

A Fully Integrated Workflow for LC-MS/MS Analysis of Labeled and Native N-Linked Glycans Released From Proteins

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Overview

Purpose: Development of a fully integrated workflow that combines novel column technology, mass spectrometry, and a bioinformatics tool for high-resolution separation and structural characterization of native and fluorescently labeled *N*-linked glycans released from various proteins including antibodies.

Methods: Native and fluorescently labeled glycans from various proteins were separated and analyzed on a Thermo Scientific™ GlycanPac AXH-1 column coupled to a Thermo Scientific™ Q Exactive™ mass spectrometer. Data analysis was performed using SimGlycan® software.

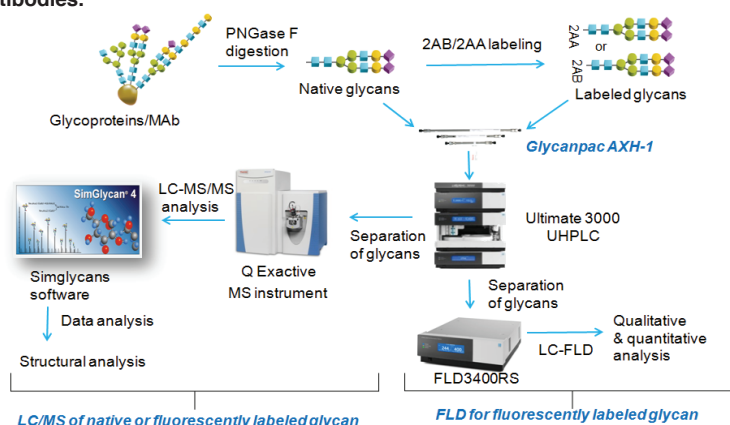
Results: A high-performance, silica-based HPLC/UHPLC column (GlycanPac AXH-1) coupled to a Q Exactive mass spectrometer or fluorescence detector enabled simultaneous separation of glycans by charge, size, and polarity. By incorporating a bioinformatics tool, a high-throughput analysis was demonstrated for biologically relevant glycans, either labeled or native, by LC-fluorescence and LC-MS methods.

Introduction

Glycans are widely distributed in biological systems in “free state” as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They are involved in a wide range of biological and physiological processes¹. The functions, efficacy and safety of protein-based drugs, including recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins². The structures of glycans are quite diverse, complex, and heterogeneous due to post-translational modifications and physiological conditions. The structural characterization, qualitative and quantitative estimation of glycans is essential in the bio-therapeutics and bio-pharmaceutical projects.³ However, it is challenging to comprehensively characterize glycan profiles and determine the structures of glycans.

We have developed a high-performance HPLC/UHPLC column (GlycanPac AXH-1 column) specifically designed for structural, qualitative, and quantitative analysis of glycans. It is designed for high-resolution and high-throughput analysis with unique selectivity for biologically relevant glycans including glycans from antibodies, either labeled or native, by LC-fluorescence or LC-MS methods. Because glycans are highly hydrophilic and polar substances, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterionic-based packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size- and composition-based separation. However, the limitation of these columns is that identification of the glycan charge state is not possible because glycans of different charge states are intermingled in the separation envelope. The GlycanPac AXH-1 column, based on advanced mixed-mode chromatography technology, overcomes these limitations and can separate glycans based on charge, size, and polarity configuration. In addition, each glycan charge state can be quantified. The GlycanPac AXH-1 column provides both greater selectivity and higher resolution with faster quantitative analysis. The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra generated. We incorporated a bioinformatics tool to simplify data analysis, enabling the development of a true high-throughput workflow. We extended this workflow (Scheme 1) to characterize glycans from various biological sources.

SCHEME1. Fully integrated workflow for analysis of glycans in proteins or antibodies.



Methods

Sample Preparation: Glycans were released from glycoproteins with PNGase F enzyme (New England BioLabs). The released glycans were conjugated with 2-aminobenzamide (2-AB) label group with reported procedure of Bigge *et. al.*⁴ 2-AA-labeled *N*-glycans from Human IgG, 2-AB-A1, 2-AB-A2, and 2-AB-A3 were purchased from ProZyme. Prior to analysis, samples were dissolved in 100% buffer (100 mM ammonium formate, pH = 4.4) and diluted further with acetonitrile to make 30% buffer and 70% acetonitrile.

Liquid Chromatography: All the glycans were separated on a GlycanPac AXH-1 (1.9 μ m, 2.1 150 mm) column by a Thermo Scientific™ Dionex™ Ultimate™ 3000 UHPLC instrument with either a fluorescence or MS detector.

Mass Spectrometry: MS analysis was performed using a Q Exactive hybrid quadrupole-Orbitrap™ mass spectrometer in negative ion mode. The following MS and MS/MS settings were used: MS scan range 380–2000. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of 1e⁶ and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of 2e⁵.

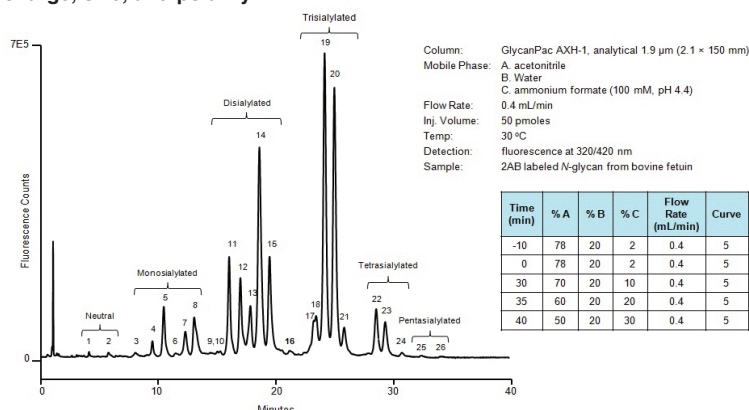
Data Analysis: SimGlycan software (PREMIER Biosoft) was used for MS/MS data analysis.⁵

Results

Separation of Labeled Glycans Based on Charge, Size, and Polarity

The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis, and characterization of neutral and charged glycans present on proteins. Figure 1 shows the separation of 2-AB-labeled bovine fetuin *N*-linked glycans on the GlycanPac AXH-1 column using fluorescence detection. The separation and elution of glycans are based on charge: the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated to penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. The retention time of each glycan charge state was confirmed using 2-AB-labeled glycan standards (not shown). Separation of glycans based on charge, size, and polarity provides significant structural and quantitative information. The chromatographic profiles (Figure 1) detected by fluorescence detector, provide qualitative information about the separation of *N*-linked glycans. The structure of glycans present in each peak were determined from the LC-MS/MS study shown in the following section.

FIGURE 1. Separation of 2-AB-labeled *N*-linked glycans from bovine fetuin by charge, size, and polarity.



LC-MS and LC-MS/MS Analysis of 2-AB-Labeled *N*-linked Glycans from Bovine Fetuin using GlycanPac AXH-1 Column

We also explored the coupling of GlycanPac AXH-1 column to MS. This is particularly attractive because MS can provide structural information about complex glycans. 2-AB-labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer. Data-dependent MS/MS spectra were acquired on all precursor ions ($z \leq 2$), and SimGlycan software was used for structural elucidation. A representative example of the analysis is shown in Figure 2. The primary advantage of the Q Exactive MS for glycan analysis is its ability to generate HCD HR/AM fragment ions. This allows for differentiation of near mass fragment ions, which we observed to be useful for correctly assigning branching and linkage. The variation of collision energy can provide different fragment ions within the mass spectrometer. In order to maximize both glycosidic and cross-ring fragments, we incorporated step-collision energy (SCE).

This provided optimum conditions for generation of a maximum number of both cross-ring and glycosidic cleavages in a single spectrum, thereby increasing confidence in our identification (Figure 3). The detailed structural information obtained from the MS/MS data further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity. However, co-elution of different charge-state glycans is common with other commercially available HILIC columns (Figure 4).

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

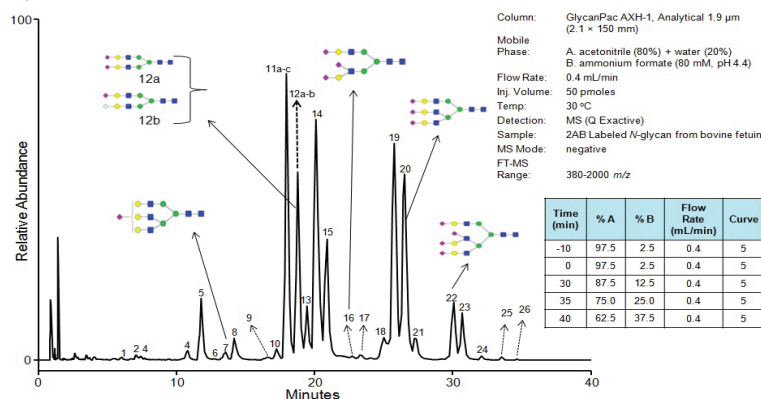


FIGURE 3. HCD MS/MS spectrum of 2-AB-labeled monosialylated triantennary *N*-linked glycans from bovine fetuin.

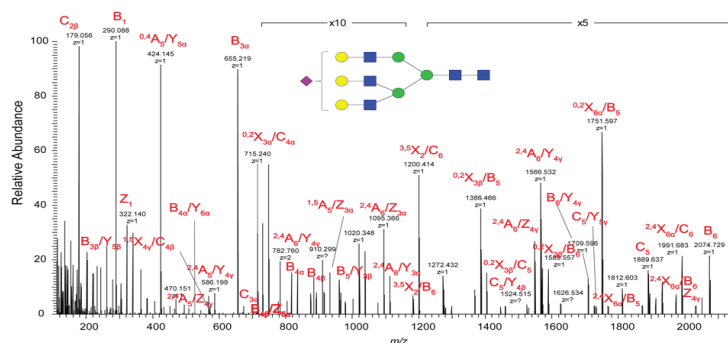
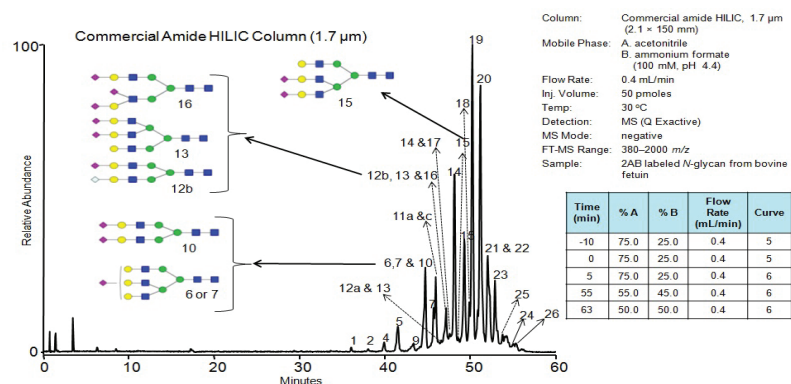


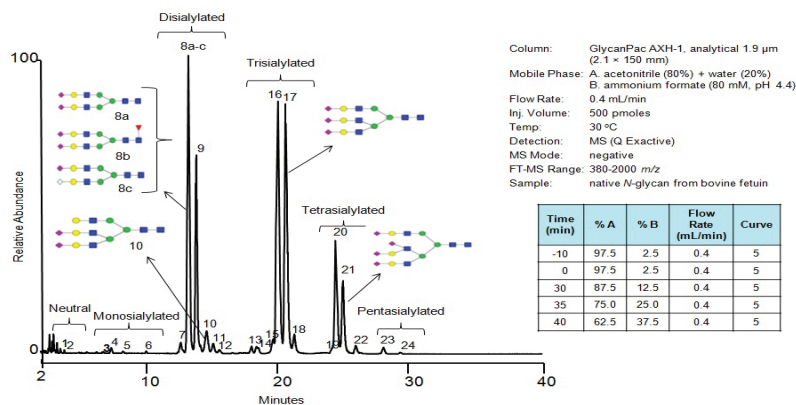
FIGURE 4. LC-MS analysis of 2-AB-labeled *N*-linked glycans from bovine fetuin by a commercial amide HILIC column (1.7 μ m) with MS detection.



LC-MS Analysis of Native Glycans Released from Proteins

The GlycanPac AXH-1 column is also suitable for analysis of native glycans. Analyzing unlabeled glycans not only eliminates the extra reaction step and cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction. Figure 5 shows the LC/MS analysis of native *N*-linked glycans from bovine fetuin using the GlycanPac AXH-1 column. The native glycans were separated based on charge, size, and polarity. Using an ammonium formate/ACN gradient highly compatible with MS detection, the separation enables excellent MS and MS/MS data for accurate confirmation of the glycan structures. Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially higher sialic acid glycans.

FIGURE 5. LC-MS analysis of native *N*-linked glycans from bovine fetuin. All the peaks are detected by MS in the negative ion mode.



Analysis of *N*-linked Glycans from Antibodies by LC-MS using GlycanPac AXH-1 Column

Glycosylation of antibodies is a prime source of product heterogeneity with respect to both structure and function. Variation in glycosylation is one of the main factors in batch-to-batch product variation,^{2,3} affecting product stability *in vivo* and significantly influencing Fc effector functions *in vivo*. A representative example of the separation of antibody glycans is shown in Figure 6, where 2-AA-labeled *N*-linked glycans from human IgG were separated using the GlycanPac AXH-1 column. Characterization of glycans in each peak was performed by LC-MS/MS, with the results in Table 1. Three different glycan charge states were found in this human IgG, the majority of glycans are neutral or mono-sialylated with minor amounts of di-sialylated glycans.

FIGURE 6. Analysis of 2-AA-labeled *N*-glycans from human IgG.

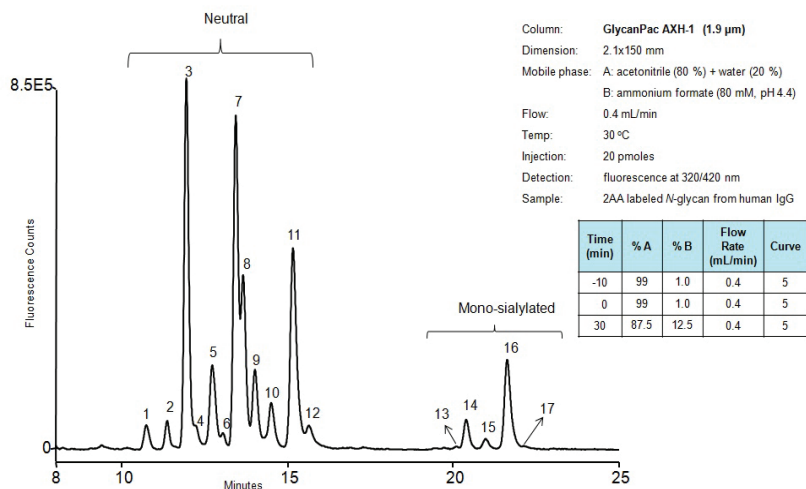
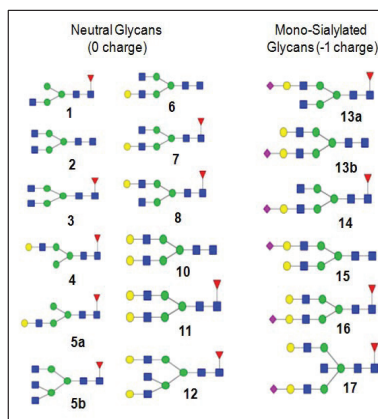


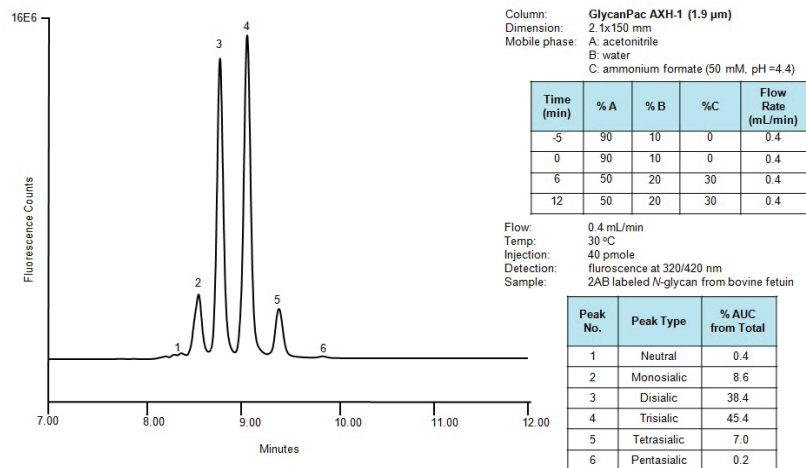
TABLE 1. Glycan structures identified from LC-MS/MS analysis of 2-AA-labeled *N*-linked glycans from human IgG.



Quantitative Determination of Glycans Based on Charge

Quantitative analysis of each glycan is essential for quick assessment of glycan variation in protein batch comparisons as well as for comparison of glycosylation profiles between normal and diseased cells. In addition, quantitative analysis of glycans separated based on charge state also provide a tool for calculating the relative amounts of different sialic acid linkages after enzymatic digestion with sialidase S and sialidase A. Figure 7 shows the quantitative analysis of 2-AB-labeled *N*-linked glycans based on charge using the GlycanPac AXH-1 column with fluorescence detection. The relative amount of each charge state glycan was estimated using a standard curve. The standards curve was drawn using the data from the chromatographic analysis of 2-AB-A2 glycan standard with the injection of different amount of samples, start from 0.1 pmoles to 5 pmoles.

FIGURE 7. Quantitative estimation of each charge state glycan in 2-AB-labeled *N*-linked glycans from bovine fetuin.



Conclusion

- A fully integrated workflow (Scheme 1) for structural characterization of native and fluorescently labeled *N*-linked glycans released from various proteins was demonstrated.
- The GlycanPac AXH-1 column separates glycans based on charge, size, and polarity, which is impossible for commercial HILIC columns. The GlycanPac AXH-1 column is also useful for the separation of reduced *O*-linked glycans from proteins and mucins, and the analysis of charged and neutral glycosylaminoglycans and glycolipids.
- LC-MS and MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.

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