Identifying Specific and Differentially Linked Glycosyl Residues in Mammalian Glycans by Targeted LC-MS Analysis

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Abstract

Glycans, which are widespread in nature, consist of a large number of monosaccharides linked via glycosidic bonds. Due to the complex nature of glycan structures on glycoproteins, assessing the configuration and position of the glycosidic linkages of a glycan is a subject of considerable interest. In this study, a method for accomplishing this using partially O-methylated alditols (PMAs) from glycans combined with LC-MS analysis is reported. N-glycans were first per-methylated with methyl iodide, and the levels of methylation were further confirmed by MALDI-TOF. PMAs were then produced via complete hydrolysis and reduction. PMAs derived from Fetuin N-glycan and Lewis^a antigen carbohydrates were successfully detected by LC-MS analysis. This analysis can be performed without the need for an additional derivatization step for GC analysis, and should be suitable for use in conjunction with a LC-MS-based analysis platform.

Keywords: Linkage analysis, Partially methylated alditol, LC-MS
Introduction

Protein glycosylation is one of the major post-translational modifications, occurs in roughly 50% of proteins in mammals. Glycosylation is formed at nitrogen or oxygen atoms on the peptide backbone, is regulated by enzymes and occurs at specific amino acids in proteins. Most glycans have complex structures, and are comprised of a large number of monosaccharides linked together by glycosidic bonds. The complexity is the result of various monosaccharides being linked in different sequences, linkage positions and branching. Protein glycosylation can be categorized as either N-linked glycosylation and O-linked glycosylation, resulting in glycans being attached to proteins via N-linkages and O-linkages respectively. Protein glycosylation has very important functions and plays significant roles in numerous biological mechanisms and pathways, including fertilization, immune response, inflammation, viral replication, cell growth, cell–cell adhesion, and glycoproteins clearance.

The analysis of N-linked glycans on glycoproteins usually involves the use of a PNGase enzyme F. The N-linked glycan released from the protein when treated with PNGase F, is then reduced with NaBH₄, followed by methylation of the hydroxyl and amine groups of the glycan. The reducing form of a methylated N-glycan can be further hydrolyzed to monosaccharides by cleaving the glycosidic linkages with concentrated acid. Monosaccharides can be characterized by total methylation and gas chromatography (GC). In order to use GC, hydrophilic monosaccharides need to be derivatized by trimethylsilylation or acetylation. GC-MS analyses of derivatized monosaccharides usually allows the excellent chromatographic resolution of monosaccharides, thus providing structural information for identification.

Partially methylated alditol acetates (PMAA) was first introduced by Björndal, et al. for the analysis of glycosidic linkages. In this procedure, free hydroxyl groups are completely methylated on the glycan with the glycosidic linkages remaining intact. The resulting methylated
glycan is then further hydrolyzed to monosaccharides, which are then converted to PMAA by reduction and acetylation. The resulting PMAA can be easily analyzed and unambiguously identified by GC-EI-MS.\textsuperscript{10}

In this study, we propose an alternative approach using an LC-ESI-MS platform to facilitate the use of conventional GC-MS. The method eliminates the tedious derivatization step generally required by GC analysis and permits specific and differentially linked glycosyl residues to be determined.

**Experimental**

**Reagents and chemicals**

A Lewis\textsuperscript{a} standard was obtained from ELICITYL (OligoTech\textsuperscript{b}; Crolles, France). Sodium borohydride, sodium hydroxide, methyl iodide, 2,5-dihydroxybenzoic, pyridine and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water (Milli-Q) was manufactured from Millipore system (Merk Millipore, Burlington, MA). Acetonitrile (ACN) and methanol (Merk Millipore, Burlington, MA) were of HPLC grade, and trifluoroacetic acid (J.T. Baker\textsuperscript{b}; Avantor, Center Valley, PA) was of ACS grade. The reduced glycans were fractionated by means of a Dowex 50W-X8 cartridge (BIO-RAD, Hercules, CA).

**Samples**

Fetuin from fetal calf serum was obtained from Sigma-Aldrich (St. Louis, MO).

**Reduction, Per-methylation, Glycan hydrolysis and Acetylation**

Fifty microgram of Lewis\textsuperscript{a} standard and 1.3 mg of fetuin were weighted, and PNGase was used to release N-glycan from these two glycoproteins. Reduction is accomplished with sodium borohydride (10 mg NaBH\textsubscript{4} in 1 mL 2 M NH\textsubscript{3}) in an aqueous solution. This process can be completed within three hours at room temperature at 37°C. The reduced glycans are fractionated
by means of a Dowex 50W-X8 (BIO-RAD, Hercules, CA) cartridge. The residual borane is removed by the repeated evaporation of methanol using a centrifugal vacuum.

All samples were per-methylated in glass tubes with Teflon-lined screw caps containing NaOH/dimethyl sulfoxide (DMSO) slurry. Five pellets of sodium hydroxide were placed in a dry mortar and approximately 3 mL of dry DMSO added. The pellets were then ground to a slurry. Roughly 0.5-1.0 mL of the DMSO-NaOH slurry was then added to the sample. Methyl iodide (0.5 mL) was added and the suspension mixed vigorously. The reaction mixture was placed on an automatic shaker for 10 min at room temperature. The reaction was quenched by the dropwise addition of 1 mL of water. Two mL of chloroform was then added, the solution mixed thoroughly and then allowed mixture to settle into two layers. The upper aqueous layer was removed, and the lower chloroform layer was washed several times with water.

Two hundred μL of 2 M TFA was added to the per-methylated glycan, and the resulting sample incubated for two hours at 110°C, after which it was dried with a stream of nitrogen.

The PMA was acetylated by treatment with 1:1 Acetic anhydride-pyridine (500 μL), 2 hr at 100°C. The PMAAs were extracted with CHCl₃ and the resulting solution with H₂O, and the organic layer containing PMAAs was dried at room temperature.

**Analysis of a permethylated glycan by MALDI-TOF MS**

Per-methylated glycans (roughly 1.25 nmole from Lewisα and 0.11 nmole from fetuin) were profiled on SCIEX TOF/TOF™ 5800. The per-methyl derivatives in ACN were mixed 1:1 with a 2,5-dihydroxybenzoic acid matrix (10 mg/mL in ACN), spotted on the target plate, air-dried, and recrystallized on-plate with ACN. Data acquisition in the reflectron mode was performed manually. The coarse laser energy control was set at high and finely adjusted using a % slider according to sample amount and spectral quality. Laser shots (5 Hz, 10 shots/spectrum) were accumulated until a satisfactory S/N was achieved.

**Analysis of the partially methylated alditols by LC-MS**
Partially methylated alditols were analyzed by an ACQUITY UPLC (Waters, Milford, MA) interfaced with a ESI Orbitrap mass spectrometer (Orbitrap ELITE, Thermo Fisher Scientific). PMA from per-methylated Lewis\(^a\) 243.9 μM in 0.1% FA in 2% ACN, PMA from per-methylated fetuin N-glycan 65.03 μM in 0.1% FA in 2% ACN. Samples were injected on a C18 capillary column (nanoACQUITY UPLC Symmetry C18 Trap Column, 5 μm particle size, 100Å pore size, 180 μm i. d. × 20 mm L, Waters, Milford, MA) and the PMA was separated using a gradient of 0–80% (v/v) 0.1% FA in ACN for 55 min at a constant flow rate of 0.4 μL/min. The sample injection volume was 2 μL. The acquisition range was \(m/z\) 200-500. Acquisition was performed in positive ionization mode. Mass spectra were viewed and analyzed using Thermos Xcalibur 3.0 (Thermo Fisher Scientific).

**Results and Discussion**

The overall scheme for this study is illustrated in Figure 1. The glycoprotein (e. g. fetuin) was treated with PNGase F to release the N-glycans, which were then further reduced with NaBH\(_4\) and permethylated using methyl iodide. These permethylated glycan products were confirmed by MALDI-TOF analysis. The partially methylated monosaccharides were generated from permethylated glycan by acidic hydrolysis. The partially methylated alditols are then produced by reducing the partially methylated monosaccharides with sodium borohydride.

The degrees of methylation will depend on number of glycosidic linkages on the monosaccharides, which will result in differences in their mass (monitored by XIC of LC-MS). By matching the calculated \(m/z\) values of possible partially methylated alditols with the observed permits ones to specifically predict and identify the various linkages for the monosaccharides.

The methylation of glycans is advantageous not only for the structure stabilization but also because it increases the hydrophobicity of the glycan, thus permitting it to be analyzed by reversed
phase liquid chromatography more easily. In this study, the completeness of glycan methylation was investigated by MALDI-TOF analysis. The spectrum of a methylated Lewis\textsuperscript{a} is shown in Figure S1A. The peak at \( m/z \) 1290 indicates the mass of the protonated material, and the corresponding -14 Da signal represents its under-methylation counterpart where not all of the free hydroxyl groups had been methylated. This under-methylated product can be completely methylated using a second methylation process. The result for fetuin N-glycan methylation is also demonstrated in Figure S1B and the results are in good agreement with previously reported findings, indicating that the glycan methylation was efficient and complete.\textsuperscript{14}

**Analysis of PMAA**

Partially methylated alditol acetates (PMAA) are widely employed for the analysis of glycosidic linkages of a glycan, and is done on a GC-MS system. Since the signals of GlcNAc, Neu5Ac are relatively low in the case of GC-MS, the use of LC-MS for such an analysis was investigated. Two compounds, Lewis\textsuperscript{a} and fetuin N-glycan, were selected for the analysis and the corresponding calculated \( m/z \) values of the ionized PMAA analogs including \([\text{M+H}]^+\), \([\text{M+NH}_4]^+\), \([\text{M+Na}]^-\) are illustrated in the tables below.

The PMAA products from the Lewis\textsuperscript{a} carbohydrate were 1,2,5,6-Me\textsubscript{4}-glucitol acetate (from glucose at the reducing end), 2,3,4,6-Me\textsubscript{4}-galactitol acetate (from galactose at the non-reducing end), 2,3,4-Me\textsubscript{3}-fucitol acetate (from fucose), 2,4,6-Me\textsubscript{3}-galactitol acetate and 2,6-Me\textsubscript{2}-GlcNAcitol acetate (Table S1A). The extracted ion chromatogram (XIC) of the Lewis\textsuperscript{a} PMAAs, which is shown in Figure S2A, indicate that the peak areas of 2,3,4-Me\textsubscript{3}-fucitol acetate and 2,4,6-Me\textsubscript{3}-galactitol acetate were roughly 2 to 1 which is in consistent with the estimated molar ratio between these two products. The fact that the XIC intensity of 2,6-Me\textsubscript{2}-GlcNAcitol acetate was still relatively low can be attributed to the fact that the hydrolyzed GlcNAc had undergone partial degradation before the acetylation treatment.

The ESI mass spectra of the Lewis\textsuperscript{a} PMAAs with adduct ions is shown in Figure S2B. Both
[M+H]$^+$ and [M+Na]$^+$ ions were observed with [M+NH$_4$]$^+$, being the predominate ion. The only exception was GlcNAcitol acetate in which a higher [M+H]$^+$ signal was observed.

The PMAA products from fetuin were (OH)GlcNAcitol acetate, (OH)2GlcNAcitol acetate, (OH)3Hexitol acetate (from mannose), (OH)2Hexitol acetate, (OH)Hexitol acetate and (OH)Neu5Acitol acetate; their corresponding adduct ions are listed in Table S1B. The extracted ion chromatogram (XIC) of the fetuin PMAAs is shown in Figure S3A. The PMAAs were eluted within 35-40 min, while (OH)2GlcNAcitol acetate was retained longer than (OH)GlcNAcitol acetate since it was more extensively methylated.

The ESI mass spectra of fetuin PMAAs is shown in Figure S3B. Similar to the phenomenon observed for the Lewis$^a$, [M+NH$_4$]$^+$ was the predominated adduct ion with the exception of Neu5Acitol acetate in which the [M+H]$^+$ ion dominated. It can be concluded that these hydrolyzed PMAAs derived from glycans can be successfully detected and analyzed by LC-ESI MS; [M+H]$^+$ ions was generated for HexNAc and the majority of [M+NH$_4$]$^+$ adduct ions were produced from hexoses.

Analysis PMA

Partially methylated alditols (PMA) were produced as the result of the hydrolysis of the permethylated glycans. The hydroxyl groups on glycosidic linkages of glycan would be preserved and not methylated. The direct analysis of the PMA was investigated without the acetylation step that generates the PMAA.

The overall theoretical MW of the Lewis$^a$ PMAs with their associated adduct ions fare listed in Table 1A, including 1,2,5,6-Me4-glucitol, 2,3,4,6-Me4-galactitol (reducing ends), 2,3,4-Me3-Fucitol, 2,4,6-Me3-galactitol and 2,6-Me2-GlcNAcitol. The extracted ion chromatogram (XIC) of the Lewis$^a$ PMAs is shown in Figure 2A, the retention times were close because they contained similar hexose core structure. Since underivatized hydroxyl groups were present on the PMAs,
they eluted earlier than their PMAA counterparts.

The ESI mass spectra of the Lewis\(^a\) PMAs is shown in Figure 2B, the predominated adduct ions were \([\text{M}+\text{Na}]^+\), except for 2,6-Me2-GlcNAcitol for which an \([\text{M}+\text{H}]^+\) peak was observed although the signal was relatively weak. It has been reported that HexNAc produced during the hydrolysis of glycans tends to be degraded without derivatization.\(^{15}\) This assumes that only two methyl groups will be generated on GlcNAc from the Lewis\(^a\), which leads to a lower signal for HexNAc. In summary, the \([\text{M}+\text{Na}]^+\) ion was the predominate ion for hexose PMAs and \([\text{M}+\text{H}]^+\) ions was observed for the GlcNAc PMA.

The theoretical MW of fetuin N-glycan PMAs with their associated adduct ions is listed in Table 1B, including (OH)GlcNacitol (reducing end GlcNAc, 1,2,3,5,6-Me5-GlcNAcitol), (OH)2GlcNAcitol (2,3,6-Me3-GlcNAcitol), (OH)3Hexitol, (OH)2Hexitol, (OH)Hexitol, and (OH)Neu5Acitol. The (OH)3Hexitol PMA from fetuin contained a hexose with three hydroxyl groups from un-methylated glycosidic linkages, including mannose in the N-glycan core structure or mannose with a triantennary N-Glycan. This hydrophilic (OH)3Hexitol PMA was the least or non-retained component on the C18 column. (OH)2Hexitols represent hexoses that contained two hydroxyl groups, including two mannoses with an N-glycan core structure or galactose from glycans. This (OH)2Hexitol can be compared with 2,4,6-Me3-galactitol from the Lewis\(^a\) PMA, since they have an identical structure and the same MW with similar LC retention times. A similar phenomenon was also observed between (OH)Hexitol of the fetuin N-glycan PMA and the Me4-glucitol of the Lewis\(^a\). In addition, as shown in XIC, the (OH)2Hexitol eluted earlier than the (OH)Hexitol from the C18 column due to its degree of hydrophilicity (Figure 3A).

The ESI mass spectra of the fetuin N-glycan PMAs is shown in Figure 3B, in which the predominate ions were \([\text{M}+\text{Na}]^+\), similar to the ones observed for the Lewis\(^a\) sample. The lone difference was the PMA from the N-glycan end with a sialic acid, in which a \([\text{M}+\text{H}]^+\) peak was the most intense signal. For their adduct ions formed in ESI, the MS results indicate that
the majorities of PMAs from the Lewisα and fetuin N-glycans were [M+Na]+ ions, which is quite different from the [M+NH₄]+ ions observed for the PMAAs.¹⁶

Quantitation of the Lewisα PMA was also conducted with serial dilutions of the Lewisα sasmple with concentrations in the 100-0.1 ppm range. A linear regression of the three PMAs including 1,2,5,6-Me₄-glucitol, 2,3,4,6-Me₄-galactitol, 2,4,6-Me₃-galactitol and 2,3,4-Me₃-fucitol are provided in Table 2. The semi-quantitative analysis of PMAs can be successfully achieved using this methodology and would be useful for the quantitation of GlcNAc at the reducing end of N-glycan contents of glycoproteins.

Conclusions

In this study, monosaccharides with different glycosidic linkages were successfully analyzed by LC-MS. The derivatized PMAAs and PMAs from glycans can be specifically identified by means of a combination of extracted ion chromatography and high resolution mass spectrometry. The findings also indicate that [M+Na]+ peaks were the major adduct ions of ESI MS for the PMAs, and [M+NH₄]+ peaks were the predominated ions for PMAAs.

This study demonstrates a convenient LC-MS-based platform that can be used for the analysis of monosaccharide composition and linkages. It can further assist in the absolute and relative quantitation of glycans from an analysis of monosaccharides at the reducing end of the glycan chain. This LC-MS-based platform represents a complementary assay for the analysis of glycoproteins, glycans, monosaccharide position and carbohydrate branch linkages.

Acknowledgements

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Supporting Information

This Supporting Information is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

Supplemental figures and tables (PDF)

References

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### Table 1A  Calculated m/z values of partially methylated alditol (PMAs) from the Lewis\(^a\)

<table>
<thead>
<tr>
<th>PMA</th>
<th>([\text{M+H}]^+)</th>
<th>([\text{M+NH}_4]^+)</th>
<th>([\text{M+Na}]^+)</th>
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</thead>
<tbody>
<tr>
<td>1,2,5,6-Me4-glucitol</td>
<td>239.1416</td>
<td>256.1755</td>
<td>261.1309</td>
</tr>
<tr>
<td>2,3,4,6-Me4-galactitol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-Me3-fucitol</td>
<td>209.1389</td>
<td>226.1649</td>
<td>231.1203</td>
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<tr>
<td>2,4,6-Me3-galactitol</td>
<td>225.1338</td>
<td>242.1598</td>
<td>247.1152</td>
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<tr>
<td>2,6-Me2-GlcNAcitol</td>
<td>252.1447</td>
<td>269.1707</td>
<td>274.1261</td>
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</table>

### Table 1B  Calculated m/z values of partially methylated alditol (PMAs) from Fetuin N-glycans.

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<th>PMA</th>
<th>([\text{M+H}]^+)</th>
<th>([\text{M+NH}_4]^+)</th>
<th>([\text{M+Na}]^+)</th>
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</thead>
<tbody>
<tr>
<td>(OH)GlcNAcitol</td>
<td>294.1911</td>
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<td>(OH)2GlcNAcitol</td>
<td>266.1598</td>
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<td>(OH)3Hexitol</td>
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<td>(OH)2Hexitol</td>
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<td>(OH)Hexitol</td>
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<td>256.1755</td>
<td>261.1309</td>
</tr>
<tr>
<td>(OH)Neu5Acitol</td>
<td>396.2228</td>
<td>413.2494</td>
<td>418.2047</td>
</tr>
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</table>

### Table 2  Linear regression of three PMAs from the Lewis\(^a\)

<table>
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<tr>
<th>PMA</th>
<th>Linear range (ppm)</th>
<th>(R^2)</th>
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<tbody>
<tr>
<td>1,2,5,6-Me4-glucitol</td>
<td>0.1-100</td>
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<td>2,3,4,6-Me4-galactitol</td>
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<td></td>
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<tr>
<td>2,3,4-Me3-fucitol</td>
<td>0.1-100</td>
<td>0.9966</td>
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<tr>
<td>2,4,6-Me3-galactitol</td>
<td>0.1-100</td>
<td>0.9905</td>
</tr>
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</table>
Figure Captions

Fig. 1 Overall scheme for this study.
Fig. 2A  Extracted ion chromatogram (XIC) of Lewis\textsuperscript{a} PMAs.

(a) 1,2,5,6-Me\textsubscript{4}-glucitol & 2,3,4,6-Me\textsubscript{4}-galactitol  
(b) 2,3,4-Me\textsubscript{3}-fucitol  
(c) 2,4,6-Me\textsubscript{3}-galactitol  
(d) 2,6-Me\textsubscript{2}-GlcNAcitol
**Fig. 2B** ESI mass spectra of Lewis^a^ PMAs with adduct ions.

(a) 1,2,5,6-Me4-glucitol & 2,3,4,6-Me4-galactitol (b) 2,3,4-Me3-fucitol (c) 2,4,6-Me3-galactitol (d) 2,6-Me2-GlcNAcitol
**Fig. 3A** Extracted ion chromatogram (XIC) of fetuin N-glycan PMAs.

(a) (OH)GlcNAcitol (b) (OH)2GlcNAcitol (c) (OH)3Hexitol (d) (OH)2Hexitol (e) (OH)Hexitol
(f) (OH)Neu5Acitol
Fig. 3B  ESI mass spectra of fetuin N-glycan PMAs with adduct ions.

(a) (OH)GlcNAcitol (b) (OH)2GlcNAcitol (c) (OH)3Hexitol (d) (OH)2Hexitol (e) (OH)Hexitol

(f) (OH)Neu5Acitol
Graphical Index

1. NaBH₄ Reduction
2. Methyl iodide Permethylation
3. Hydrolysis, 2M TFA
4. NaBH₄ Reduction

Partially methylated alditols, PMA

LC-MS analysis