Application Note 20909

Separation of 2AA-Labeled *N*-Linked Glycans from Human IgG on a High Resolution Mixed-Mode Column

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Key Words

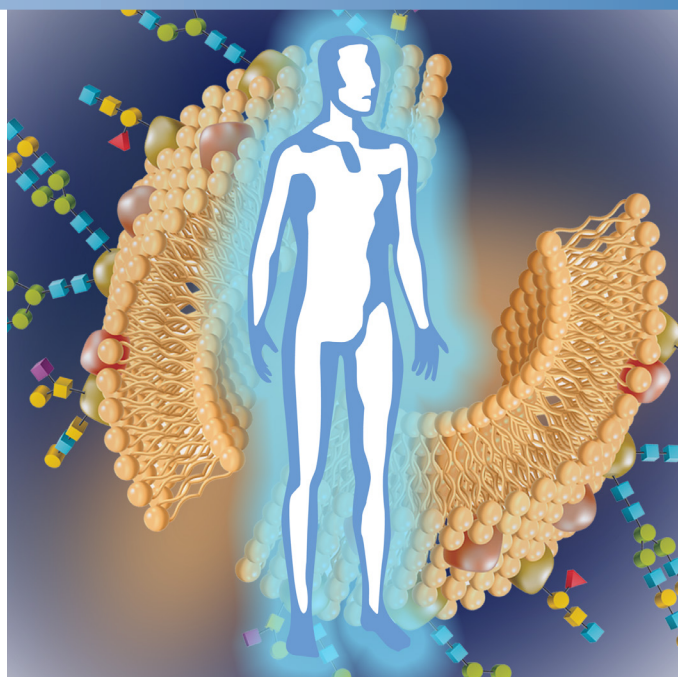
GlycanPac AXR-1, mixed-mode chromatography, isomeric separation, *N*-linked glycans, glycoproteins, reversed phase, anion-exchange, 2AA-labeled *N*-linked glycans, human IgG, charge based separation, glycan analysis

Abstract

This application note demonstrates the separation of 2AA-labeled *N*-linked glycans released from human immunoglobulin G (IgG) using a reversed phase / weak anion exchange mixed-mode column (Thermo Scientific™ GlycanPac™ AXR-1, 1.9 µm, 150 × 2.1 mm) with fluorescence detection. The method exhibits excellent separation of glycans based on isomeric structure, charge and size.

Introduction

Glycosylation is one of the most common post translational protein modifications (PTMs), that impacts therapeutic protein development [1]. Most of the protein drug candidates in preclinical and clinical development, such as recombinant proteins and monoclonal antibodies (MAbs), are glycosylated. Their biological activites are often dependent on the structure and types of glycans attached to these proteins [2]. Due to processing after en-bloc oligosaccharide transfer to the antibody, their structures can be extremely heterogeneous. Glycosylation variation contributes significant heterogeneity to antibody products with respect to both structure and function. Glycan variation is a major factor in MAb batch-variation; impacting product stability, Fc effector functions, biological activity, immunogenicity [3,4], pharmacokinetics and therapeutic MAb clearance. Glycan variation can be contributed by expression cell type, processes involved in fermentation, and purification protocols as well as during formulation and storage. Elucidation of the role(s) of antibody glycan structures is necessary for control of development and manufacturing processes for clinically important antibodies and related pharmaceuticals. Because charge, size and isomer variants may be present, structural characterization of glycan charge, size and isomers (linkage and branching) is essential for bio-therapeutic and bio-pharmaceutical projects [3]. Along with monosaccharide composition, these attributes help identify and thus control the panoply of potential glycan structures.



Various HPLC separation modes have been developed for glycan analysis, including normal phase (or hydrophilic interaction, HILIC), ion-exchange and reversed-phase [2,3]. Because glycans are very hydrophilic (polar), a common separation mode utilizes amide HILIC columns; as exemplified by the Thermo Scientific™ Accucore™ 150-Amide-HILIC column [5] that separates glycans based on hydrogen bonding, resulting in size and composition-based separations. Amide HILIC columns are particularly useful for the separation of 2AB-labeled *N*-linked glycans released from antibodies, for example MAbs, in which the majority of glycans are neutral. However, amide HILIC columns do not provide adequate separations when glycans contain 2 or more charge states (e.g., neutral and mono- or di-sialylated *N*-linked glycans) because glycan isoforms with different charge states are intermingled in the separation envelope.

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Recently we developed a mixed-mode column (GlycanPac AXH-1) with both weak anion-exchange (WAX) and hydrophilic interaction (HILIC) properties [6,9] which separates *N*-linked glycans based on charge, polarity, and size. The GlycanPac AXH-1 supports improved characterization of charge-states (sialylation) compared to the amide HILIC phases. The GlycanPac AXH-1 separations are broadly applicable for qualitative, quantitative and structural analysis of both labeled (2AB and 2AA) and native, unlabeled. *N*-linked glycans from proteins using fluorescence and/or mass spectrometry (MS) detection [6]. This is particularly useful for antibodies (e.g. human IgG).

Here we describe the new GlycanPac AXR-1 mixed-mode column that further improves separations by resolving glycans into different charge groups, and further separates glycans within each charge group based on isomerization and size. This substantially increases resolution of complex *N*-linked glycan structures, and helps differentiate isomeric structures not resolved by other approaches. This application note demonstrates the separation of 2AA-labeled *N*-linked glycans from human IgG on the GlycanPac AXR-1 column.

Experimental Details

Consumables	Part Number
Deionized (D.I.) water, 18.2 MΩ-cm resistivity	
Fisher Scientific acetonitrile HPLC grade	AC610010040
Fisher Scientific LC-MS grade formic acid	A117-50
Fisher Scientific ammonium formate (≥99 %)	AC-401152500
Thermo Scientific™ Premium 2 mL vial convenience kit	60180-600
PNGase F, New England BioLabs	P0705L
Human IgG	
2-amino benzoic acid (Anthranilic acid, 2AA-)	
Fisher Scientific glacial acetic acid	AA36289AP

Buffer Preparation
Ammonium formate (100 mM, pH 4.4): Dissolve 6.35 ± 0.05 g of ammonium formate and 70 ± 0.05 g formic acid in 999.6 g of D.I. water. Mix the eluent well and filter through a 0.2 µm pore filter

Sample Preparation
Dissolve the 2AA-labeled *N*-linked glycans from human IgG (or individually labeled standards at approximately 5000 pmol each) in 100 µL D.I. water in a 250 µL autosampler vial
Inject 1–5 µL
Note: store the standard at -20 ºC

Instrumentation
Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system; including pump (LPG-3400RS) thermal compartment (TCC-3000RS), split-loop well plate auto sampler (WPS-3000TRS) fluorescence detector with Dual-PMT (FLD3400RS), biocompatible 2 µL micro flow cell (6078.4330)

Separation Conditions	
Column:	GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm)
Mobile phase:	A: Acetonitrile
	B: D.I. water
	C: Ammonium formate (50 mM, pH 4.4)
Flow rate:	300 µL/min
Column temperature:	40 °C
Injection volume:	1 µL
Sample amount:	100 pmoles
Fluorescence detector:	λ_{Ex} = 320 nm & λ_{Em} = 420 nm

Data Processing	
Software:	Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results

The GlycanPac AXR-1 column provides qualitative structural characterization of neutral and charged glycans present in glycoproteins. For this Application Note the *N*-linked glycans were released from the antibody by PNGase-F treatment and labeled using a modification of the procedure detailed by Bigge et.al., [7].

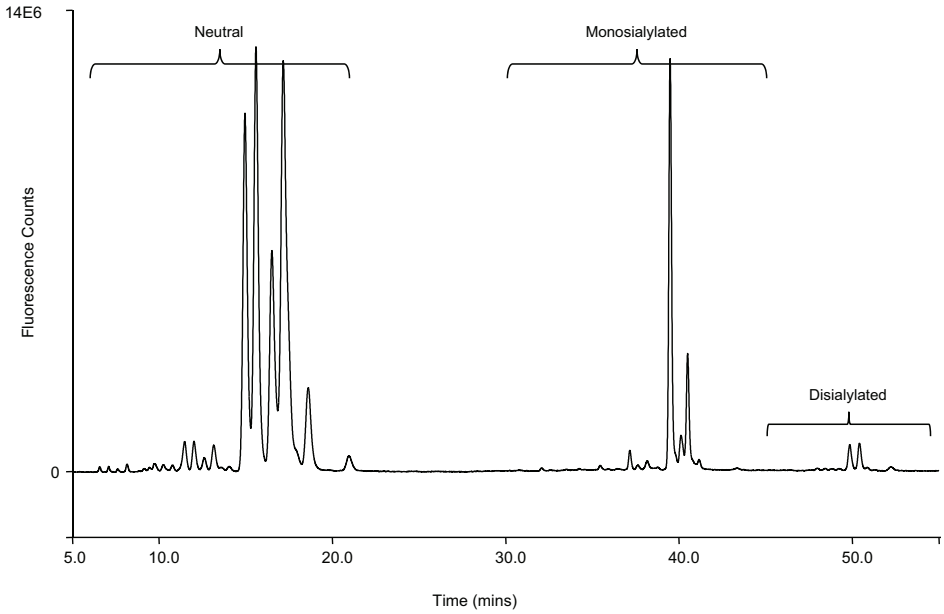

Figure 1: Separation of 2AA-labeled *N*-linked glycans from human IgG by charge, size and isomerization using the GlycanPac AXR-1 (150 × 2.1 mm, 1.9 µm) column

Time (min)	%A	%B	%C
-10	0	92	8
0	0	92	8
1	0	92	8
25	0	92	8
26	0	50	50
60	10	40	50

Table 1: Separation gradient

Figure 1 shows the separation of neutral and acidic 2AA-labeled *N*-glycans from human IgG using a GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm) column. The glycan elution profile consists of a series of peaks grouped into clusters with neutral glycans first, followed by monosialylated, then disialylated species. Analytes in each cluster represent glycans with the same charge. Within each cluster the glycans having the same charge are further separated according to their isomeric structure and size by reversed phase interactions. As shown in figure 1, 2AA-labeled neutral glycans elute between 5 and 22 minutes, monosialylated glycans elute between 30 and 45 minutes and disialylated glycans elute between 45 and 55 minutes. More than 40 peaks are identified from the separation of 2AA-labeled *N*-linked glycans from this human IgG sample.

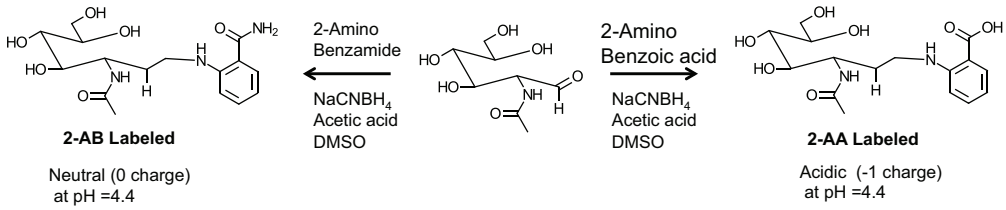

Figure 2: Schematic representation of labeling of 2AB and 2AA for *N*-linked glycans

GlycanPac AXR-1 works well with 2AB-labeled *N*-linked glycans from highly sialylated proteins where the majority of glycans are negatively charged [8]. However, most antibody *N*-linked glycans are neutral, so we improved retention and resolution here by introducing an additional charge. Labeling neutral glycans with 2AA introduces a single negative charge (Figure 2), and thus promotes increased binding to the GlycanPac AXR-1 column.

Conclusion

- The GlycanPac AXR-1 is a high-resolution, silica-based HPLC column for simultaneous separation of glycans by isomeric structure, charge and size.
- The GlycanPac AXR-1 column provides exceptional selectivity and excellent resolution of 2AA-labeled *N*-linked glycans released from human IgG.
- The GlycanPac AXR-1 works well for the direct injection of purified 2AA-labeled *N*-glycans from IgG under fully aqueous conditions.

References

[1] Varki, A. Biological Roles of Oligosaccharides: All the Theories Are Correct. Glycobiology 1993, 3, 97–130.

[2] Bertozzi, C.R.; Freeze, H.H.; Varki, A.; Esko, J.D. Glycans in Biotechnology and the Pharmaceutical Industry, Essentials of Glycobiology, Second Edition; Cold Spring Harbor Laboratory Press: New York, 2009; Chapter 51.

[3] Guidance for Industry, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product, Draft Guidance; U.S. Department of Health and Human Services Food and Drug Administration, February 2012 [Online] www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM291128.pdf (accessed Jan. 18, 2013).

[4] (a) Anal Bioanal Chem (2010) 397:3457–3481 (b) Analytical Biochemistry Volume 370, Issue 2, 15 November 2007, Pages 147–161

[5] Accucore 150-Amide-HILIC Application Note: Analysis of Human IgG Glycans on a Solid Core Amide HILIC Stationary Phase http://www.separatedbyexperience.com/documents/AN-LC-Accucore-HILIC-Human-IgG-Glycans-AN20703_E.pdf

[6] GlycanPac AXH-1 Product Specification: http://www.dionex.com/en-us/webdocs/114170-PS-GlycanPac-AXH1-Column-PS20695_E.pdf

[7] Bigge, J. C. et al., Non-selective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Analytical Biochemistry, 1995, 230, 229-238.

[8] GlycanPac AXR-1 Application Note: Separation of 2AB-Labeled *N*-Linked Glycans from Bovine Fetuin on a Novel Ultra High Resolution Mixed-Mode Column http://www.separatedbyexperience.com/documents/AN-LC-GlycanPac-AXR1-Glycans-Bovine-Fetuin-AN20908_E.pdf

[9] GlycanPac AXH-1 Application Notes: Structural Analysis of Labeled *N*-Glycans from Proteins by LC-MS/MS Separated Using a Novel Mixed-Mode Stationary Phase http://www.separatedbyexperience.com/documents/AN-LC-GycanPac-N-Glycans-Proteins-AN20786_E.pdf

Separation of 2AB-Labeled *N*-Glycans from Bovine Fetuin on a Novel Mixed-Mode Stationary Phase http://www.separatedbyexperience.com/documents/AN-LC-GlycanPac-N-Glycans-Bovine-Fetuin-AN20754_E.pdf

Structural Analysis of Native *N*-Glycans Released from Proteins Using a Novel Mixed-Mode Column and a Hybrid Quadrupole-Orbitrap Mass Spectrometer http://www.separatedbyexperience.com/documents/AN-LC-GlycanPac-Native_N-Glycans-Bovine-Fetuin-AN20827_E.pdf

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