Application Note 20910

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

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Key Words

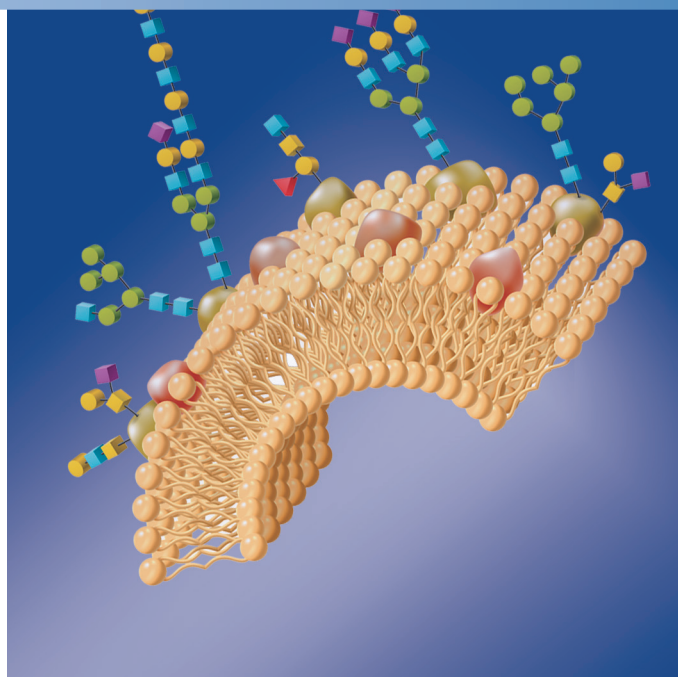
GlycanPac AXR-1, mixed-mode chromatography, *N*-linked glycans, glycoproteins, reversed-phase, anion-exchange, 2AA-labeled *N*-linked glycans, bovine fetuin, isomeric separation, charge based separation, glycan analysis

Abstract

This application note demonstrates the separation of 2AA-labeled *N*-linked glycans released from bovine fetuin 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 charge, isomeric structure and size.

Introduction

Glycosylation is a common post-translational modification (PTM) on proteins, and is often found on therapeutic proteins [1]. In fact, most of the protein pharmaceutical candidates in preclinical and clinical development are glycosylated. The efficacy of recombinant protein based drugs (e.g. erythropoietin [EPO] and Follicle-Stimulating Hormone [FSH]) is often dependent on the structure and the types of glycans attached to the protein [2]. Further, protein glycosylation is a prime source of therapeutic protein heterogeneity with respect to both structure and function, and variation in glycosylation is one of the main factors in product batch-to-batch variation. This affects product stability *in vivo* and significantly influences biological activity, pharmacokinetics, and clearance, as well as immunogenicity [2,3]. Glycan structures are diverse, complex and heterogeneous. Variation in glycosylation can be attributed to several factors including the type of cell in which the glycoprotein is produced as well as processes involved in cell culture, purification, formulation, and storage. Thus, understanding the structure of glycans in proteins provides detailed information necessary for control of reproducible production during development and manufacturing of clinically useful proteins. Structural characterizations of glycans, including monosaccharide composition, linkage and branch isomerism, charge, and size variations are essential for bio-therapeutics and bio-pharmaceutical projects [3].



Various HPLC separation modes have been developed for glycan analyses, including normal phase or hydrophilic interaction (HILIC), ion-exchange, and reversed-phase (RP) [4]. Because glycans are very hydrophilic (polar), a common separation mode employs amide-HILIC columns; as exemplified by the Thermo Scientific™ Accucore™ 150-Amide-HILIC column, that resolves glycans by hydrogen-bonding, producing a size and composition-based separation. Amide-HILIC columns are particularly useful for the separation of 2-aminobenzamide-(2AB) labeled *N*-linked glycans released from antibodies, for example MAbs, in which the majority of glycans harbor no charge. However, amide-HILIC columns do not provide adequate separations where glycans that harbor 2 or more charge states are present (e.g., neutral and sialylated glycans) because glycan isoforms with different charge states co-elute 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 [5, 6] which separates *N*-linked glycans based on charge, polarity, and size. The GlycanPac AXH-1 improves characterization of charge states (sialylation) compared to the amide-HILIC phases. The GlycanPac AXH-1 separations support broad applicability for qualitative, quantitative and structural analysis of both labeled (2AB and 2AA) and unlabeled *N*-linked glycans from proteins using fluorescence and/or mass spectrometry (MS) detection [5]. This is particularly useful for antibodies (including 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 separating 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 bovine fetuin on the GlycanPac AXR-1 column using both binary and ternary gradient eluent systems.

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
Bovine fetuin	
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 0.70 ± 0.05 g of 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 2AA-labeled *N*-linked glycans from bovine fetuin or individual labeled standards (approximately 5000 pmol each) in 100 µL D.I. water in a 250 µL autosampler vial, Thermo Scientific 055428.

Inject 1–5 µL to introduce 100 pMol per injection.

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, flow cell: 2 µL micro flow cell: 6078.4330

Separation Conditions	
Column:	Thermo Scientific™ GlycanPac™ AXR-1 column (1.9 µm, 150 × 2.1 mm)
Mobile Phase:	A: Acetonitrile
	B: D.I. Water
	C: Ammonium formate (100 mM, pH =4.4)
Flow rate:	400 µL/min
Column temperature:	30 °C
Injection volume:	1–5 µL
Sample Amount:	100 pmoles
Samples:	2AA-labeled *N*-linked glycans from bovine fetuin
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 is used for qualitative and structural characterization of neutral and charged glycans present in glycoproteins. For this Application Note the *N*-linked glycans were released from bovine fetuin by PNGase-F treatment and labeled using a modification of the procedure detailed by Bigge et.al, [7].

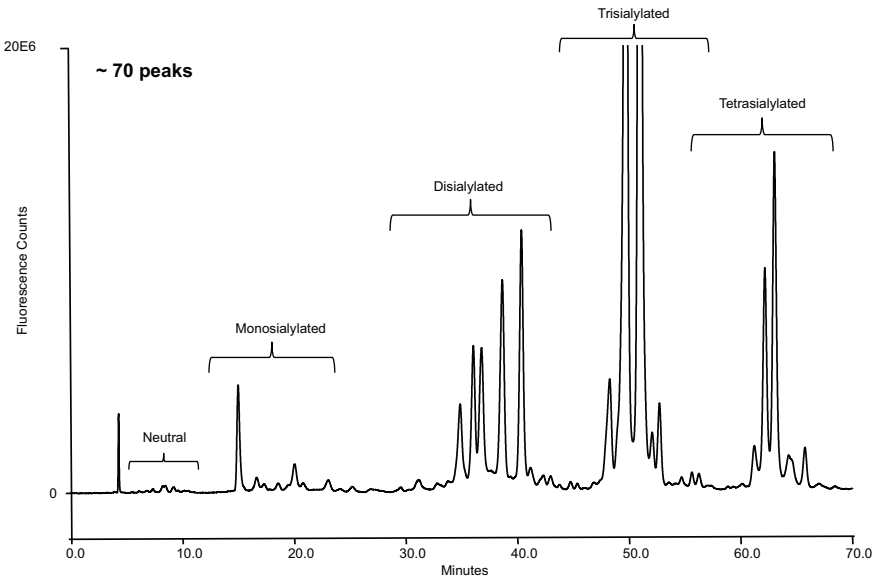

Figure 1: Separation of 2AA-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a ternary gradient with late introduction of acetonitrile on a GlycanPac AXR-1 column

Time (min)	%A	%B	%C
-10	0	85	15
0	0	85	15
1	0	85	15
25	0	70	30
70	10	40	50

Table 1: Ternary gradient conditions for Figure 1

Figure 1 shows the separation of neutral and acidic 2AA-labeled *N*-linked glycans from bovine fetuin using a GlycanPac AXR-1 (1.9 µm, 150 × 2.1 mm) column with a ternary gradient. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, followed by monosialylated, disialylated, trisialylated and tetrasialylated species. Analytes in each cluster represent glycans of the same charge. Within each cluster, glycans having the same charge are further separated according to their isomeric structure, sized and polarity by reversed-phase mechanisms. Note: The gradient was completed before elution of the few penta-sialylated glycans known to be present in bovine fetuin.

As shown in Figure 1, 2AA-labeled neutral glycans elute between 4 and 12 min, monosialylated glycans elute between 12 and 27 min, disialylated glycans elute between 27 and 45 min, trisialylated glycans elute between 45 and 58 min, and tetrasialylated glycans elute between 58 and 70 min. More than 70 peaks are identified in less than 70 minutes in this chromatogram of 2AA-labeled *N*-linked glycans from bovine fetuin.

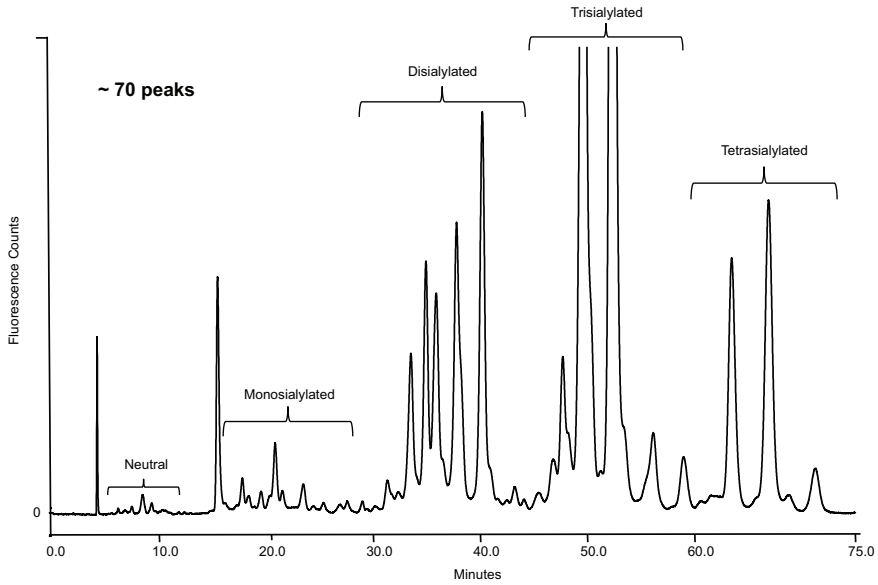

Figure 2: Separation of 2AA-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a binary gradient without acetonitrile on a GlycanPac AXR-1 column

Time (min)	%B	%C
-10	90	10
0	90	10
1	90	10
45	40	60
60	30	70
75	30	70

Table 2: Binary gradient conditions for Figure 2

Figure 2 repeats the chromatography using a binary gradient, beginning with a lower ammonium formate concentration, employing a shallower (slower) gradient rate and eliminating acetonitrile from the eluent system. Here, slightly better resolution of several peaks is observed due to the lower initial salt concentration and gradient slope. In this case the chromatogram was completed in 60 minutes.

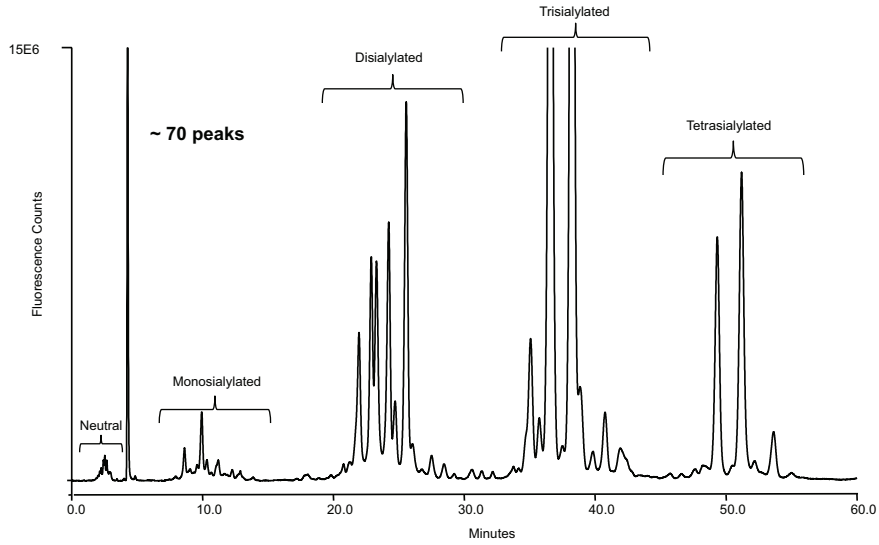

Figure 3: Separation of 2AB-labeled *N*-linked glycans from bovine fetuin by charge, isomerism and size using a binary gradient without acetonitrile on a GlycanPac AXR-1 column

Time (min)	%B	%C
-10	95	5
0	95	5
1	95	5
50	50	50
60	50	50

Table 3: Binary gradient conditions for Figure 3

Due to 2AA-labeling, a formal negative charge was introduced onto each glycan, increasing all glycan negative charges by 1. For example, glycans that are neutral when labeled with 2AB become negatively charged when labeled with 2AA. This produces greater retention on the GlycanPac AXR-1 column. Similarly mono-sialylated glycans acquire a negative-2 charge; di-sialylated glycans acquire a negative-3 charge, etc. Thus 2AA-labeled *N*-linked glycans (Figure 2) from fetuin require higher ionic strength for elution than the 2AB-labeled *N*-linked glycans from fetuin. However, 2AA-labeling promotes better retention and thus resolution of neutral glycans than the same glycans labeled with 2AB, using the GlycanPac AXR-1 column. For this comparison, Figure 3 depicts the chromatography of 2AB-labeled *N*-linked glycans from bovine fetuin using significantly reduced ammonium formate concentrations. In figure 2, the ammonium formate concentration runs from 10 to 70 mM, while in Figure 3, the range is 5 to 50 mM.

In Figure 1, the ammonium formate concentration runs from 15 to 30 mM in 25 min, and later-eluting glycans are eluted with an acetonitrile gradient. This limits the volatile salt concentration and supports better performance of the MS system. Thus, the ternary gradient condition is preferred for mass spectrometric applications with 2AA-labeled *N*-linked glycans.

Conclusion

- The GlycanPac AXR-1 is a high- resolution, silica-based mixed-mode HPLC column for simultaneous separation of glycans by isomeric structure, charge and size.
- The GlycanPac AXR-1 column provides improved selectivity and excellent resolution of 2AA-labeled *N*-linked glycans released from bovine fetuin.
- Binary and ternary gradient conditions for the separation of both 2AA-labeled *N*-linked glycans from fetuin are illustrated.
- The GlycanPac AXR-1 works well for the direct injection of purified 2AA-labeled *N*-linked glycans from bovine fetuin 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] Thermo Scientific Poster Note: Novel Glycan Column Technology for the LC-MS Analysis of Labeled and Native *N*-Glycans Released from Proteins and Antibodies.

http://www.dionex.com/en-us/webdocs/114197-PN-LC-MS-N-Glycans-Proteins-Antibodies-PN70513_E.pdf

[6] Thermo Scientific 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. Anal. Biochem., 1995, 230, 229-238.

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