4} \quad \text{(S3)}$$

where $E_{complex}$, $E_{CB[7]}$, E_{NH4} represent the energies of the optimized complex ion, CB[7], and ammonium cation, respectively, which are directly obtainable from each DFT output file. The $E_{protonated\ Sia}$ represents the energy of the optimized structure of protonated sialic acid ion. Each representative energy was the value of the lowest-energy structure among the five optimized candidate structures. The calculated binding energies are presented in Table S5.

Discussions

Quantification of Neu5Gc and Neu5Ac in Glycoproteins. The relative error of the quantification results obtained by our method with the values in previous studies are in a narrow range from 0.4 to 26.5%, except bF (Table S6). These errors are comparable to the relative standard deviation between the quantification results of the previous studies, implying a high accuracy and precision of the present method (Table S6). For bF, the maximum relative error was 23.5% for the case when the concentration unit is μg analyte/mg glycoprotein, but 51.2% when the unit is mol analyte/mol glycoprotein despite the results obtained by processing the same experimental data. Since we confirmed the protein sequence of bF (uniprot number P12763) with the sequence coverage of 75.7% by performing LC-ESI-MS/MS experiments, the bF extinction coefficient used herein is reliable. Therefore, we estimated that the concentration difference of bF is caused by the method difference between the UV-Vis spectroscopy of our method and bicinchoninic acid (BCA) protein assay of the previous studies[S1,S2].

Investigation of Free Neu5Gc and Neu5Ac in the Binary Mixture Using LC-MRM-MS. LC-MRM-MS experiments were performed to confirm whether Neu5Gc and Neu5Ac affect each other's absolute intensity when their retention times are overlapped. Neu5Gc and Neu5Ac solutions with different concentrations (0.1, 0.5, 1, and 10 μM) were investigated both in the positive and negative ion modes. We selected protonated/deprotonated sialic acid ions as parent ions since the mass difference between Neu5Gc and Neu5Ac is approximately 16 Da (calculated: 15.9949) which is similar with the mass difference between sodium and potassium cation (calculated: 15.9739). Three product ions of each sialic acid ion were selected, and the collision energies for each product ion were also optimized using a LC-MS software (LabSolutions, Shimadzu, Japan). Figure S5 shows that chromatographic peaks of two sialic

acids are highly overlapped. We confirmed that both sialic acids can be determined in this range when two sialic acids are not mixed (Figure S6). The linearity of each sialic acid in range of 0.1–100 μM was observed with a confidence level of 95% (Figure S7). Thus, we investigated absolute intensities of Neu5Gc and Neu5Ac in their mixtures in this condition.

Figure S8 shows that the absolute intensities of Neu5Ac in range from 0.1 to 1 μM increase as the solution-phase concentration of Neu5Gc increases. Interestingly, the absolute intensity of 10 μM Neu5Ac is decreased despite the concentration increase of Neu5Gc. In contrast to Neu5Ac, the absolute intensities of Neu5Gc at all concentrations decrease with increasing concentrations of Neu5Ac. The same phenomena were observed regardless of the ion mode (Figure S8). To understand these phenomena, further experiments of Neu5Gc solutions were performed using the MRM method optimized for Neu5Ac. The abundant chromatographic peaks were observed despite the use of the MRM method optimized for Neu5Ac (Figure S9a). Thus, we further investigated MS spectra of Neu5Gc to confirm whether peaks of Neu5Gc fragment ions are overlapped with those of protonated/deprotonated Neu5Ac ions (Figure S9b). It is observed in positive ion mode that the tri-isotope mass peak of dehydrated Neu5Gc ions (M+2, observed: *m*/*z* 310.10; calculated: *m*/*z* 310.1024) have a similar mass-to-charge ratio (*m*/*z*) as that of the monoisotopic peak of protonated Neu5Ac ions (observed: *m*/*z* 310.10; calculated: *m*/*z* 310.1138). In addition, we also observed that the $[Neu5Gc\text{-}H\text{-}CH_4]^-$ generated during ionization processes (observed: *m*/*z* 308.10; calculated: *m*/*z* 308.0618) has highly similar *m*/*z* with that of deprotonated Neu5Ac (observed: *m*/*z* 308.10; calculated: *m*/*z* 308.0982). These findings imply that the overlapping of the precursor peaks of Neu5Gc and Neu5Ac fragments strongly induce the intensity increase of Neu5Ac in the range from 0.1 to 1 μM. In the case of the intensity decrease of Neu5Ac (10 μM) and Neu5Gc (all concentrations), we estimated that the decrease occurs by competitive ionization between Neu5Gc and Neu5Ac as their concentration increases. Consequently, the precise quantification of two sialic acids using

LC-MS/MS requires great caution without the complete chromatographic separation of Neu5Gc and Neu5Ac.

Figure S1 Mass spectra of the CB[7]-Neu5Gc and CB[7]-Neu5Ac solutions. The concentrations of CB[7], sialic acid, and ammonium acetate are 10, 10, 200 μM, respectively.

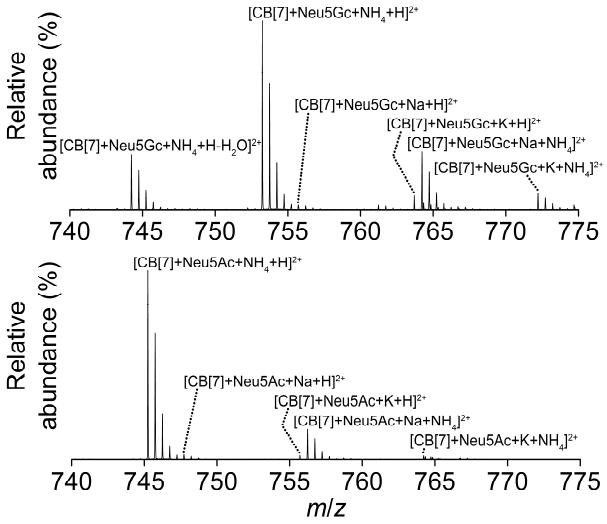

Figure S2 ITC thermograms of Neu5Gc and Neu5Ac with CB[7] in (a) pH 2 and (b) pH 7 buffer solutions.

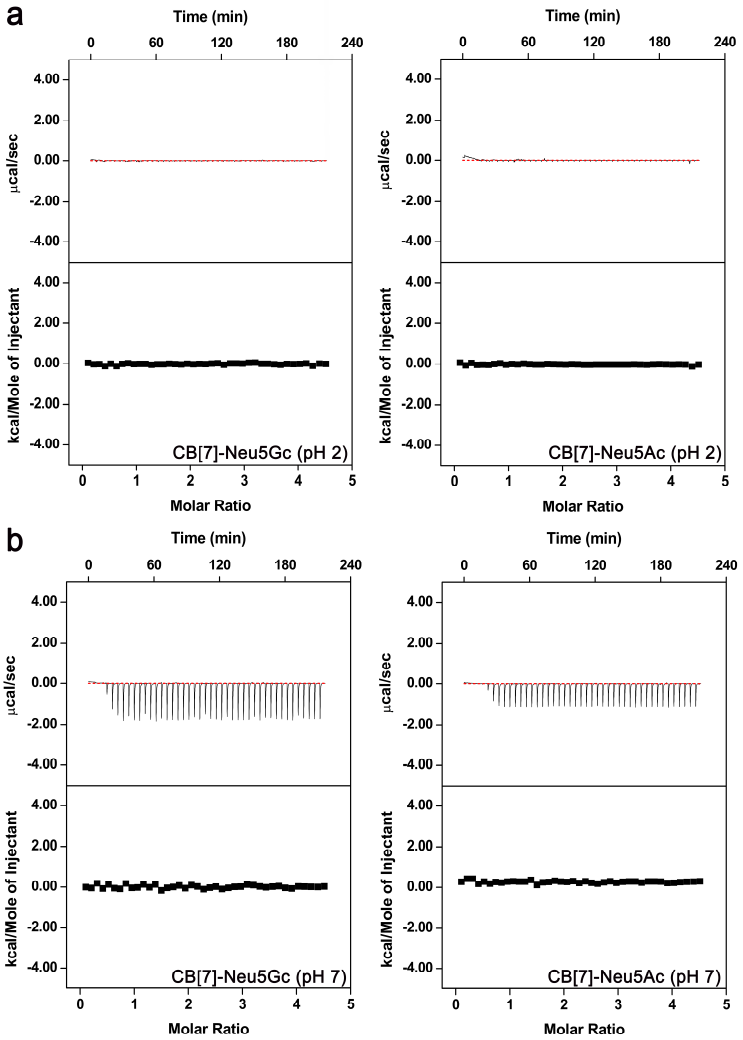

Figure S3 Complexation proportion between Neu5Gc and Neu5Ac toward CB[7] depending on the different concentrations of CB[7].

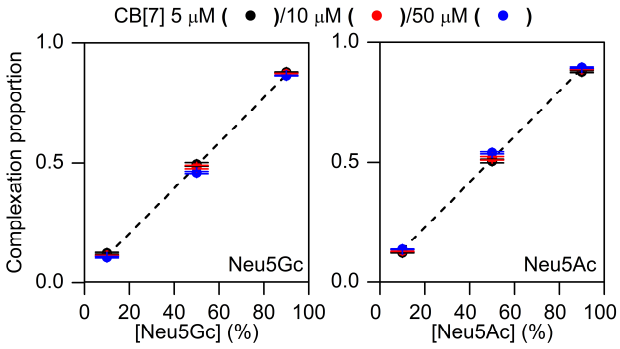

Figure S4 Host-guest interactions of representative structures of CB[7]-sialic acid complex ions.

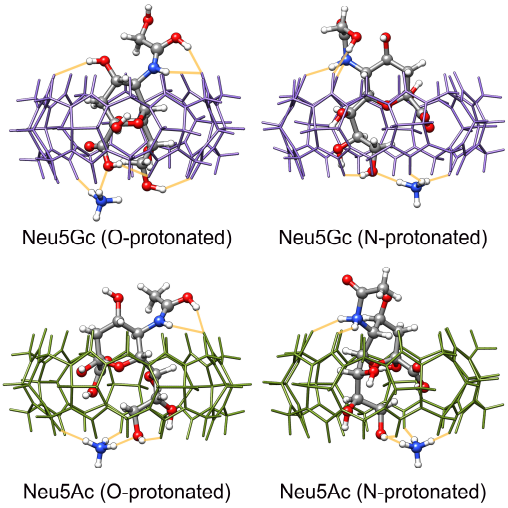

Figure S5 Extracted ion chromatograms of two sialic acids.

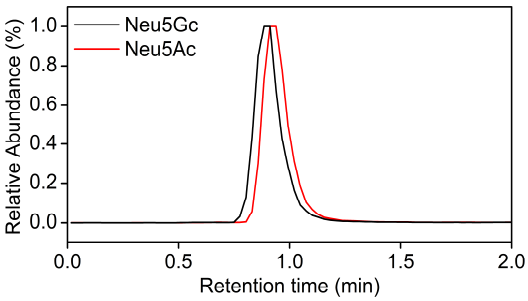

Figure S6 Calibration curves of Neu5Gc and Neu5Ac when they are not mixed.

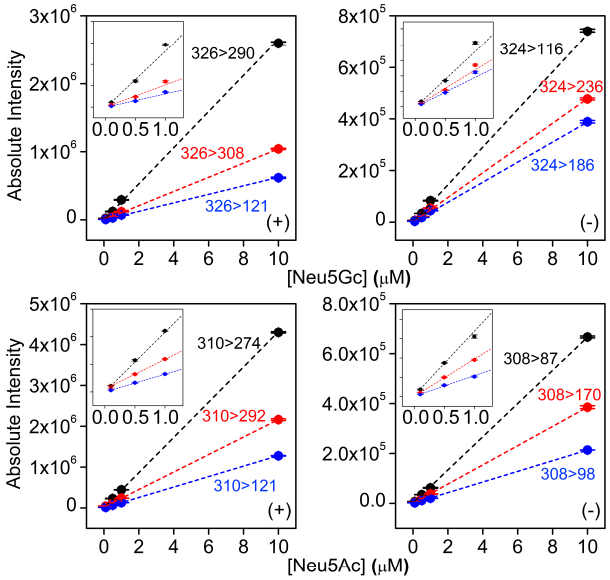

Figure S7 Calibration curves (dots) of (a) free Neu5Gc and (b) free Neu5Ac in the range of 0.1–100 μM. Each range of the confidence level of 95%, which is shown as the solid line, was obtained by treatment of the raw data in OriginPro 8.5.

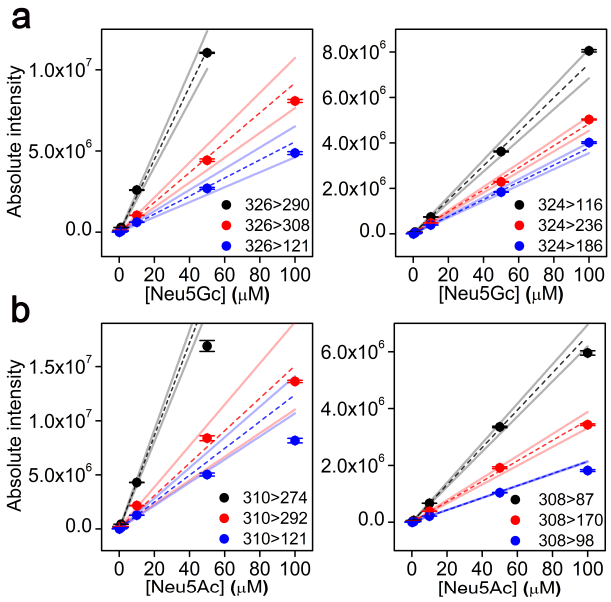

Figure S8 Calibration curves of (a) free Neu5Gc and (b) free Neu5Ac when they are mixed together. The calibration curves were observed in both positive and negative ion modes using LC-MRM-MS.

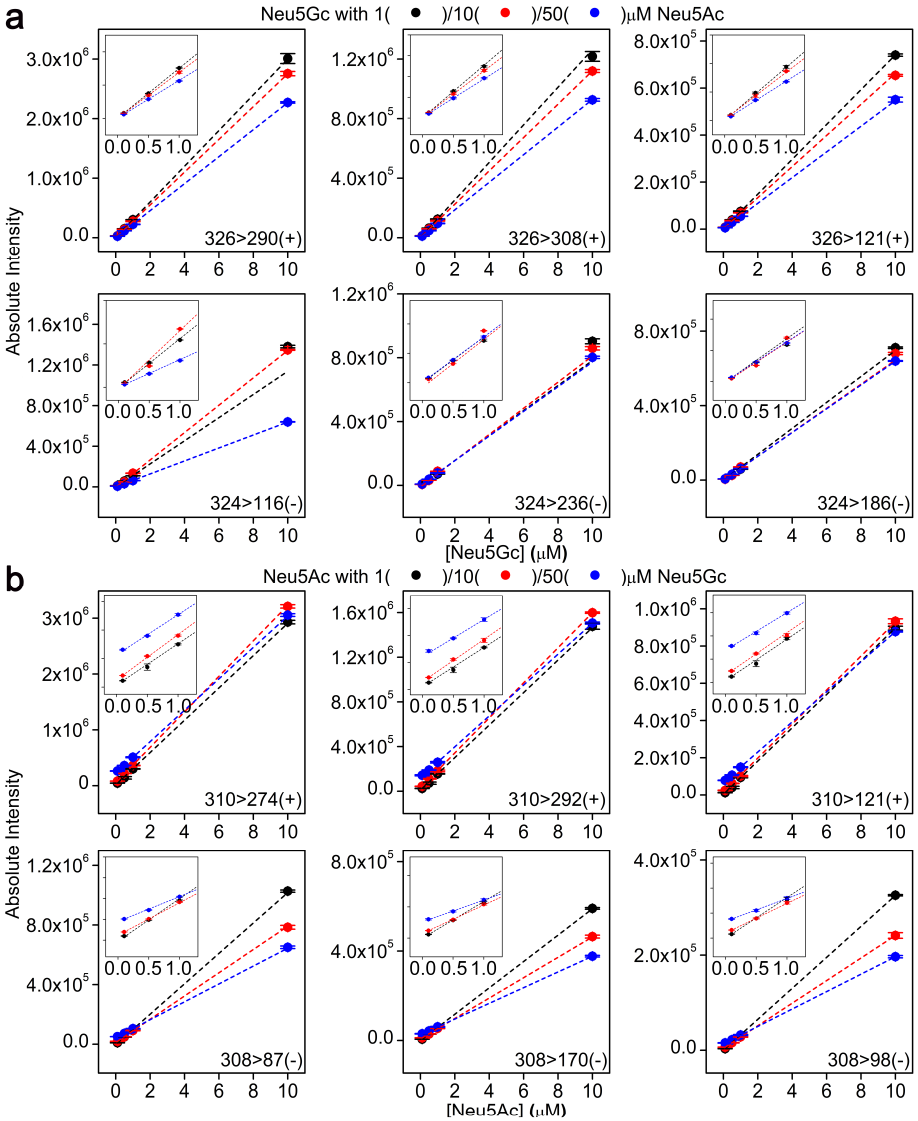

Figure S9 Chromatograms and mass spectra of Neu5Gc fragment ions. (a) Extracted ion chromatograms of Neu5Gc when Neu5Gc are investigated using the MRM method optimized for Neu5Ac. (b) Mass spectra of Neu5Gc in both the positive and negative ion modes.

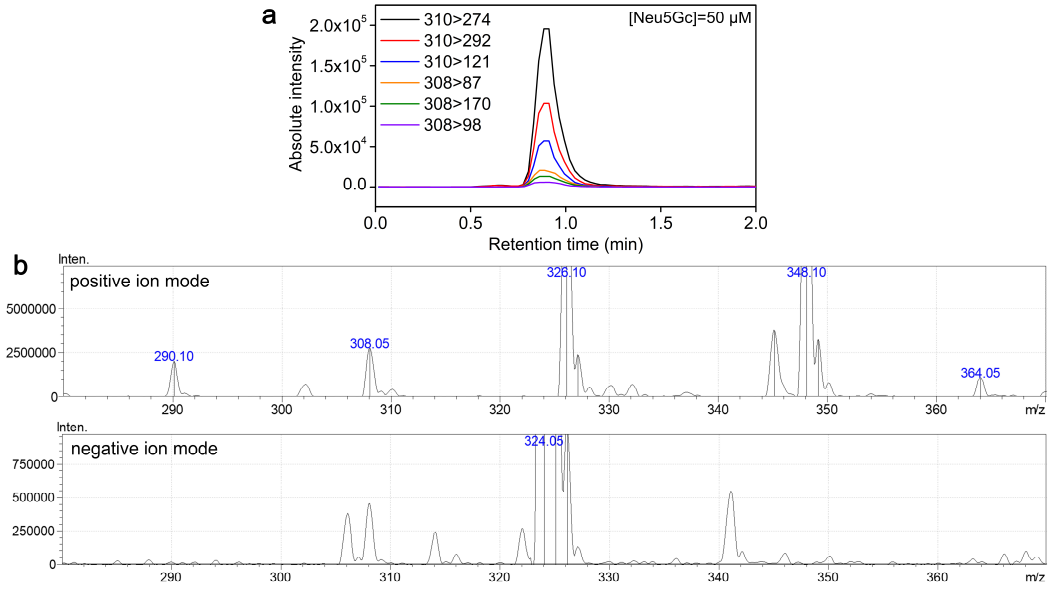

Table S1 The peaks of CB[7]-sialic acid complex ions shown in mass spectra.

Complex	*m*/*z* (calculated)	*m*/*z* (observed)
$[CB[7]+Neu5Gc+NH_4+H-H_2O]^{2+}$	744.2381	744.2398
$[CB[7]+Neu5Ac+NH_4+H]^{2+}$	745.2458	745.2442
$[CB[7]+Neu5Ac+Na+H]^{2+}$	747.7236	747.7205
$[CB[7]+Neu5Gc+NH_4+H]^{2+}$	753.2344	753.2409
$[CB[7]+Neu5Ac+K+H]^{2+}$	755.7105	755.7130
$[CB[7]+Neu5Gc+Na+H]^{2+}$	755.7210	
$[CB[7]+Neu5Ac+Na+NH_4]^{2+}$	756.2369	756.2331
$[CB[7]+Neu5Gc+K+H]^{2+}$	763.7080	763.7022
$[CB[7]+Neu5Ac+K+NH_4]^{2+}$	764.2238	764.2236
$[CB[7]+Neu5Gc+Na+NH_4]^{2+}$	764.2343	
$[CB[7]+Neu5Gc+K+NH_4]^{2+}$	772.2213	772.2200

Table S2 LODs, LOQs, linear ranges, and concentration ratios of Neu5Gc with Neu5Ac in the present and previous studies.

Ref	Neu5Gc			Neu5Ac		
	LOD (pmol)	LOQ[a] (pmol)	Linear range (pmol)	LOD (pmol)	LOQ[a] (pmol)	Linear range (pmol)
Our method	0.48	1.60	5–9,000	0.20	0.65	5–9,000
S1	0.5-2	1.7-6.7	2–2000	1-2	3.3-6.7	2–2000
S2	0.06	0.20	0.23–11.25	0.11	0.37	0.54–67.5
S3[b],[c]	35.3-104	176.5-520		35.3-104	176.5-520	
S4[d]	0.000028	0.000056	0.000140–0.140	0.000028	0.000056	0.000140–0.140
S5[c]	25	125	~160,000	30	150	~160,000
S6[c]	0.023	0.115		0.025	0.125	

[a] If LOQs were not described in a previous study, we obtained these values by multiplying LODs of the study by 10/3.
[b] The exact LODs and LOQs of sialic acids were not described in Ref S3. The authors in Ref S3 stated that *N*-acylmannosamines obtained from cleavage of sialic acids have similar sensitivities of aldoses in their study. Therefore, we entered the values of aldoses in Ref S3.
[c] The LODs in Ref S3, S5, and S6 are expressed as twice the baseline noise. Therefore, in this case, we obtained LOQs of these studies by multiplying the LODs by 10/2.
[d] The LODs in Ref S4 are expressed as five times the baseline noise. Therefore, in this case, we obtained LOQs of this study by multiplying the LODs by 10/5.

Table S3 Determined concentrations of two sialic acids in their binary mixtures using the gas-phase host-guest chemistry. All units are in μM, and the standard deviations were calculated for three replicated experiments.

$[Neu5Gc]_{sol}$[a]	$[Neu5Ac]_{sol}$[a]	$[Neu5Gc]_{det}$[b]	$[Neu5Ac]_{det}$[b]	$[Neu5Gc]_{sol}$	$[Neu5Ac]_{sol}$	$[Neu5Gc]_{det}$	$[Neu5Ac]_{det}$
0.05	0.45	0.05 ± 0.01	0.45 ± 0.01	2.00	18.00	2.02 ± 0.10	18.01 ± 0.11
0.15	0.35	0.15 ± 0.01	0.34 ± 0.01	6.00	14.00	5.71 ± 0.01	14.40 ± 0.16
0.25	0.25	0.25 ± 0.01	0.25 ± 0.01	10.00	10.00	9.93 ± 0.07	10.06 ± 0.07
0.35	0.15	0.35 ± 0.01	0.15 ± 0.01	14.00	6.00	14.16 ± 0.05	5.83 ± 0.05
0.45	0.05	0.45 ± 0.01	0.05 ± 0.01	18.00	2.00	17.89 ± 0.01	2.09 ± 0.01
0.10	0.90	0.10 ± 0.01	0.90 ± 0.01	5.00	45.00	4.41 ± 0.02	45.57 ± 0.02
0.30	0.70	0.30 ± 0.01	0.69 ± 0.01	15.00	35.00	14.31 ± 0.16	35.67 ± 0.16
0.50	0.50	0.50 ± 0.02	0.48 ± 0.01	25.00	25.00	24.85 ± 0.06	25.13 ± 0.06
0.70	0.30	0.70 ± 0.01	0.30 ± 0.01	35.00	15.00	34.94 ± 0.30	15.03 ± 0.30
0.90	0.10	0.89 ± 0.01	0.10 ± 0.01	45.00	5.00	45.05 ± 0.01	4.93 ± 0.01
0.20	1.80	0.20 ± 0.01	1.80 ± 0.01	7.50	67.50	7.13 ± 0.10	68.46 ± 0.10
0.60	1.40	0.60 ± 0.02	1.39 ± 0.01	22.50	52.50	21.74 ± 0.23	53.50 ± 0.22
1.00	1.00	1.00 ± 0.01	0.99 ± 0.01	37.50	37.50	37.59 ± 0.15	37.73 ± 0.15
1.40	0.60	1.40 ± 0.01	0.59 ± 0.02	52.50	22.50	52.50 ± 0.13	22.46 ± 0.13
1.80	0.20	1.80 ± 0.01	0.20 ± 0.01	67.50	7.50	67.50 ± 0.19	7.46 ± 0.19
0.50	4.50	0.50 ± 0.02	4.50 ± 0.02	10.00	90.00	9.76 ± 0.05	90.36 ± 0.05
1.50	3.50	1.51 ± 0.01	3.49 ± 0.01	30.00	70.00	29.96 ± 0.31	70.09 ± 0.29
2.50	2.50	2.49 ± 0.03	2.49 ± 0.01	50.00	50.00	49.83 ± 0.29	50.40 ± 0.29
3.50	1.50	3.51 ± 0.03	1.46 ± 0.01	70.00	30.00	69.65 ± 0.01	30.82 ± 0.01
4.50	0.50	4.50 ± 0.01	0.50 ± 0.02	90.00	10.00	89.64 ± 0.05	10.41 ± 0.09
1.00	9.00	0.94 ± 0.02	9.05 ± 0.02				
3.00	7.00	2.95 ± 0.04	7.05 ± 0.04				
5.00	5.00	5.01 ± 0.03	4.99 ± 0.03				
7.00	3.00	7.02 ± 0.02	2.99 ± 0.01				
9.00	1.00	9.00 ± 0.01	0.99 ± 0.01				

[a] solution-phase concentrations of Neu5Gc and Neu5Ac in their binary mixture

[b] determined concentrations of Neu5Gc and Neu5Ac in their binary mixture

Table S4 Quantification results of single sialic acid solutions using the gas-phase host-guest chemistry. All units are in μM, and the standard deviations were calculated for three replicated experiments.

$[Neu5Gc]_{sol}$[a]	$[Neu5Gc]_{det}$[b]	$[Neu5Ac]_{sol}$[a]	$[Neu5Ac]_{det}$[b]
0.10	0.10 ± 0.01	0.10	0.10 ± 0.01
0.30	0.34 ± 0.01	0.30	0.29 ± 0.02
0.50	0.52 ± 0.05	0.50	0.46 ± 0.00
0.70	0.73 ± 0.02	0.70	0.70 ± 0.05
0.90	1.01 ± 0.02	0.90	0.88 ± 0.13
2.00	2.01 ± 0.16	2.00	1.95 ± 0.13
5.00	4.58 ± 0.07	5.00	4.97 ± 0.84
7.00	6.92 ± 0.03	7.00	6.98 ± 0.91
10.00	9.98 ± 0.08	10.00	10.27 ± 0.40
20.00	20.14 ± 0.05	20.00	20.02 ± 0.67
40.00	40.08 ± 0.25	40.00	41.67 ± 0.98
50.00	49.71 ± 0.90	50.00	49.78 ± 1.44
90.00	90.31 ± 0.47	90.00	91.42 ± 3.71

[a] solution-phase concentrations of Neu5Gc and Neu5Ac

[b] determined concentrations of Neu5Gc and Neu5Ac

Table S5 Experimental/theoretical CCSs and binding energies of representative structures of CB[7]-sialic acid complex ions with calculated energies of free sialic acid ions. The calculation of binding energies was performed as described in the Experimental Section of the Supporting Information.

		O-protonated	N-protonated
Experimental CCS	Neu5Gc	227.9 ± 0.9	
	Neu5Ac	226.0 ± 0.4	
Theoretical CCS ($Å^2$)	Neu5Gc	229.2 ± 2.8	227.3 ± 2.5
	Neu5Ac	229.2 ± 3.2	226.8 ± 1.9
Binding Energy[a] (kJ/mol)	Neu5Gc	-340	-410
	Neu5Ac	-389	-364
ΔE_{Sia}[a],[b] (kJ/mol)	Neu5Gc	-20	
	Neu5Ac	-10	

[a] $\Delta E < 0$: favorable

[b] $\Delta E_{Sia} = E_{\text{free sialic acid, O-protonated}} - E_{\text{free sialic acid, N-protonated}}$

Table S6 Sialic acid concentrations in various glycoproteins proposed in previous studies.

Determined concentrations	mol/mol[a]		μg/mg[b]	
	Neu5Gc	Neu5Ac	Neu5Gc	Neu5Ac
mucin[c]			76.22 ± 1.78	116.11 ± 2.69
bT	1.35 ± 0.05	1.09 ± 0.04	6.31 ± 0.21	4.86 ± 0.16
bF	0.62 ± 0.04	8.69 ± 0.05	4.20 ± 0.88	49.81 ± 2.06
hT	N.D.[d]	3.11 ± 0.10	N.D.	10.29 ± 0.32
hAGP	N.D.	22.92 ± 0.19	N.D.	106.18 ± 0.91
hEPO	N.D.	3.08 ± 0.11	N.D.	65.09 ± 2.23
cet[e]	1.70 ± 0.07	0.08 ± 0.00		

Reported concentrations	mol/mol			μg/mg		
	Neu5Gc	Neu5Ac	Ref	Neu5Gc	Neu5Ac	Ref
mucin				74 – 79	116 – 130	S5,S6
bT	1.3 – 2.3	1.0 – 1.9	S1,S2			
bF	0.30 – 0.59	14 – 20	S1,S2	N.D. – 6.8	46.2 – 62.8	S3,S4
hT	N.D.	2.7 – 4.8	S1,S2	N.D.	14.0	S3
hAGP	N.D.	24 – 32	S2	N.D.	100.2 – 106	S4,S5
hEPO[f]	N.D.	3.14 ± 0.07	our study	N.D.	66.21 ± 1.39	our study
cet	1.77		S7			

Relative error (%)[g]	mol/mol		μg/mg	
	Neu5Gc	Neu5Ac	Neu5Gc	Neu5Ac
mucin			0.4	5.6
bT	12.9	3.5		
bF	51.2	45.7	23.5	8.6
hT	·	12.9	·	26.5
hAGP	·	15.7	·	3.0
hEPO	·	1.9	·	1.7
cet	4.0			

Relative standard deviation (%)	mol/mol			μg/mg		
	Neu5Gc	Neu5Ac	Ref	Neu5Gc	Neu5Ac	Ref
mucin				12.9	2.8	S5,S6
bT	16.8	8.8	S1,S2			
bF	26.8	14.0	S1,S2	141.5	21.5	S3,S4
hT		19.3	S1,S2			
hAGP		13.1	S2		4.0	S4,S5

[a] moles of each sialic acid/moles of the target protein

[b] weight of each sialic acid/weight of the target protein

[c] Because the amino acid sequence of mucin is unknown, it is impossible to determine the concentrations (mol/mol) of two sialic acids in this protein.

[d] Because we receive the solution state of cetuximab from Korea University College of Medicine, we cannot determine the concentrations (μg/mg) of two sialic acids.

[e] Not detected
[f] The quantitative analysis for human erythropoietin expressed in HEK293 was performed using the LC-MS/MS technique with MRM since the Neu5Ac concentration of the protein has not been previously reported.
[g] The reference concentration used for obtaining the relative errors was the average value of the concentrations reported previously.

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