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Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography–mass spectrometry

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Abstract

We have previously demonstrated that liquid chromatography/mass spectrometry equipped with a graphitized carbon column (GCC-LC/MS) is useful for the structural analysis of carbohydrates in a glycoprotein. Here, we studied the monosaccharide composition analysis and quantitative oligosaccharide profiling by GCC-LC/MS. Monosaccharides were labeled with 2-aminopyridine and then separated and monitored by GCC-LC/MS in the selective ion mode. The use of tetradeuterium-labeled pyridylamino (d_4-PA) monosaccharides as internal standards, which were prepared by the tagging of standard monosaccharides with hexadeuterium-labeled 2-aminopyridine (d_6-AP), afforded a good linearity and reproducibility in ESIMS analysis. This method was successfully applied to the monosaccharide composition analysis of model glycoproteins, fetuin, and erythropoietin. For quantitative oligosaccharide profiling, oligosaccharides released from an analyte and a standard glycoprotein were tagged with d_0- and d_6-AP, respectively, and an equal amount of d_0- and d_4-PA oligosaccharides were coinjected into GCC-LC/MS. In this procedure, the oligosaccharides that existed in either analyte or a standard glycoprotein appeared as single ions, and the oligosaccharides that existed in both analyte and a standard glycoprotein were detected as paired ions. The relative amount of analyte oligosaccharides could be determined on the basis of the analyte/internal standard ion-pair intensity ratio. The quantitative oligosaccharide profiling enabled us to make a quantitative and qualitative comparison of glycosylation between the analyte and standard glycoproteins. The isotope tag method can be applicable for quality control and comparability assessment of glycoprotein products as well as the analysis of glycan alteration in some diseases.

Keywords: Monosaccharides; Oligosaccharides; Pyridylamination; Isotope tag

1. Introduction

A variety of recombinant glycoproteins and modified glycoproteins are developed as medical agents, and most of them exist in heterogeneous forms because of the various combinations of oligosaccharides. Alteration of glycosylation is known to affect the biological activity, mobilization, and biophysical properties of glycoproteins [1], so assessments of their carbohydrate structure and heterogeneity are essential in many stages of development and quality control of glycoprotein products. Since glycosylation varies in response to changes in the manufacturing condition, monosaccharide composition analysis and/or oligosaccharide profiling are needed for the characterization and as a test for constancy and comparability assessments of glycosylation [2]. Several analytical procedures using HPLC have been reported for oligosaccharide profiling and structural analysis of carbohydrates [3–5]. The oligosaccharide profiling using liquid chromatography/mass spectrometry (LC/MS) is especially known to provide structural information from their chromatographic behavior and molecular mass [6–8]. We have developed mass spectrometric oligosaccharide profiling using a graphitized carbon column (GCC), which can separate

Abbreviations: AP, 2-aminopyridine; d_0, non-deuterium-labeled; d_4, tetradeuterium-labeled; d_6, hexadeuterium-labeled; Fuc, fucose; Gal, galactose; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; GCC, graphitized carbon column; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; PA, pyridylamino; R.S.D., relative standard deviation; SIM, selected ion mode; TFA, trifluoroacetic acid; TIC, total ion chromatogram

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oligosaccharides based on subtle differences in branch, position, and linkage with volatile solution [9,10]. This method enables us to distinguish the glycosylation among some glycoprotein products produced in different cells [11].

A use of internal standards is known to improve the precision and linearity in quantitative analyses. Isotopic analogs of the analytes are currently the preferred internal standards for quantification by mass spectrometry (MS) procedures. For instance, Gygi et al. [12] demonstrated the approach for the accurate quantification of the proteins within complex mixture using isotope-coded affinity tags (ICATs). The use of the isotope-labeled carbohydrates as internal standards can make it possible to quantify the carbohydrates by LC/MS. Reductive pyridylamination is frequently used for the tagging of carbohydrates in HPLC analysis [13,14]. This derivatization is known to afford higher sensitivity in MS analysis [15], and PA oligosaccharides were reported to be separated by GCC [16]. Here, we study quantitative analysis of carbohydrates using tetradeuterium-labeled pyridylamino (d_4-PA) carbohydrates as internal standards. First, we study the monosaccharide composition analysis by using d_4-PA monosaccharides as internal standards. Next, the isotope tag method is used for the quantitative oligosaccharide profiling using recombinant human chorionic gonadotropin (rhCG) and human chorionic gonadotropin (hCG) as an analyte and standard glycoproteins, respectively.

2. Materials and methods

2.1. Materials

All monosaccharide standards were purchased from Seikagaku-kogyo (Tokyo, Japan). The pyridylamination apparatus (PALSTATION), reagents for the pyridylamination reaction, and PA monosaccharide standards were available from TaKaRa Biomedicals (Otsu, Japan). The hexadeuterium-labeled 2-aminopyridine (d_6-AP) was purchased from Wako (Osaka, Japan). Human chorionic gonadotropin (hCG) and recombinant hCG (rhCG) were bought from Sigma (St. Louis, MO, USA). *N*-glycosidase F was purchased from Roche Diagnostics. All other chemicals and reagents were of analytical grade and were commercially available.

2.2. Pyridylamination of monosaccharides

For the pyridylamination of amino sugars, free amino groups of monosaccharides (GlcN, GalN, 1–1000 pmol) were acetylated by incubation in 50 μl of methanol/pyridine/distilled water (30/15/10, v/v/v) with 2 μl of acetic anhydride for 30 min at room temperature. The mixture was dried using a vacuum centrifuge evaporator without heating. Acetic acid (50 μl), methanol (60 μl), and 10 μl of coupling reagent prepared by mixing 100 mg of AP was added to monosaccharides (Fuc, Gal, Glc, Man, GlcNAc, GalNAc, 1–1000 pmol). The mixture was heated at 90 °C for 20 min by PALSTATION, and the excess reagents were removed by evaporation under a stream of nitrogen gas at 60 °C for 20 min. Then 10 μl of a reducing reagent, prepared just before use by mixing 6 mg of borane–dimethylamine complex and 100 μl of acetic acid, was added, and the mixture was heated at 90 °C for 35 min. The reaction mixture was dried three times under a stream of nitrogen gas at 50 °C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of isotope analogs, the tetradeuterium-labeled PA (d_4-PA) monosaccharide, d_0-AP was just replaced by d_6-AP (Fig. 1).

2.3. Monosaccharide composition analysis of a glycoprotein

A glycoprotein (25 pmol) was placed in a hydrolysis tube fitted with a Teflon-lined screw cap. Fifty microliters of 2 M HCl-2M trifluoroacetic acid (TFA) was added to the sample, which was then heated at 100 °C for 6 h. Simultaneously, a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analytes. The solution obtained was freeze-dried. The monosaccharides obtained from the analyte glycoproteins and standard monosaccharides were tagged with non-deuterium-labeled 2-aminopyridine (d_0-AP) and d_6-AP, respectively. Each tagged oligosaccharide mixture was dissolved into purified water, and a mixture of d_0- and d_4-PA monosaccharides was injected into the GCC-LC/MS.

2.4. Preparation of N-linked oligosaccharides

N-linked oligosaccharides were released from hCG as described previously [17]. Briefly, hCG and rhCG (100 μg)

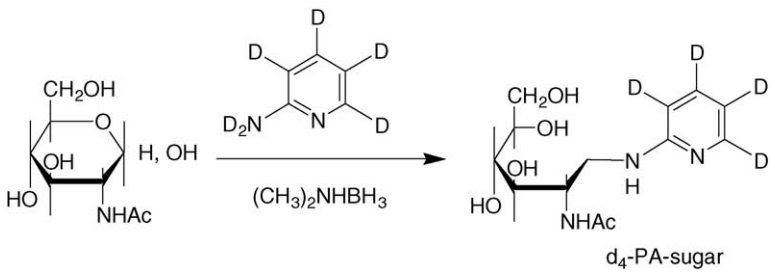


Fig. 1. Synthesis of d_4-PA monosaccharide internal standard.

were dissolved in 360 μl of 0.5 M Tris–HCl buffer (pH 8.6), containing 8 M guanidine hydrochloride and 5 mM ethylenediaminetetra-acetic acid (EDTA). After an addition of 2.6 μl of 2-mercaptethanol, the mixture was allowed to stand at room temperature for 2 h. To this solution, 7.56 mg of monoiodoacetic acid was added, and the resulting mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was applied to a PD-10 column (Amershambioscience, Uppsala, Sweden) to remove the reagents, and the eluate was lyophilized.

Carboxymethylated hCG and rhCG were dissolved in 100 μl of 0.1 M sodium phosphate buffer, pH 7.2, and incubated with 5 units of PNGase F at 37 °C for 2 days. Protein was precipitated with 340 μl of cold ethanol, and the supernatant was dried.

2.5. Pyridylamination of oligosaccharides from hCG

To the lyophilized oligosaccharides released from rhCG we added 10 μl of coupling reagent prepared by mixing 300 mg of d_0-AP, and 100 μl of acetic acid, and the reaction mixture was heated at 90 °C for 60 min. Then, 10 μl of a reducing reagent, prepared just before use by mixing 20 mg of borane–dimethylamine complex and 100 μl of acetic acid, was added, and the mixture was heated at 80 °C for 60 min. The reaction mixture was dried three times under a stream of nitrogen gas at 60 °C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of the tetradeuterium-labeled (d_4)-PA oligosaccharide isotope analogs, d_0-AP was just replaced by d_6-2-aminopyridine.

2.6. LC/MS analysis

LC was carried out using a Magic 2002 HPLC system (Michrom BioResources Inc., Auburn, CA, USA) using a Hypercarb column (0.2 mm × 150 mm, Thermoelectron, San Jose, CA, USA). The flow rate was set at 2–3 μl/min through a splitter system. The mobile phases were 5 mM ammonium acetate (pH 8.5) with 2% of acetonitrile (pump A) and 80% of acetonitrile (pump B). A gradient of 10–35% of B in 60 min was used for the monosaccharide analysis. For oligosaccharide profiling, we used a gradient of 5–20% of B in 20 min, 20–70% of B in 15 min, and 70–95% of B in 5 min. The mass spectrometer used was a TSQ 7000 (Thermoelectron) equipped with a nanoelectrospray ion source (AMR Inc., Tokyo, Japan). The ESI voltage was set to 2000 V (positive ion mode) or 1500 V (negative ion mode), and the capillary temperature was 175 °C.

3. Results

3.1. Monosaccharide composition analysis using the isotope tag method

First, we examined the possibility of the isotope-tag method for the monosaccharide composition analysis of gly-

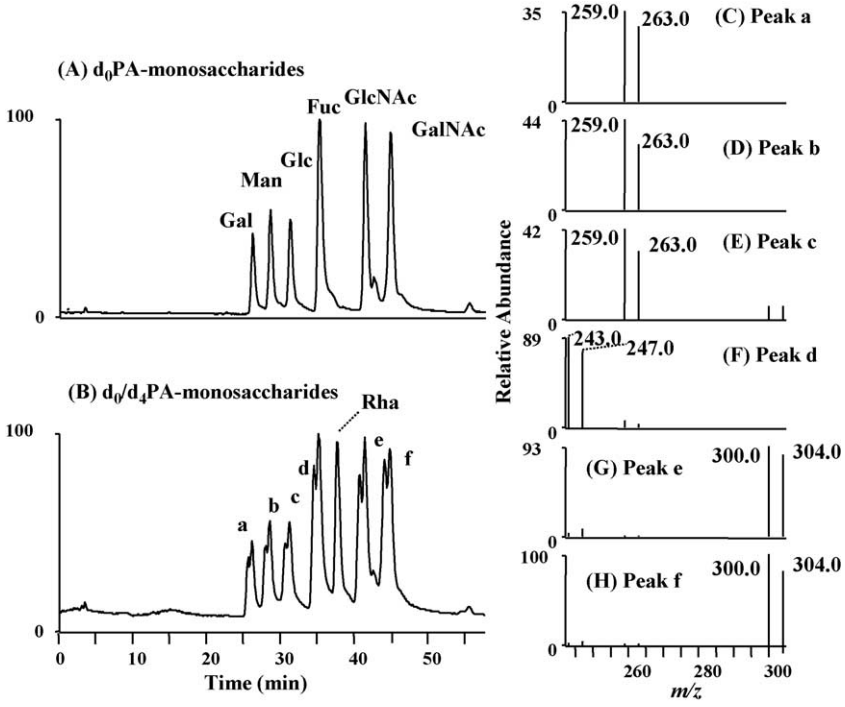


Fig. 2. (A) Extracted ion chromatogram (set *m*/*z* values, 243, 259, and 300) of d_0-PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, GlcNAc, and GalNAc). (B) Extracted ion chromatogram (set *m*/*z* values, 243, 247, 259, 263, 300, and 304) of a mixture of d_0- and d_4-PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, Rham, GlcNAc and GalNAc). (C) Mass spectra of peaks a (C), b (D), c (E), d (F), e (G), and f (H).

coproteins. An equal molar of each d_0-PA monosaccharide (Gal, Man, Glc, Fuc, GlcNAc, and GalNAc, 1 pmol each) was analyzed by GCC-LC/MS in the positive ion mode. The ions monitored were *m*/*z* 259 (for d_0-PA-Gal, d_0-PA-Man, and d_0-PA-Glc), *m*/*z* 243 (d_0-PA-Fuc), and *m*/*z* 300 (d_0-PA-GlcNAc and d_0-PA-GalNAc). Fig. 2A shows the mass chromatogram of the d_0-PA monosaccharides. All six d_0-PA monosaccharides were retained and separated by GCC. The detection limit at a signal-to-noise ratio of 3 was 45 fmol.

The d_4-PA monosaccharides were prepared as internal standards by tagging of standard monosaccharides with d_6-AP and combined with d_0-PA monosaccharides. Fig. 2B shows the chromatogram of a mixture of d_0-, d_4-PA monosaccharides and PA-labeled Rhamnose, which is frequently used as an internal standard in the monosaccharide composition analysis. Paired ions with a difference of *m*/*z* 4 were detected in the mass spectra of peaks a–f (Fig. 2C–H). When 0.5 pmol d_0-PA monosaccharides were determined in the presence of d_4-PA monosaccharides or Rhamnose by GCC-LC/MS, the relative standard deviation ($n = 5$) was 1.8–4.8% or 5.6–8.3%, respectively.

To assess the linearity and reproducibility of the whole procedure, including reacetylation, pyridylamination, the removal of excess derivatization reagents, and GCC-LC/MS, we tagged different amounts of monosaccharides (Gal, Man, Glc, Fuc, GlcN, and GalN, 1–1000 pmol) with d_0-AP, and d_4-PA monosaccharides (4 or 20 pmol) were added to the d_0-PA monosaccharides (1–10 pmol or 10–1000 pmol, respectively). The whole process of the isotope tag method was found to be linear for all six monosaccharides over the tested range of 1–1000 pmol (Fig. 3). The accuracy of this method was approximately 80–100% (Fig. 3), and the relative standard deviations (%R.S.D.) were less than 7.2% for all monosaccharides (based on the peak area ratio of monosaccharides from five samples).

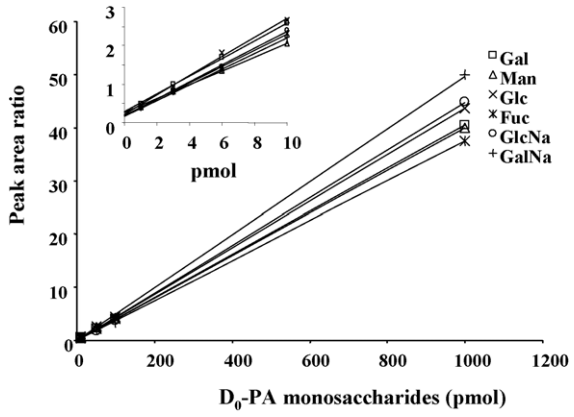


Fig. 3. Linearity on the isotope-tag method for monosaccharide quantification. For the internal standards, 1000 pmol monosaccharides were derivatized to d_4-PA monosaccharides. Different amounts of monosaccharides were derivatized to d_0-PA monosaccharides and co-injected with 4 pmol (A) or 20 pmol (B) internal standards into GCC-LC/MS.

We used this method for the monosaccharide composition analysis of fetuin and erythropoietin. Accuracy in the monosaccharide composition analysis of a glycoprotein relies on the condition of hydrolysis. Fan et al. [18] studied the hydrolysis of N-linked oligosaccharides and recommended 4 h with 2 M TFA at 100 °C for neutral sugars, and 6 h with 4 M HCl at 100 °C for amino sugars. While these hydrolysis conditions result in the complete release of neutral and amino sugars with no degradation, it takes two hydrolyses for a single sample. To quantify both neutral and amino sugars in glycoproteins in the same run, fetuin and erythropoietin (25 pmol) were heated in 2 M HCl-2M TFA at 100 °C for 6 h [19], and a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analyte glycoproteins. After hydrolysis, the analyte and standard monosaccharides were tagged with d_0- and d_6-AP, respectively. Fig. 4A and E show the mass chromatogram of monosaccharides prepared from fetuin and erythropoietin in the presence of d_4-PA monosaccharides, respectively.

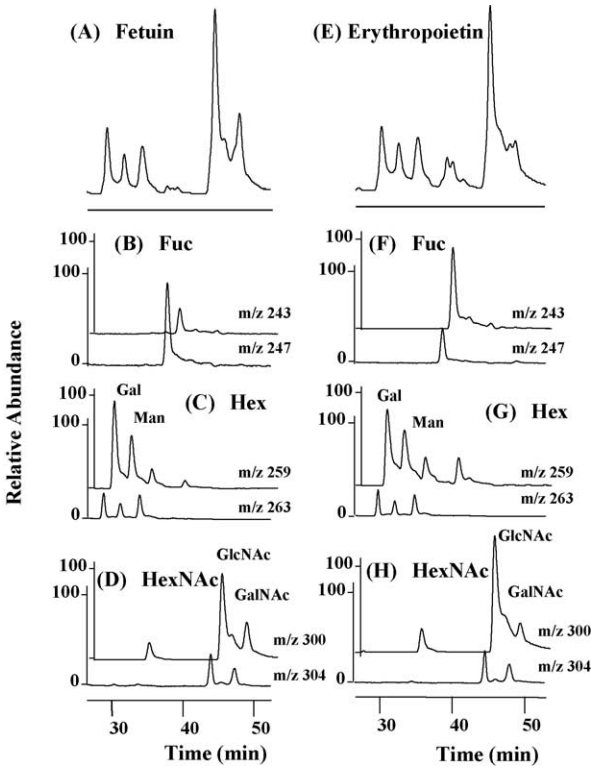


Fig. 4. Monosaccharide composition analysis of glycoproteins. Extracted ion chromatograms of d_0-PA monosaccharides from fetuin and d_4-PA standard monosaccharides (set *m*/*z* values, 243, 247, 259, 263, 300, and 304) (A), d_0-PA Fuc from fetuin and d_4-PA standard Fuc (set *m*/*z* values, 243 and 247) (B), d_0-PA Hex from fetuin and d_4-PA standard Hex (set *m*/*z* values, 259 and 263) (C), and d_0-PA HexNAc from fetuin and d_4-PA standard HexNAc and (set *m*/*z* values, 300 and 304) (D). Extracted ion chromatograms of d_0-PA monosaccharides from erythropoietin and d_4-PA standard monosaccharides and (set *m*/*z* values, 243, 247, 259, 263, 300, and 304) (E), d_0-PA Fuc from erythropoietin and d_4-PA standard Fuc (set *m*/*z* values, 243 and 247) (F), d_0-PA Hex from erythropoietin and d_4-PA standard Hex (set *m*/*z* values, 259 and 263) (G), and d_0-PA HexNAc from erythropoietin and d_4-PA standard HexNAc (set *m*/*z* values, 300 and 304) (H).

Table 1
Monosaccharide composition analysis by isotope-tag method

Glycoprotein	Monosaccharide	mol/mol[a]	mol/mol
Fetuin	Fuc	0.3	0 [20]
	Gal	10.4	12
	Man	7.6	9
	GlcNAc	14.7	15
	GalNAc	3.4	3
Erythropoietin	Fuc	3.4	4.1 [21]
	Gal	12.8	13.8
	Man	8.1	8.7
	GlcNAc	15.6	17.2
	GalNAc	1.5	0.9

[a] Values were expressed as mol detected in 1 mol glycoprotein.

Fig. 4B, and F show the mass chromatograms of d_0-, and d_4-PA fucose, Fig. 4C and G indicate those of d_0-, d_4-PA hexose, and Fig. 4D and H show those of d_0-, d_4-PA HexNAc. The monosaccharide compositions of fetuin and erythropoietin calculated from the peak area ratios (d_0-PA/d_4-PA monosaccharides) were in good agreement with the reported values (Table 1) [20,21]. By heating the standard monosaccharides simultaneously the decomposition of monosacchrides during hydrolysis can be corrected, and a use of isotope analogs as the internal standards can reduce deviation in ESIMS analysis.

3.2. Quantitative oligosaccharide profiling using the isotope tag method

Next, we explored the capability of the isotope-tag method for the quantitative oligosaccharide profiling. When d_0-PA oligosaccharides prepared from an analyte glycoprotein are analyzed with an equal part of d_4-PA oligosaccharides prepared from a standard glycoprotein, oligosaccharides which link to both the analyte and the standard glycoproteins are expected to appear as paired ions with a difference of 4 Da, and the individual oligosaccharides in the analyte glycoprotein can be quantified based on the analyte/internal standard ion-pair intensity ratio. On the other hand, any oligosaccharides that link to either the analyte or the standard glycoprotein ought to be detected as single ions. Oligosaccharides released from rhCG and hCG were tagged with d_0- and d_6-AP, respectively, and the tagged oligosaccharides were analyzed by GCC-LC/MS in both positive and negative ion modes.

Fig. 5A and B show the mass spectra of the peak which was detected at 21.5 min in the positive and the negative ion mode, respectively. In the positive ion mode, ions at *m/z* 863.0, 1359.4 and 1197.2 were detected (Fig. 5A), and they can be assigned to d_4-PA $[Hex]_5[HexNAc]_4^{2+}$ (an asialobiantennary oligosaccharide), d_4-PA$[Hex]_3[HexNAc]_4^{+}$ (a fragment of the asialobiantennary form) and d_4-PA$[Hex]_4[HexNAc]_4^{+}$ (a fragment of the asialobiantennary form), respectively. In contrast, only an ion at *m/z* 860.9 (d_4-PA$[Hex]_5[HexNAc]_4^{2-}$, asialobiantennary oligosaccharide) was detected in the negative ion mode (Fig. 5B). This result suggests that mass spectra

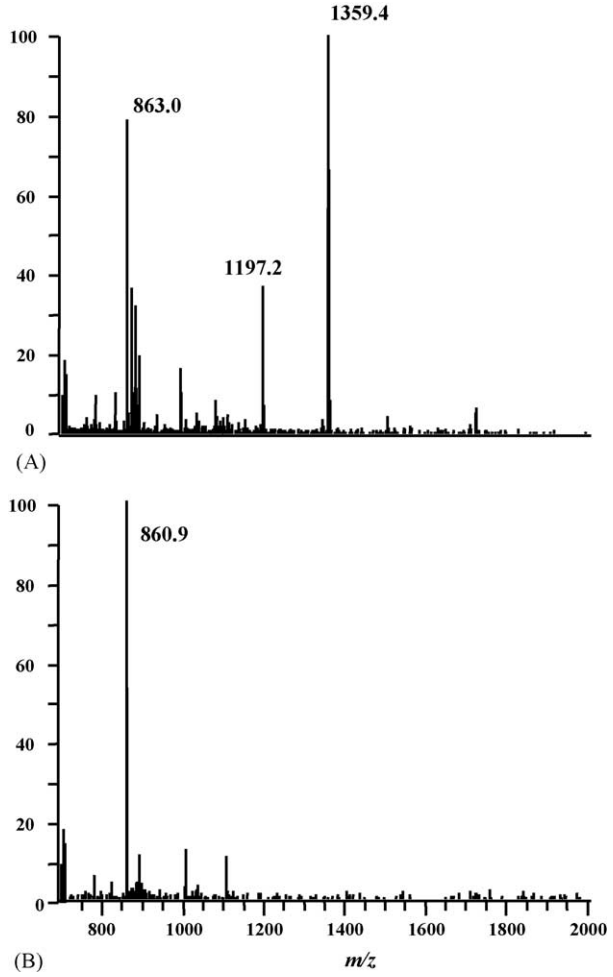


Fig. 5. Mass spectra of d_4-PA oligosaccharide. D_4-PA oligosaccharide eluted at 21.5 min from GCC was analyzed by ESIMS in the positive ion mode (A) and negative ion mode (B).

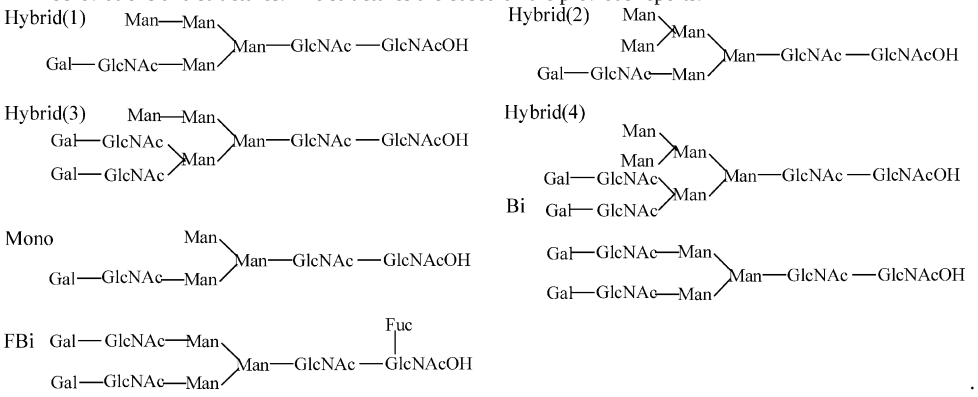
of PA oligosaccharides become complicated due to fragmentation in the positive ion mode, while only molecular ions can be detected in the negative ion mode. Therefore, ESI analysis in the negative ion mode was chosen for the PA oligosaccharide profiling.

Fig. 6A and B show the TIC of a mixture of equal parts of d_0-PA oligosaccharides prepared from rhCG and d_4-PA oligosaccharides from hCG, and its two-dimensional display (retention time versus *m/z*), respectively. The carbohydrate structures, which can be deduced from *m/z* values, are indicated in Table 2. Paired ions at *m/z* 757.5, 759.5 were observed in the mass spectrum of peak a1. Based on carbohydrate composition $[Hex]_5[HexNAc]_3$, it can be assigned to a hybrid type oligosaccharide. Likewise, peak l1, l2, l4, l5, p1, p2, and p4 consisted of paired ions and can be assigned to monosialylated (l1, l2, l4, l5) and disialylated (p1, p2) biantennary oligosaccharide without Fuc. Fig. 7 shows TIC of d_0-, d_4-PA oligosaccharides (A), extracted ion chromatograms of d_0-PA (B), d_4-PA (C), and d_0-, d_4-PA monosialylated biantennary form (D). The mass spectra of peaks l1–l5 are shown in Fig. 7E–I. Peak l3 was not observed in Fig. 7D and only

Table 2
Structural assignment of peaks in Fig. 6B

Peak nos.	Carbohydrate composition[a]	Deduced structure[b]	Theoretical mass (d_0-PA-sugar)	Observed *m/z*			Ion-pair intensity ratio d_0/d_4
				d_0-PA-rhCG	d_4-PA-hCG		
				M^{2-}	M^{3-}	M^{2-}	
a1	$[Hex]_5[HexNAc]_3$	Hybrid (1)	1517.5	757.5		759.5	0.27
b1	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	Bi + NA_2	2303.1		768.2		
c1	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_2$	FBi + NA_2	2449.3		816.7		
d1	$[Hex]_4[HexNAc]_3[NeuNAc]_1$	Mono + NA	1646.6			824.3	
d2	$[Hex]_4[HexNAc]_3[NeuNAc]_1$	Mono + NA	1646.6			824.0	
e1	$[Hex]_6[HexNAc]_3$	Hybrid (2)	1679.6	838.6			
e2	$[Hex]_6[HexNAc]_3$	Hybrid (2)	1679.6			840.6	
f1	$[Hex]_5[HexNAc]_4$	Bi	1720.7	858.9			
f2	$[Hex]_5[HexNAc]_4$	Bi	1720.7			861.2	
g1	$[Hex]_5[HexNAc]_3[NeuNAc]_1$	Hybrid (1) + NA	1807.7	902.9			
g2	$[Hex]_5[HexNAc]_3[NeuNAc]_1$	Hybrid (1) + NA	1808.7			905.0	
h1	$[Fuc]_1[Hex]_5[HexNAc]_4$	FBi	1866.8			934.0	
i1	$[Hex]_6[HexNAc]_4$	Hybrid (3)	1882.8	940.2			
j1	$[Hex]_5[HexNAc]_5$	Bi + GN	1924.9			962.7	
k1	$[Hex]_6[HexNAc]_3[NeuNAc]_1$	Hybrid (2) + NA	1970.8			986.8	
k2	$[Hex]_6[HexNAc]_3[NeuNAc]_1$	Hybrid (2) + NA	1970.8			986.2	
l1	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi + NA	2011.9	1004.7		1006.7	0.77
l2	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi + NA	2011.9	1004.6		1007.3	0.56
l3	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi + NA	2011.9	1004.6			
l4	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi + NA	2011.9	1004.6		1006.5	0.67
l5	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi + NA	2011.9	1004.6		1006.4	0.49
m1	$[Hex]_7[HexNAc]_4$	Hybrid (4)	2044.9	1021.4			
n1	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi + NA	2158.0			1079.8	
n2	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi + NA	2158.0			1079.8	
n3	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi + NA	2158.0			1079.8	
o1	$[Hex]_6[HexNAc]_4[NeuNAc]_1$	Hybrid (3) + NA	2174.0	1085.6			
o2	$[Hex]_6[HexNAc]_4[NeuNAc]_1$	Hybrid (3) + NA	2174.0	1085.7			
p1	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	Bi + NA_2	2303.1	1150.3		1152.1	5.76
p2	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	Bi + NA_2	2303.1	1150.2		1152.2	5.92
p3	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	Bi + NA_2	2303.1	1150.1			
p4	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	Bi + NA_2	2303.1	1150.3		1152.4	0.45

[a] Hex, hexsose; HexNAc, *N*-acetyl hexsosamine; NeuNAc, *N*-acetyl neuraminic acid; Fuc, fucose.
[b] Abbreviations and structures. The structures are based on the previous reports.



single ion was detected in Fig. 7G. These results suggest that one of monosialylated binantenary oligosaccharides isomers links to only rhCG.

We determined relative amounts of some oligosaccharides in rhCG on the basis of ion-pair intensity ratios (Table 2). The amount of monosialylated biantennary forms (l1, l2, l4, and l5) linked to rhCG were 50–70% of those to hCG. The amount of disialylated biantennary forms (p1 and p2) linked to rhCG was five-fold of those to hCG, and the linkage of p4 to rhCG was one-half of that of hCG. The isotope tag method clearly shows the difference in distribution of isomers between rhCG and hCG.

In this procedure, oligosaccharides linked to either rhCG or hCG were detected as single ions. As shown in Table 2, nine oligosaccharides were detected as single ions in rhCG, and they are reduced to hybrid type and complex type.

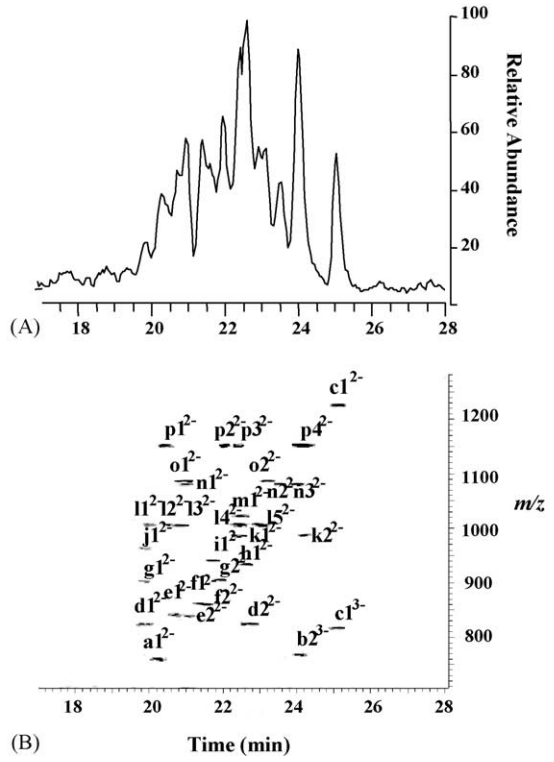


Fig. 6. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A), and its 2D display (B). Oligosaccharides (from 2 μg rhCG and hCG) were analyzed by GCC-LC/MS in the negative ion mode.

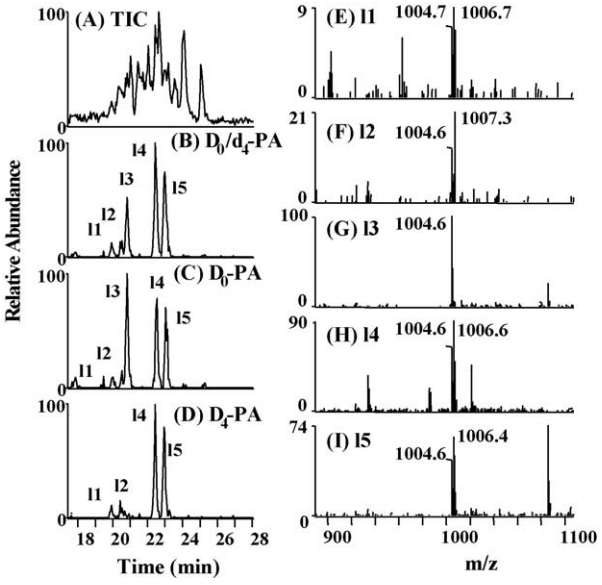


Fig. 7. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A). Extracted ion chromatograms of d_0-, and d_4-PA monosialylated biantennary (set *m*/*z* values, 1004–1007) (B), d_0-PA monosialylated biantennary (set *m*/*z* values, 1004–1005) (C), and d_4-PA monosialylated biantennary oligosaccharides (set *m*/*z* values, 1006–1007) (D). Mass spectra of peak l1–l5 (E–I).

Fourteen oligosaccharides were detected only in hCG, and most of them were fucosylated complex type. These results show the differences in glycosylation between rhCG and hCG and suggest that many hybrid type oligosaccharides linked to rhCG, while fucosylated oligosaccharides attach to hCG.

4. Discussion

Alteration of glycosylation is known to cause many changes in the biological activity as well as the physical properties of proteins. Several procedures of oligosaccharide profiling have been reported for the assessment of alteration of glycosylation, however, most of them can be used for only either qualitative or quantitative analysis. Although mass spectrometric oligosaccharide profiling is useful for the qualitative analysis, it has a problem on precision, and some isomers are still indistinguishable if their retention times are closed to others. In this study, we demonstrated that the use of isotope-tagged internal standards and GCC-LC/MS made it possible to do both quantitative and qualitative carbohydrate analysis.

First, we demonstrated the monosaccharide composition analysis using the isotope tag method. The use of internal standards that were heated under the same hydrolysis condition as an analyte glycoprotein resulted in good precision and accuracy in the monosaccharide composition analysis. Several HPLC methods for determination of monosaccharides have been reported. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been widely used for monosaccharide composition analysis [20,22–25]. Although HPAEC-PAD gives high resolution of all common monosaccharides and has the advantage of not requiring derivatization, this method is also known to have a disadvantage of limited selectivity [26]. The isotope tag method with SIM mode is equal to the HPAEC-PAD in sensitivity and is better than it in selectivity.

Next, we demonstrated the potentiality of the isotope tag method for quantitative oligosaccharide profiling using rhCG and hCG as model glycoproteins. hCG consists of an α subunit (MW 14.7 kDa) and a β subunit (MW 23.0 kDa), and oligosaccharides link to Asn52, and 78 in the α subunit and Asn13 and 30 in the β subunit. It has been reported that the majority of N-linked oligosaccharides in rhCG and hCG are fucosylated or non-fucosylated di-, tri-, and tetra-antennary forms with a various level of sialylation [27–30]. We prepared d_0-PA oligosaccharides and d_4-PA oligosaccharides from rhCG and hCG, respectively, and an equal part of d_0-PA and d_4-PA oligosaccharides was injected into LC/MS. We demonstrated that the oligosaccharides existing in one side protein were detected as single ions, whereas common oligosaccharides were detected as paired ions. We could easily realize that monosialo-, and disialobiantennary oligosaccharides linked to both hCG and rhCG, while fucosylated oligosaccharides and some hybrid type oligosaccharides linked to only hCG and rhCG, respectively. In addition, we demonstrated the pos-

sibility of the quantitative comparison the oligosaccharides between two quite similar glycoproteins. This quantitative oligosaccharide profiling is expected to be a powerful tool in various stages, including quality control and comparability assessment of glycoprotein products, and elucidation of glycan alteration in some diseases.

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