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アプリケーションノート

DMB-Labeled Sialic Acid Analyses Using HPLC-, UHPLC-, and UPLC-Based, BEH C_{18} Columns

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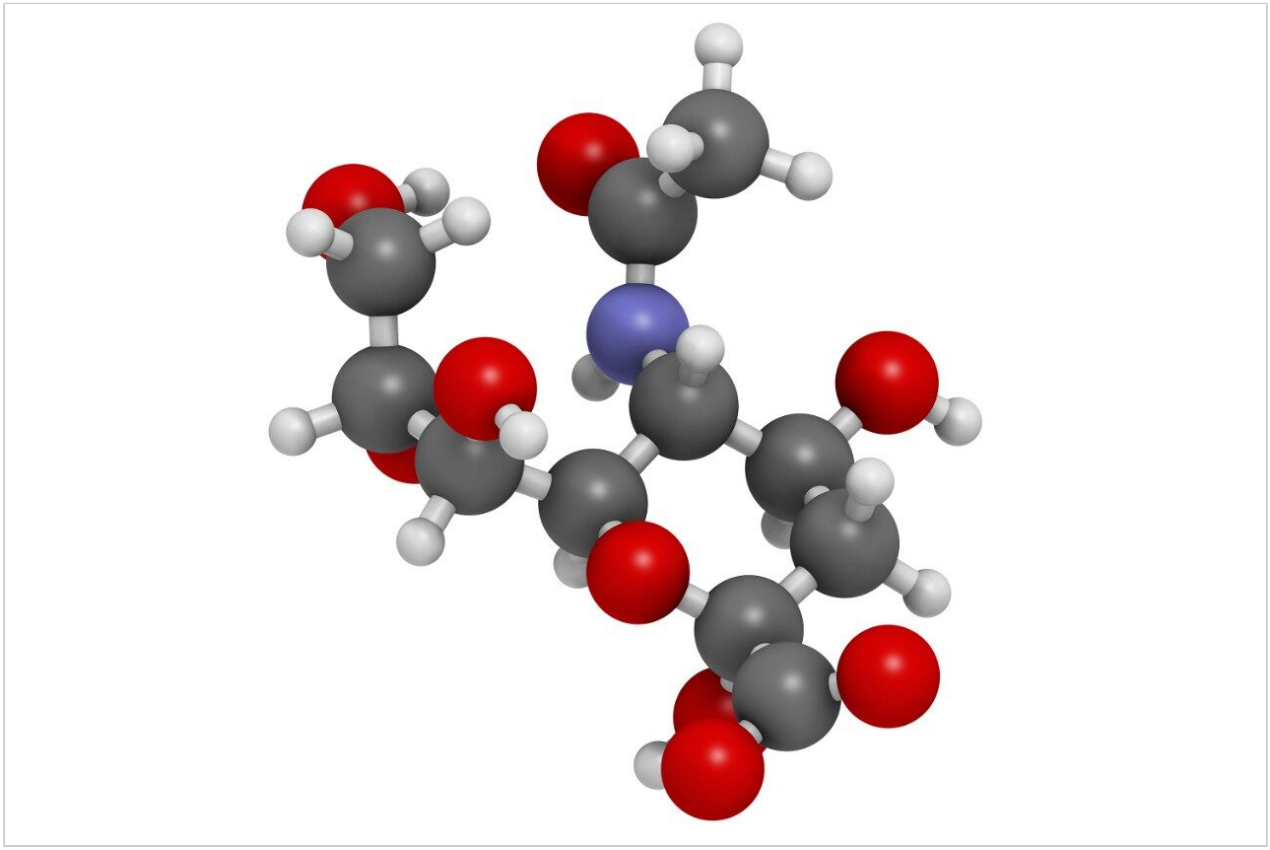

Abstract

This application extends on Waters technologies for the analysis of intact glycoproteins, glycoprotein subunits, glycopeptides, as well as released N-Linked glycans from biotherapeutic glycoproteins and related compounds.

Benefits

- The analysis of DMB-labeled sialic acids derived from biotherapeutics glycoproteins is a well established and accepted method by various international regulatory agencies
- Waters Bridged-Ethylene Hybrid (BEH) 130.-based chemistries, available in different particle size and column configurations, are well suited for the HPLC-, UHPLC-, or UPLC. -based analysis of DMB-labeled sialic acids
- This application extends on Waters technologies for the analysis of intact glycoproteins, glycoprotein subunits, glycopeptides, as well as released N-Linked glycans from biotherapeutic glycoproteins and related compounds

Introduction

Sialic acids are a family of nine-carbon acidic monosaccharides that occur naturally at the end of sugar chains attached to the surfaces of cells and soluble proteins.[1] A diverse range of sialic acids are found in nature, but the two major sialic acids found on N-glycans and O-glycans in biopharmacuticals are N-acetyl-neuraminic acid (Neu5Ac) and N-glycolyl-neuraminic acid (Neu5Gc). Sialylation can enhance serum half life as well as affecting biological activity such as increasing the anti-inflammatory activity of IgG. Humans cannot synthesize Neu5Gc and its presence on a drug can lead to immune reactions such as chronic inflammation.[2] Anti-Neu5Gc antibodies have been detected in normal human sera, and can neutralize any Neu5Gc containing biopharmaceutical lowering its efficacy. The choice of cell line can greatly influence the type of sialic acids present on a biopharmaceutical, for instance 24% of the sialic acids on mouse IgG are Neu5Gc compared to none on human IgG. It is therefore imperative to monitor both the level and types of sialic acids during all stages of the product life cycle. Consequently, ICH guideline Q6B states that the sialic acid content should be determined for glycoprotein biopharmaceuticals since it is considered a critical quality attribute (CQA).[3]

Among the chromatographic methods there are direct detection methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD),[4] and those that require pre-column sample derivatization followed by LC separation with fluorescence detection[5] such as that detailed in this application note.

This pre-column derivitization and analyses method begins with the release of the targeted sialic acids from the isolated glycoprotein using mild acid hydrolysis conditions (e.g., 2 M acetic acid for two hours at 80 °C). The freed sialic acid species are then derivitized using 1,2-diamino-4,5-methylenedioxybenzene-2HCl (DMB) dye. The DMB-labeled sialic acids are then separated via reversed-phase (RP) chromatography with subsequent on-line fluorescence detection. Of particular importance to the collection of quality data is the fact that the DMB-labeled sialic acids are light sensitive so samples should be analyzed within 24 hours post labeling to prevent sample degradation that can compromise results. This can become a significant problem if a large number of samples need to be analyzed using traditional HPLC-based techniques that can take more than 30 minutes per sample analysis.

Experimental

Methods

The Glyko Sialic Acid Reference Panel (p/n GKRP-2503) containing Neu5Ac, Neu5Gc, Neu5,7Ac2, Neu5Gc9Ac, Neu5,9Ac2, and Neu5,7(8),9Ac3 was purchased from ProZyme, Inc., 3832 Bay Center Place, Hayward, CA 94545. The DMB labeling protocol is as follows:

Preparation of 1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) labeling solution:

- Into a 2 mL glass vial, 436 µL of water and 38 µL of glacial acetic acid and were mixed.
- 26 µL of 2-mercaptoethanol was added to the 2 mL vial and mixed.
- 440 µL of the above solution was added to a separate 2 mL glass vial containing 4 mg of sodium hydrosulfite and mixed.
- This solution was added to a 2 mL glass vial containing to 0.7 mg of DMB (Toronto Research Chemicals, 2 Brisbane Road, Toronto, Ontario, Canada: p/n D416370) and mixed.
- Due to the light and moisture sensitivity this DMB-labeling solution was used immediately after preparation.

DMB Labeling of Sialic Acid Reference Panel:

- 20 µL of the DMB-labeling solution was added to the vial containing the purchased Sialic Acid Reference Panel.
- The sample was incubated in the dark using a heater block set to 50 °C.
- After three hours, the reaction was stopped by adding 480 µL of water to the reaction mixture.

- This DMB-labeled sample was injected onto the ACQUITY UPLC System with injection volumes scaled appropriately for column configurations as shown below.

LC analyses

LC system:	ACQUITY UPLC System with Fluorescence Detector
Columns:	XBridge BEH C_{18}, 130Å, 3.5 µm, 4.6 x 100 mm (p/n 186003033) XBridge BEH C_{18}, 130Å, XP 2.5 µm, 3.0 x 75 mm (p/n 186006034) ACQUITY UPLC BEH C $_{18}$, 130Å, 1.7 µm, 2.1 x 50 mm (p/n 186002350)
Column temp.:	30 °C
Sample temp.:	4 °C
Mobile phase:	Acetonitrile:Methanol:Water (9%:7%:84%)
Strong and weak needle wash:	Acetonitrile:Methanol:Water (9%:7%:84%)
FLR detection:	Excitation wavelength: 373 nm, Emission wavelength: 448 nm

Column configuration:	2.1 x 50 mm	3.0 x 75 mm	4.6 x 100 mm
Flow rate:	0.18 mL/min	0.26 mL/min	0.43 mL/min
Injection volume:	0.7 µL	2.0 µL	6.7 µL
Data management:	Empower Pro (v3) Software		

The Waters Application Note entitled: "*Future Proofing the Biopharmaceutical QC Laboratory: Chromatographic Scaling of HPLC Monosaccharide Analyses Using the ACQUITY UPLC H-Class Bio System*" (p/n 720005255EN) details the steps and considerations necessary to successfully develop a HPLC-, UHPLC-, or UPLC-based separation of hydrolyzed monosaccharides derived from glycoproteins. This application note details the application of the same scaling principles to the LC-based analysis of DMB-labeled sialic acids on the same Waters BEH C_{18}, 130Å column chemistry.

Results and Discussion

A series of experiments were performed using the XBridge BEH C_{18}, 130Å, 3.5 µm, 4.6 x 100 mm Column to determine whether a gradient or an isocratic separation could be developed to yield adequate DMB-labeled, sialic acid reference standard component resolution. Results from this work confirmed that an eluent containing acetonitrile:methanol:water (9%:7%:84%) could be used to yield baseline resolution of the sialic acid species of interest without the need to run a gradient. While the shown HPLC-based column separation (Figure 1, top) would adequately address many laboratory sample throughput needs, the next series of experiments were undertaken to demonstrate that the same high-quality separation could be performed using the same BEH C_{18}, 130Å-based chemistry but of reduced particle size and column I.D. and length for those requiring higher sample throughput (Figure 1, middle and bottom) using the scaling techniques detailed in Waters Application Note p/n 720005255EN.

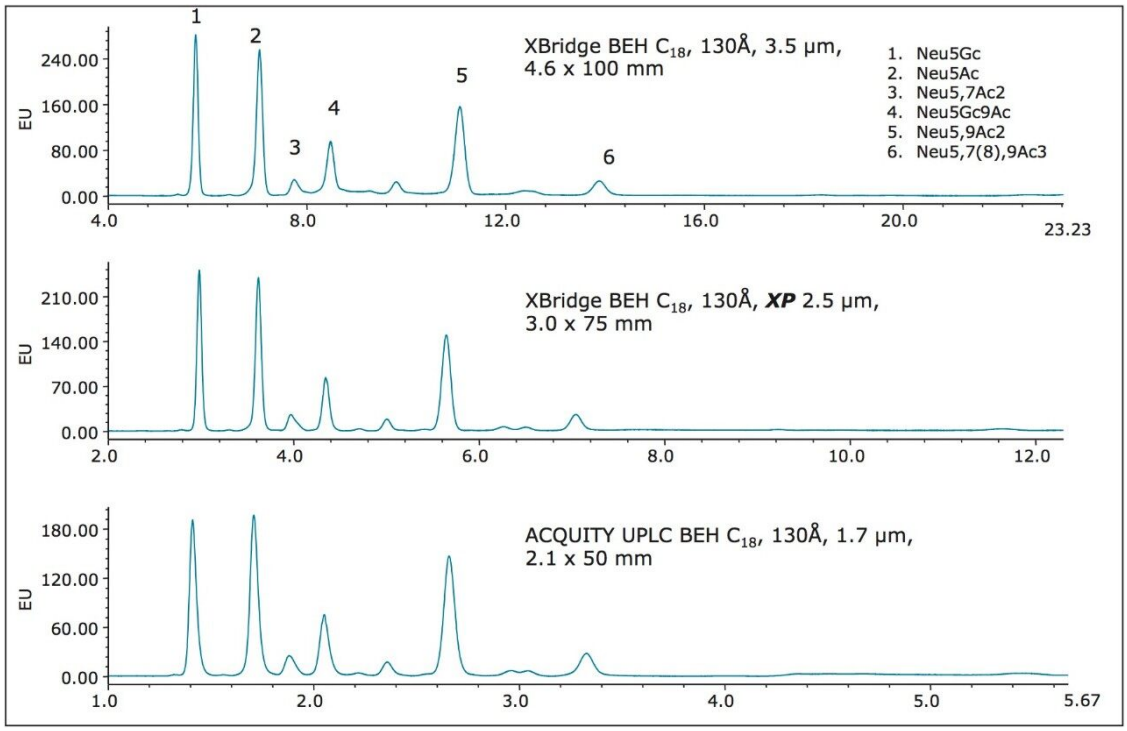

Figure 1. Geometric scaling of a DMB-labeled Sialic Acid Reference Panel on Waters XBridge BEH C_{18}, 130Å, 3.5 µm, 4.6 x 100 mm (top), XBridge BEH C_{18}, 130Å, XP 2.5 µm, 3.0 x 75 mm (middle), and ACQUITY UPLC BEH C_{18}, 130Å, 1.7 µm, 2.1 x 50 mm (bottom) Columns on the same ACQUITY UPLC System

Conclusion

This application note clearly demonstrates the ability to obtain high-quality, DMB-labeled sialic acid analyses on geometrically scaled HPLC, UHPLC, and UPLC column configurations containing same Waters BEH C_{18}, 130Å reversed-phase-based chemistry on 3.5 µm (HPLC), 2.5 µm (UHPLC), and 1.7 µm (UPLC) particles. For high sample throughput needs, the UPLC column and separation conditions provide adequate component resolution with an approximate four-fold reduction in analysis time and eluent consumption.

References

1. Wang, B.; Brand-Miller, J. The role and potential of sialic acid in human nutrition. E*uropean Journal of*

Clinical Nutrition 2003, 57, 1351–1369.

2. Nguyen, D.H.; Tangvoranuntakul, P.; Varki, A. Effects of natural human antibodies against a nonhuman sialic acid that metabolically incorporates into activated and malignant immune cells. *J Immunology* 2005, 175, 228–236.

3. Specifications: test procedures and acceptance criteria for biotechnological/biological products. *Published in the Federal Register* 1999, 64, 44928.

4. Rohrer, J.S. Analyzing sialic acids using high-performance anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem*. 2000, 283, 3–9.

5. Hara, S.; Yamaguchi, M.; Nakamura, M. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. *Anal. Biochem*.1989, 179, 162–166.

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