

An Ultra High Resolution Glycan Column for Isomeric Separation and the Structural Identification of Labeled *N*-Glycans from Proteins Including Antibodies

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Overview

Purpose: Separation and identification of various complex *N*-linked glycans from proteins 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 Thermo Scientific™ GlycanPac™ AXR-1 column coupled to a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ mass spectrometer. Data analysis was performed using SimGlycan® software.

Results: The GlycanPac AXR-1 column coupled to Orbitrap Fusion mass spectrometer enabled resolution of twice as many peaks and identification of four times as many structures for 2AB-labeled *N*-linked glycans from bovine fetuin compared to other commercially available column technologies. Furthermore, this column allows direct injection of aqueous samples.

Introduction

Glycans are involved in a wide range of biological and physiological processes including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development. The functions of glycans are often dependent on the structure and types of glycans attached to the proteins. *N*-linked glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are affected by glycosylation. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, the glycan content of glycoprotein products, as well as thorough characterization of biosimilars have become increasingly important. The structures of glycans are highly diverse, complex and heterogeneous due to post-translational modifications. Thus, it is challenging to comprehensively characterize glycan profiles and determine their structures [1].

Various modes of HPLC separation have been developed for the analysis of glycans [2-4]. Glycans are highly hydrophilic polar substances, and therefore one common separation mode utilizes amide hydrophilic interaction liquid chromatography (HILIC columns), which separates glycans based on hydrogen bonding, resulting in a size and composition-based separation. Amide HILIC columns are particularly useful for the separation of 2AA-labeled *N*-linked glycans released from antibodies, such as mAbs, where the majority of the glycans are neutral. However, amide HILIC amide columns do not provide a good separation when glycans are highly charged (charge ≥ 2) such as sialylated *N*-linked glycans. Here glycans of different charge states are intermingled in the separation envelope.

Recently, we have developed a novel mixed-mode column (GlycanPac AXH-1) with both weak anion-exchange (WAX) and HILIC properties [5], which separate *N*-linked glycans based on charge, size, and polarity. The GlycanPac AXH-1 column provides unique charge-based separation and broader applicability in term of qualitative and quantitative structural analysis of 2AB and 2AA labeled as well as native *N*-linked glycans from proteins by both fluorescence and mass spectrometry (MS) detection [6, 7]. Here we describe the new GlycanPac AXR-1 mixed-mode column which provides an even higher resolution separation based on isomeric structure along with separation based on charge, size, and polarity.

The GlycanPac AXR-1 column is based on novel mixed-mode column chemistry, combining both WAX and reversed-phase (RP) retention mechanisms for optimal selectivity and high resolving power. The WAX functionality provides retention and selectivity for negatively charged glycans, while the reversed-phase 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 exceptional resolution with more than 4 times the glycan structures identified compared to existing commercial columns, including amide HILIC columns and the GlycanPac AXH-1 column, for 2AB-labeled *N*-linked glycans released from bovine fetuin. The GlycanPac AXR-1 column is designed for HPLC and UHPLC methods using either fluorescence or MS detection, and uses volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile, presenting the eluting glycans ready for introduction into MS instruments.

Methods

Sample Preparation

Glycans were released from glycoproteins with PNGase F enzyme (New England BioLabs). The released glycans were labeled with 2-aminobenzamide (2AB) and

2-amino benzoic acid (2AA) with slight modification from the reported procedure of Bigge *et. al.*,[8] Prior to analysis, samples were dissolved in 100 μ L D.I. water in a 250 μ L auto sampler vial.

Liquid Chromatography

All glycans were separated on a GlycanPac AXR-1 column (1.9 μ m, 2.1 \times 150 mm) by a Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC instrument with either a fluorescence or MS detector.

Mass Spectrometry

MS analysis was performed using an Orbitrap Fusion Tribrid mass spectrometer in negative ion mode. LC-MS² and LC-MS³ experiments were conducted for structural elucidation.

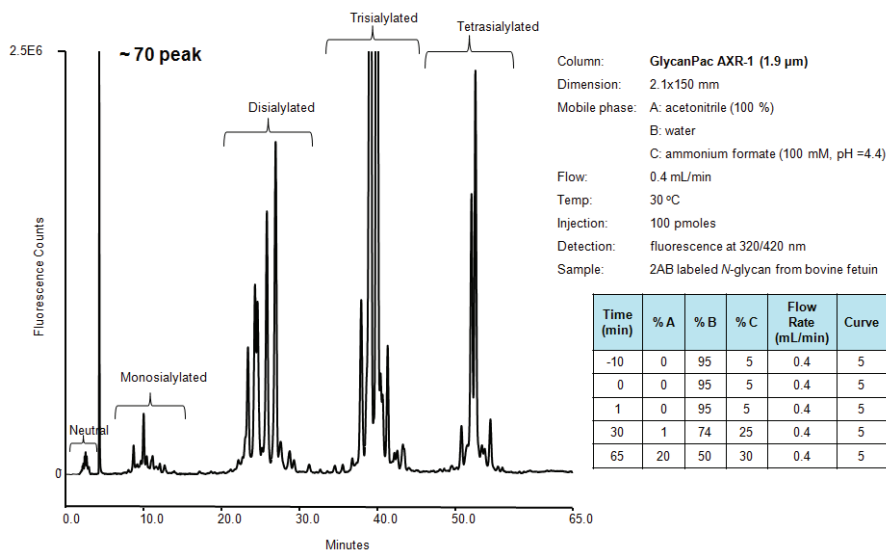
Data Analysis

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) present in glycoproteins, glycolipids and glycopolymers. 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 (1.9 μ m, 2.1 \times 150 mm) column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, close to the void, followed by monosialylated, disialylated, trisialylated, tetrasialylated, and finally 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 reversed-phase interaction. The GlycanPac AXR-1 (1.9 μ m) column provides \geq 70 resolved peaks with \geq 1% intensity for 2AB-labeled *N*-linked glycans from bovine fetuin. *N*-linked glycan structures present in each peak were identified using LC-MS² and LC-MS³ data.

FIGURE 1. Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, size, polarity and isomeric structure using GlycanPac AXR-1 (1.9 μ m) column by ternary gradient condition.



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 in-depth analysis of complex glycans due to its ability to provide structural information. 2AB-labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXR-1 column and analyzed on an Orbitrap Fusion mass spectrometer.

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 ($1.7 \mu\text{m}$, Figure 2) 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 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 using a combination of GlycanPac AXR-1 column and Orbitrap Fusion

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

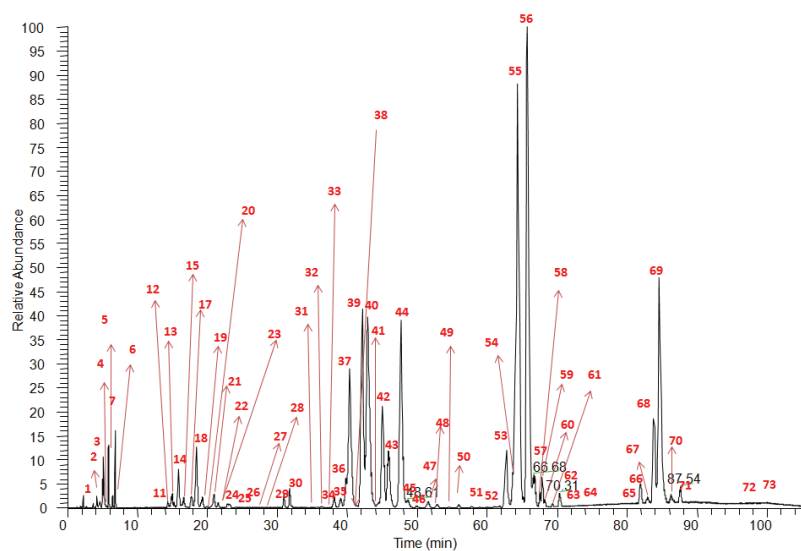


FIGURE 3. LC-MS analysis of 2AB-labeled *N*-linked glycans from bovine fetuin by a commercial amide HILIC column ($1.7 \mu\text{m}$) with MS detection.

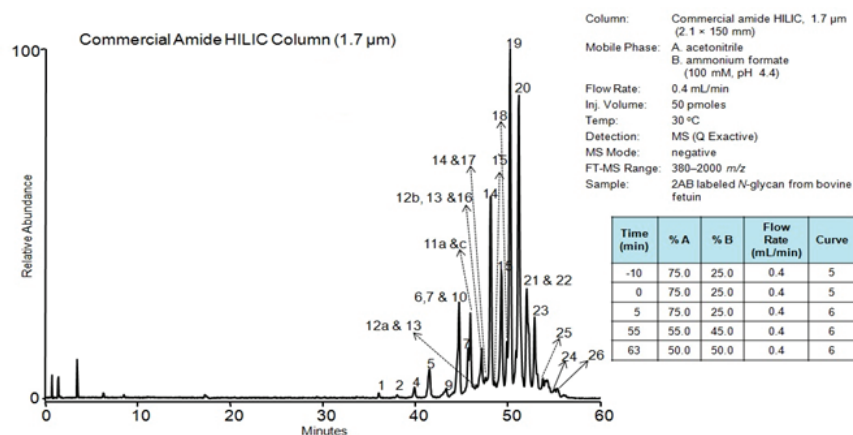


FIGURE 4. Separation of disialylated biantennary glycans from bovine fetuin based on α 2-3 and α 2-6 sialic acid linkage by the GlycanPac AXR-1 column.

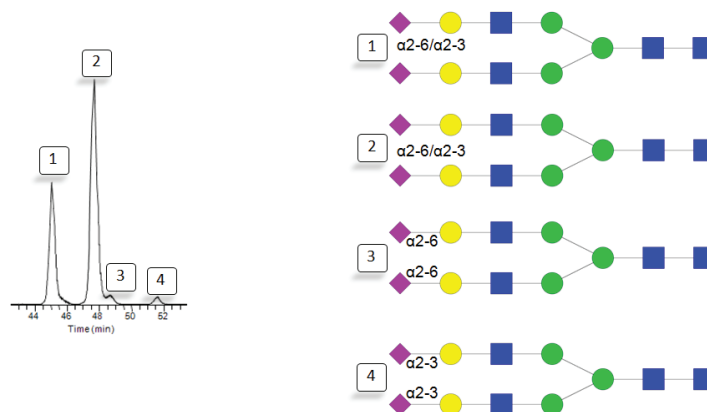
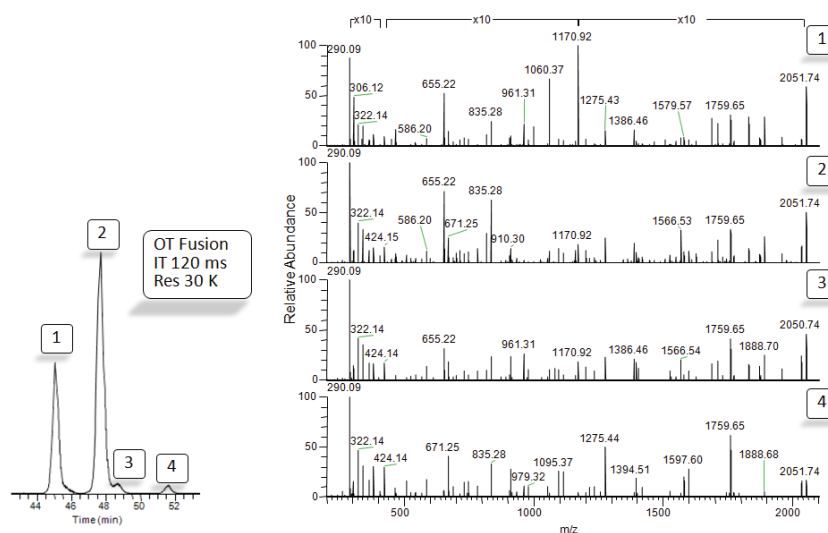


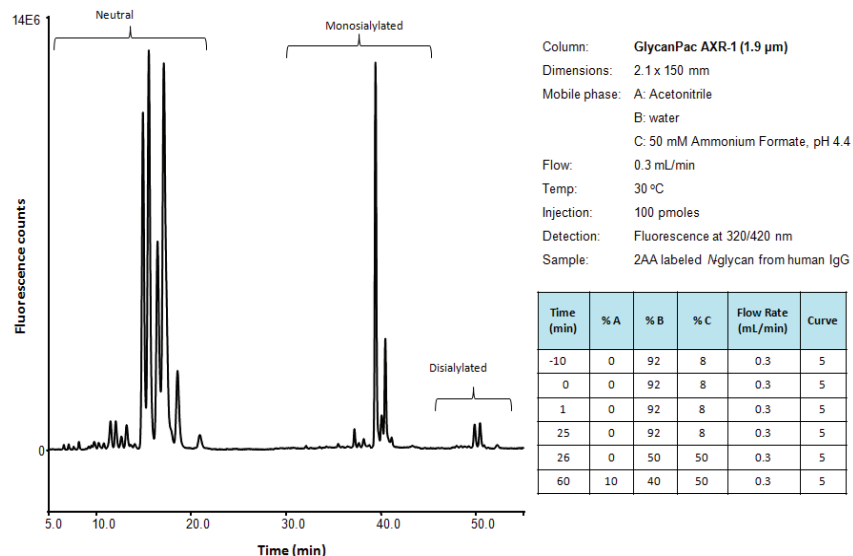
FIGURE 5. LC-MS/MS (HCD) spectra of 2AB-labeled *N*-linked glycans from bovine fetuin.



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. Antibodies are the most common proteins developed for therapeutics, and are under development for the treatment of numerous diseases. However, antibody glycosylation is a major source of heterogeneity with respect to both structure and therapeutic function. Glycosylation variants are primary factors in batch-to-batch antibody variation, altering product stability in vivo, and significantly influencing Fc effector functions in vivo. Both the U.S. FDA and European regulations require understanding of glycan profiles in these proteins because of their profound influence on safety and efficacy of biopharmaceuticals. 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 150 mm) 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. This also enables LC-MS³ workflows, providing additional stage of information for glycans structural elucidation

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