

An Ultra High Resolution Column and Mass Spectrometer for Isomeric Separation and the Structural Identification of Labeled N-linked Glycans

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Overview

Purpose: Separation and identification of various complex *N*-linked glycans using a novel high resolution mixed-mode column and an Orbitrap Fusion Tribrid mass spectrometer.

Methods: Fluorescently labeled glycans from various proteins were separated and analyzed on a mixed-mode column coupled to an Orbitrap Fusion MS. Data analysis was performed using SimGlycan software.

Results: The mixed-mode column coupled to Orbitrap Fusion enabled resolution of twice as many peaks and identification of four times as many 2AB-labeled *N*-linked glycans from bovine fetuin compared to commercially available column technologies.

Introduction

Various modes of HPLC separation have been developed for the analysis of glycans¹⁻³. One common separation mode utilizes amide HILIC columns, which separates glycans based on hydrogen bonding, resulting in a size and composition-based separation. Amide HILIC columns are useful for the separation of *N*-linked glycans released from antibodies, such as mAbs, where the majority of the glycans are neutral. However, these columns do not provide a good separation when glycans are highly charged (charge ≥ 2) such as sialylated *N*-linked glycans. Here we describe the new mixed-mode column (GlycanPac AXR-1) which provides separation based on isomeric structure along with separation based on charge, size, and polarity. This column is based on mixed-mode column chemistry, combining both WAX and RP retention mechanisms. The WAX functionality provides retention and selectivity for negatively charged glycans, while the RP mode facilitates the separation of glycans of the same charge according to their isomeric structure, polarity, and size. As a result, the GlycanPac AXR-1 column provides twice the resolution with more than four times the glycan structures identified compared to existing commercial columns for 2AB-labeled *N*-linked glycans released from bovine fetuin.

Methods

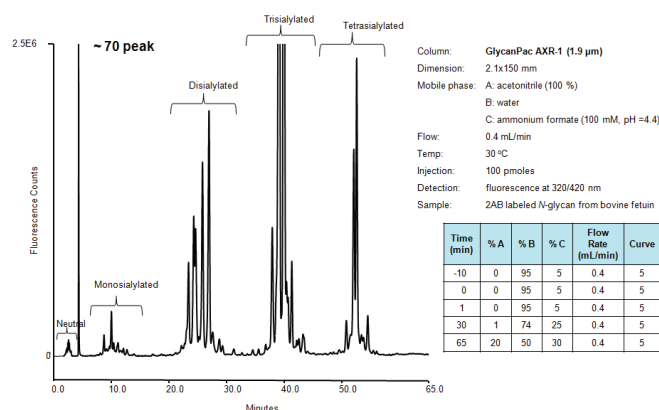
Glycans were released from glycoproteins with PNGase F enzyme (New England BioLabs). The released glycans were labeled with 2AB and 2AA. All glycans were separated on a GlycanPac AXR-1 column (1.9 μm , 2.1 \times 150 mm) column by a Thermo Scientific™ Dionex™ Ultimate™ 3000 UHPLC instrument with either a fluorescence or MS detector. MS analysis was performed using an Orbitrap Fusion MS in negative ion mode. SimGlycan® 4.5 software (PREMIER Biosoft) was used for MS/MS data analysis.

Results

The GlycanPac AXR-1 column is designed for high-resolution separation of neutral and charged glycans (native and labeled). However, it should be noted that for neutral glycans, such as those released from antibodies, it is advantageous to use 2AA-labeling technique to enhance retention as well as selectivity on the GlycanPac AXR-1 column. Figure 1 shows the separation of neutral and acidic 2AB-labeled *N*-linked glycan from bovine fetuin using a GlycanPac AXR-1 column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, followed by mon-, di-, tri-,

tetra- and pentasialylated species. Peaks in each cluster represent the glycans of the same charge separated by ion exchange interaction. Within each cluster, glycans containing the same charge are further separated according to their isomeric structures, sizes, and polarity by RP interaction. The GlycanPac AXR-1 column provides ≥ 70 resolved peaks with $\geq 1\%$ intensity for 2AB-labeled *N*-linked glycans from bovine fetuin.

FIGURE 1. Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using GlycanPac AXR-1 column .



LC-MS/MS Analysis of 2AB-Labeled *N*-linked Glycans from Bovine Fetuin Using GlycanPac AXR-1 Column

The coupling of the GlycanPac AXR-1 column to MS is particularly attractive because MS enables detailed structural information. 2AB-labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXR-1 column and analyzed on an Orbitrap Fusion MS. The LC-MS profile of the GlycanPac AXR-1 column showed the highest number of resolved peaks (≥ 70) for bovine fetuin glycans ever achieved (Figure 2), more than doubling the number the existing commercially available stationary phases can resolve. The commercially available HILIC amide column (1.7 μm , Figure 3) was only able to resolve 26 peaks. Most commercial stationary phases are poor for separating glycan structural isomers. A single LC peak using these columns can have many structural isomers. So in most instances mixed MS^2 spectrum are generated that contain fragment ions from multiple glycans making it extremely difficult to assign correct structures. The GlycanPac AXR-1 column can resolve structural isomers (Figure 4). The ability to resolve structural isomers introduces complexity to analysis. Namely, far more MS/MS^2 spectra need to be triggered in a single LC- MS^2 analysis. Additionally, wider dynamic range and sensitivity are needed from MS to detect and generate good quality MS^2 spectra not only for the most abundant glycans but the low abundant species as well (Figure 5). Orbitrap Fusion with its wide dynamic range and ultrahigh mass resolution of makes it the ideal platform for looking deeper into the glycome and confidently identifying low-abundance glycans. Overall, 135 unique glycan structures were identified.

FIGURE 2. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by GlycanPac AXR-1 column with MS detection.

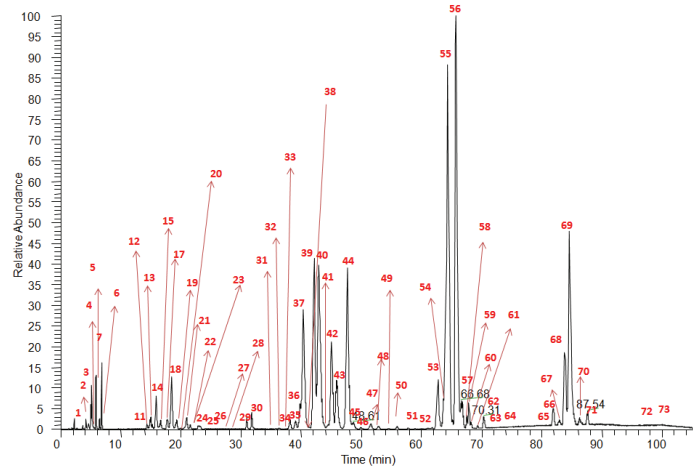


FIGURE 3. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by a commercial amide HILIC column (1.7 μ m) with MS detection.

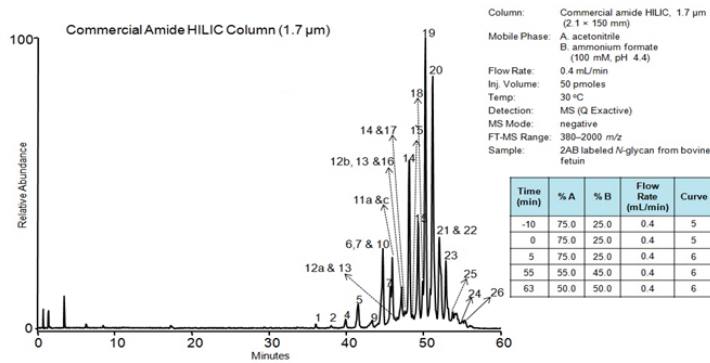


FIGURE 4. Separation of disialylated biantennary glycans from bovine fetuin based on α 2-3 and α 2-6 sialic acid linkage by the GlycanPac AXR-1 column.

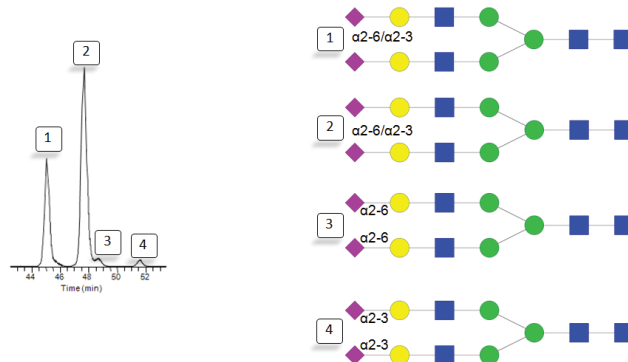
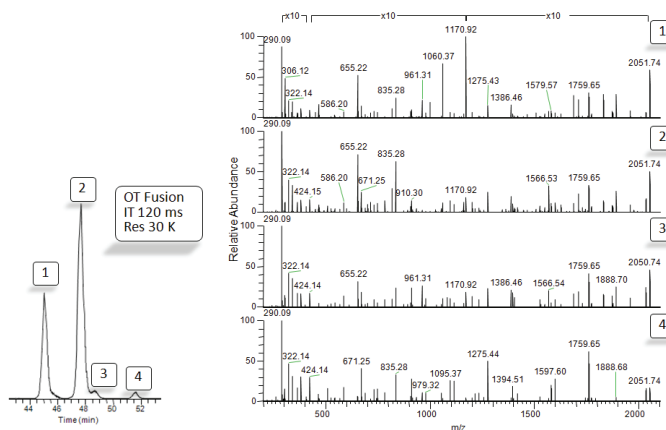


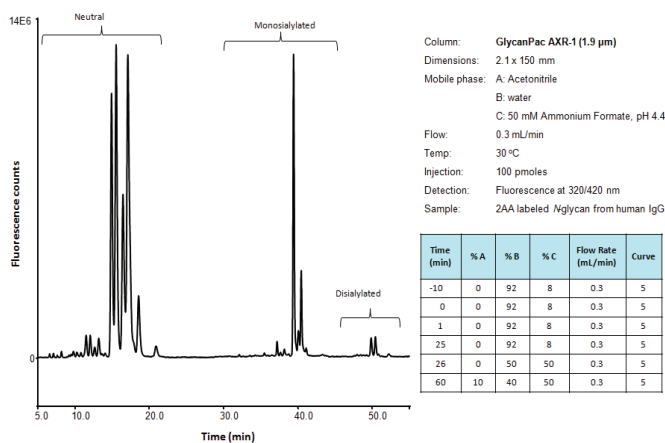
FIGURE 5. LC-MS/MS (HCD) spectra of 2AB-labeled *N*-linked glycans from bovine fetuin.



Analysis of 2AA-Labeled Antibody Glycans

Unlike 2AB, 2AA labeling introduces a formal negative charge to each glycan. This promotes greater binding to the GlycanPac AXR-1 column, thus improving retention of both neutral and negatively charged glycans. Figure 6 shows the separation of neutral and acidic 2AA-labeled *N*-Linked glycans from a human IgG using a GlycanPac AXR-1 (1.9 μ m, 2.1 x 150mm) column. As with the fetuin sample in the previous figures, the IgG-derived glycan elution profile consists of clusters of peaks in which the neutral glycans elute first, followed by monosialylated and disialylated forms. Analytes in each cluster represent the glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their isomerism and size by reversed-phase interactions. As shown in Figure 6, 2AA-labeled neutral glycans elute between 5 and 22 min, 2AA-labeled monosialylated glycans elute between 30 and 45 min and 2AA-labeled disialylated glycans elute between 45 and 55 min. More than 40 peaks are identified from the separation of 2AA-labeled *N*-glycans from this human IgG.

FIGURE 6. Separation of 2AA-labeled *N*-linked glycans from human IgG by charge, isomers, and size using a GlycanPac AXR-1 (1.9 μ m) column



Conclusion

- The GlycanPac AXR-1 column separates glycans based on charge, isomeric structure, size, and polarity, providing twice the number of resolved peaks and more than 4 times the glycan structures identified compared to existing commercial amide HILIC columns for 2AB-labeled *N*-linked glycans released from bovine fetuin.
- Faster Orbitrap enables higher scan rates at higher resolution. This translates to increased sensitivity and better quality MS/MS data for both abundant and low abundance glycans.

References

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