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Rapid High-Throughput Profiling and Quantitation of Sialic Acids in Biotherapeutics

Anna Fong[1]; Ace G. Galermo[1]; John Yan[1]; Tom Rice[1]; Aled Jones[1]; Archana Datt[1]; Hamutal Bonen[1]; Gregory Staples[1, 2]; Ted Haxo[1]

[1]Agilent Technologies, Hayward, CA; [2]Agilent Technologies, Santa Clara, CA

Introduction

The composition of glycans present on biotherapeutic glycoproteins can affect immunogenicity, pharmacokinetics and pharmacodynamics.[1] Glycans are carbohydrates composed of monosaccharides arranged into many different possible oligosaccharide structures based on composition and linkage position. Sialic acid capping at the non-reducing terminal of N- or O-glycans can serve a key role in mediating the effectiveness of therapeutic glycoproteins.[2] Depending on the molecule and the application, terminal sialic acid may reduce the rate of clearance, reduce antibody-dependent cellular cytotoxicity (ADCC) activity, or can be anti-inflammatory.[3-5] Two forms commonly found in biotherapeutics are N-acetylneuraminic acid (Neu5Ac) and N-glycoylneuraminic acid (Neu5Gc). Neu5Ac is usually the predominant species while Neu5Gc is not synthesized by humans and its presence on biotherapeutics can be immunogenic. Therefore, it is essential to monitor not only the absolute quantity of sialic acid, but also the levels of different sialic acid species present in therapeutic glycoproteins.

Here we present a new high-throughput workflow based on a 96-well plate format for the release, labeling, and analysis of sialic acids from therapeutic glycoproteins using rituximab, etanercept, and NISTmAb as examples. Sialic acid residues are released then labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in a two-step procedure. The DMB-labeled sialic acids are then separated and analyzed using a rapid 10-minute method based on reversed-phase ultra high-performance liquid chromatography (UHPLC) coupled with fluorescence and optional mass spectrometry detection. The workflow offers both qualitative characterization of Neu5Ac, Neu5Gc and other sialic acid species using a sialic acid reference panel (SARP), as well as absolute quantitation with picomol level sensitivity using included Neu5Ac and Neu5Gc quantitative standards. The workflow enables reliable and reproducible high-throughput profiling and quantitation of sialic acids, providing a broad detection range and improved sensitivity for molecules with low levels of sialylation.

Sample Preparation

Samples were prepared using a developmental protocol using a 96-well plate format. Sialic acids were released from rituximab (Rituxan, lot # M190170), etanercept (Enbrel, lot # M190088), NISTmAb (lot # 14HB-D-002) and erbitux (Cetuximab, lot # MI60886) through an acid hydrolysis reaction. The method eliminates the need for a dry down step, thereby, decreases overall sample preparation time by 1-2 hours. The sample amount is typically 200 µg of glycoprotein with low level sialyation and 5 µg of highly sialylated glycoprotein. Serial dilutions of sialic acid reference standards were used to prepare a standard curve for Neu5Ac and Neu5Gc. Released sialic acids, SARP, and standards were then derivatized with DMB. Sialic acid release and labeling steps were performed in a thermocycler. The workflow is illustrated in Figure 1.

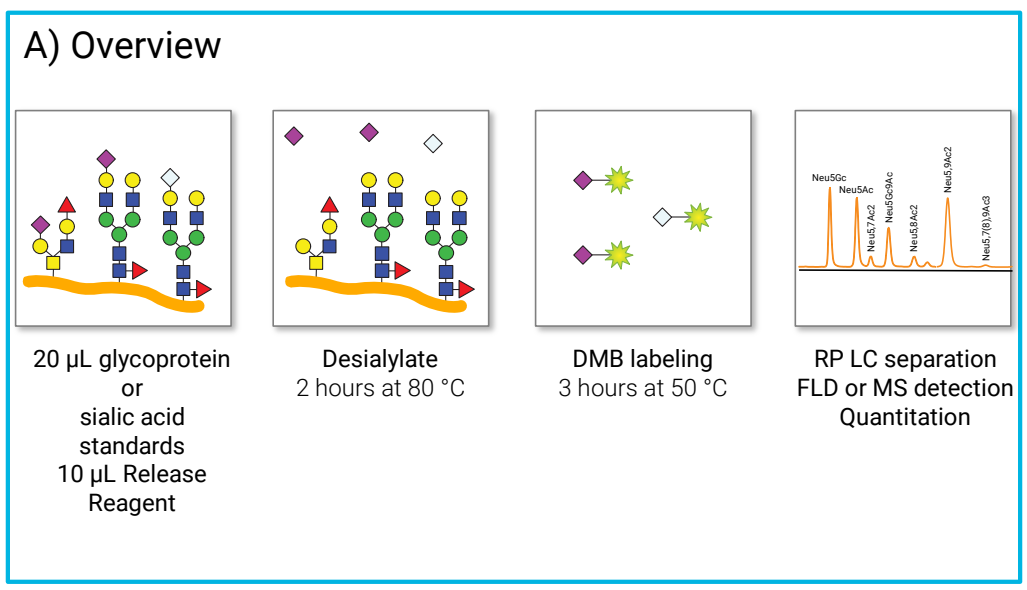

Figure 1A. Sialic acid release and DMB labeling workflow A) overview B) DMB labeling mechanism.

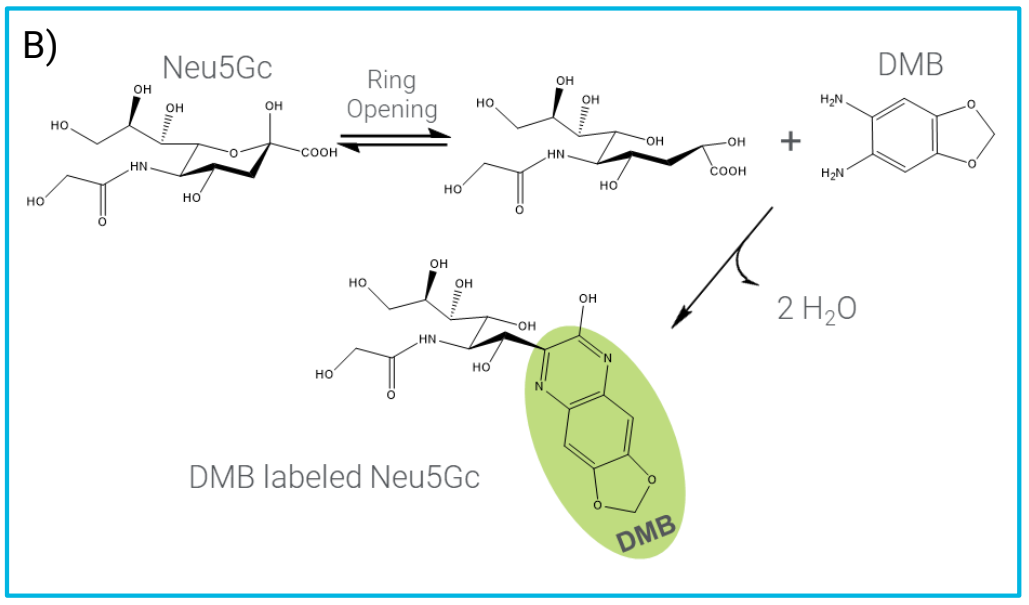

Figure 1B. Sialic acid release and DMB labeling workflow A) overview B) DMB labeling mechanism of sialic acid Neu5Ac

LC/FLD/MS Analysis of DMB Labeled Sialic Acids

DMB labeled sialic acids from Rituxan, Enbrel, NISTmAb and Cetuximab were analyzed using reversed-phase (RP) separation with an Agilent 1290 Infinity II UHPLC system in conjunction with fluorescence detection (FLD) for quantitation. All RP-UHPLC separations were conducted under the conditions described in Table 1. Additional in-line analysis using a 6545XT AdvanceBio LC/Q-TOF (Table 2) was performed to confirm elution order of the DMB-labeled sialic acids present in the SARP. A fixed flow splitter was utilized post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. The data was analyzed with Agilent OpenLab CDS and MassHunter Qualitative Analysis 10.0 software. Neu5Gc and Neu5Ac were quantified using the calibration curves.

Parameter	Value
Instrument	Agilent 1290 Infinity II LC System
Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µm (p/n 697775-902).
Column Temp	30 °C
Mobile Phase	A) Methanol:acetonitrile:water (4:8:88) B) Acetonitrile
Gradient Program	Time (min) / %A / %B / Flow rate (mL/min) 0.00 / 100 / 0 / 0.4 — Isocratic elution 6.00 / 100 / 0 / 0.4 6.25 / 20 / 80 / 0.4 — Wash 7.30 / 20 / 80 / 0.4 7.50 / 100 / 0 / 0.4 — Re-equilibration 10.00 / 100 / 0 / 0.4
Injection Volume	10 µL (Equivalent to 0.25 pmol of Enbrel-10pmol of Rituxan)
Detection	Agilent 1260 Infinity II FLD λ_{Ex} 373 nm, λ_{Em} 448 nm

Table 1. Reversed-phase UHPLC conditions

6545XT AdvanceBio LC/ Q-TOF	
Source	Dual AJS ESI
Gas Temperature	350 °C
Drying Gas Flow	11 L/min
Nebulizer	15 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Vcap	1400 V
Nozzle Voltage	1800 V
Fragmentor	120 V
Skimmer	65 V
Oct 1 VF Vpp	600 V
Mass Range (MS)	*m/z* 400-1000
Mass Range (MS/MS)	*m/z* 100-550
Acquisition Mode	High resolution (4 GHz)

Table 2. 6545XT AdvanceBio LC/Q-TOF parameters

LC/FLD/MS analysis of DMB Labeled SARP

RP-UHPLC analysis of DMB labeled SARP results in the separation and detection of six sialic acid derivatives: Neu5Gc, Neu5Ac, Neu5,7Ac2, Neu5Gc,9Ac, Neu5,8Ac2, Neu5,9Ac2, and Neu5,7(8),9Ac3. While differences in retention times may be observed with different columns, flow rate, solvents or laboratory conditions, the elution order of DMB derivatized sialic acids remain consistent. The reference panel is used to evaluate the resolution and accuracy of the chromatographic system at the beginning of the sample sequence. A typical chromatogram of DMB-labeled SARP is shown in Figure 2. Identification of the DMB-sialic acid derivatives were confirmed by mass spectrometry (Figure 2b).

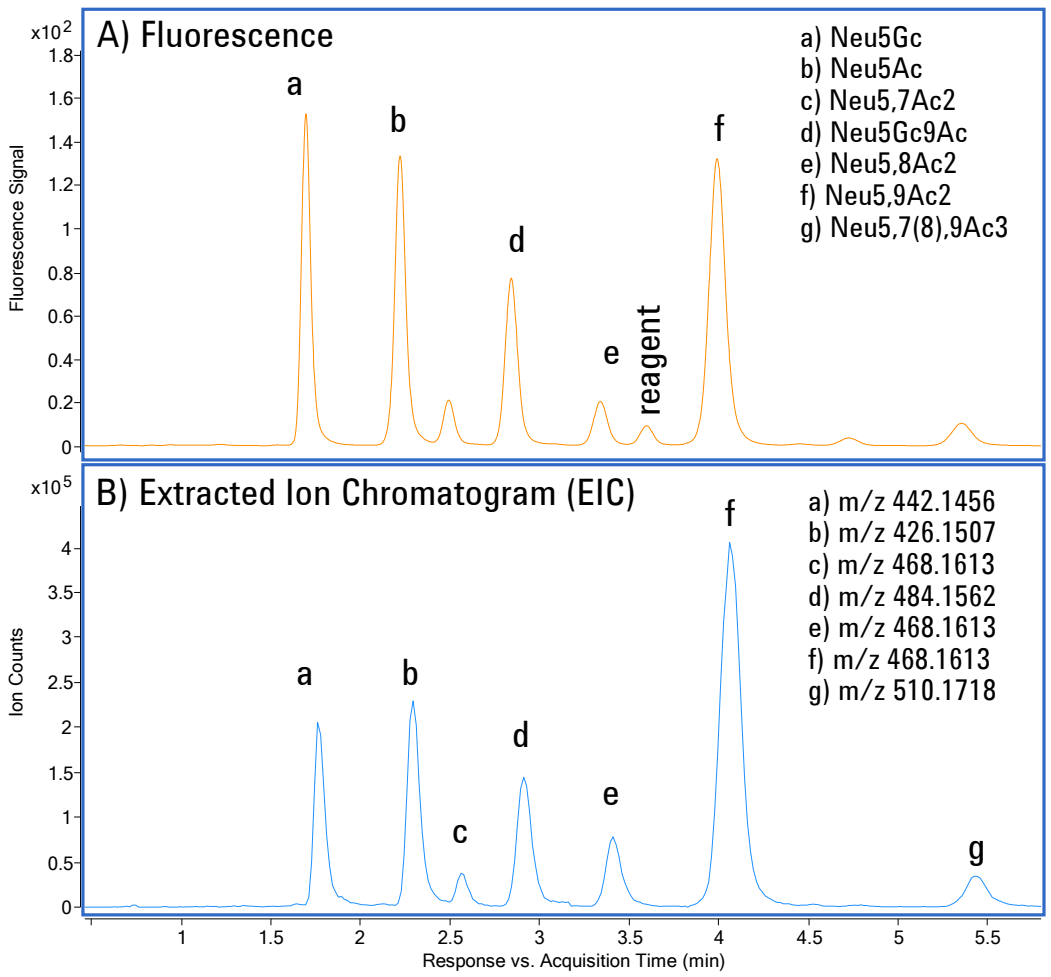

Figure 2. UHPLC chromatogram of DMB labeled SARP. A)fluorescence B) Extracted ion chromatogram of DMB labeled sialic acid species, $[M+H]^{1+}$.

Analysis of Sialic Acid Content of Biotherapeutics and NISTmAb

DMB labeled sialic acids identified by applying the workflow to Rituxan, Enbrel, Cetuximab and the NISTmAb are shown in Figure 3. Both Rituxan (Figure 3A) and Enbrel (Figure 3B) contain primarily Neu5Ac while NISTmAb (Figure 3C) and Cetuximab (Figure 3D) contains primarily Neu5Gc. Mass spectra of major peaks in DMB labeled samples from Enbrel and Cetuximab confirm their identities as Neu5Ac and Neu5Gc respectively (Figure 4).

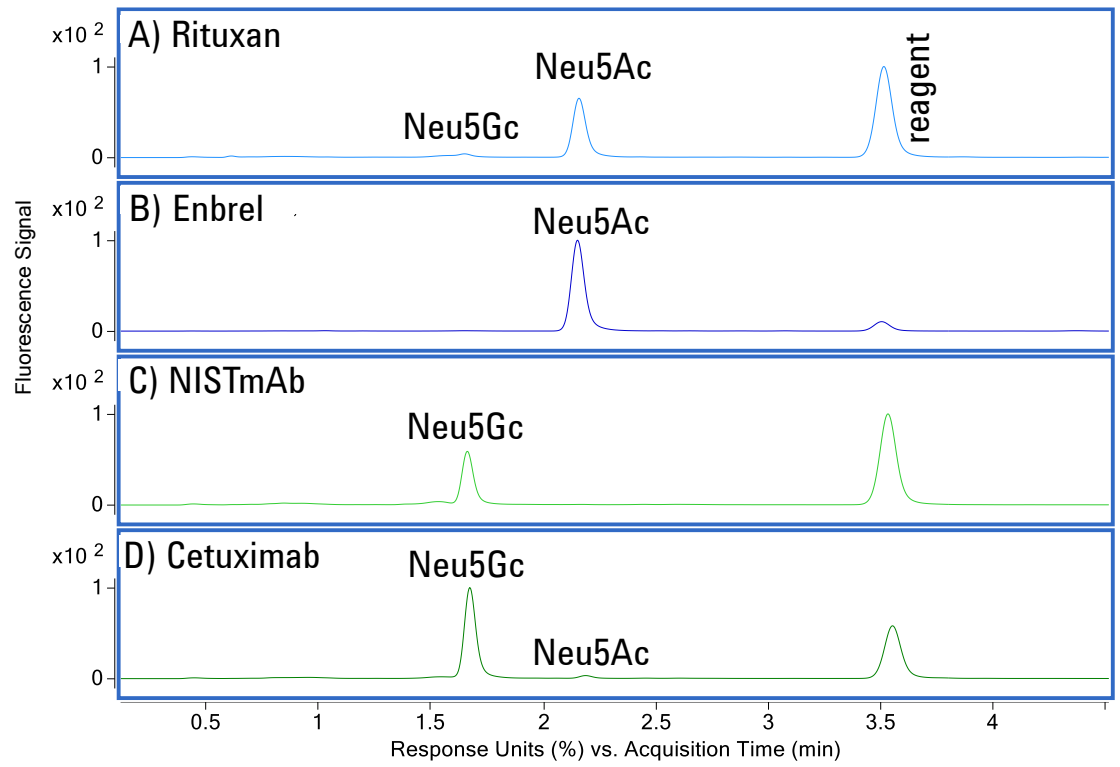

Figure 3. UHPLC fluorescence profiles of DMB labeled sialic acids from different glycoproteins. A) Rituxan, B) Enbrel, C) NISTmAb and D) Cetuximab.

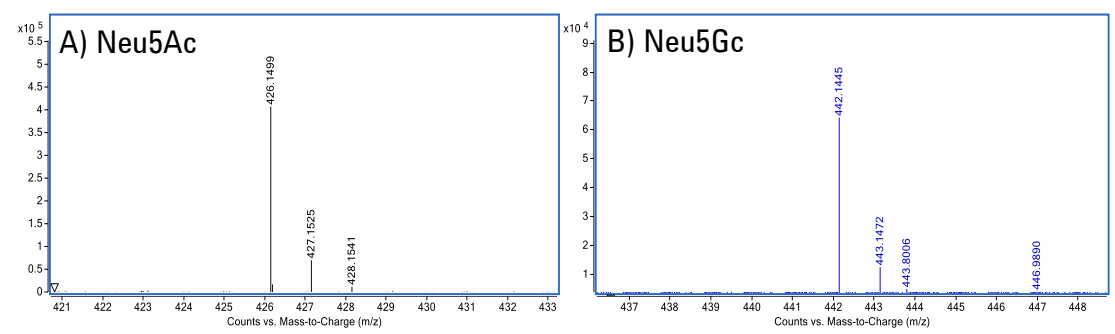

Figure 4. Mass spectra of DMB labeled sialic acid A) Neu5Ac from Enbrel and B) Neu5Gc from Cetuximab.

Quantitative Analysis of Sialic Acid Content

Based on the chromatographic separation and fluorescence response of Neu5Gc and Neu5Ac standards labeled with DMB, a quantitative calibration curve was generated (Figure 5). The LOD and LOQ was calculated using the noise determined by OpenLab CDS using P2P noise calculation (Table 3). The detected molar quantities of Neu5Gc and Neu5Ac from Rituxan, Enbrel, NISTmAb and Cetuximab was determined based on integrated peak areas and are listed in Table 4.

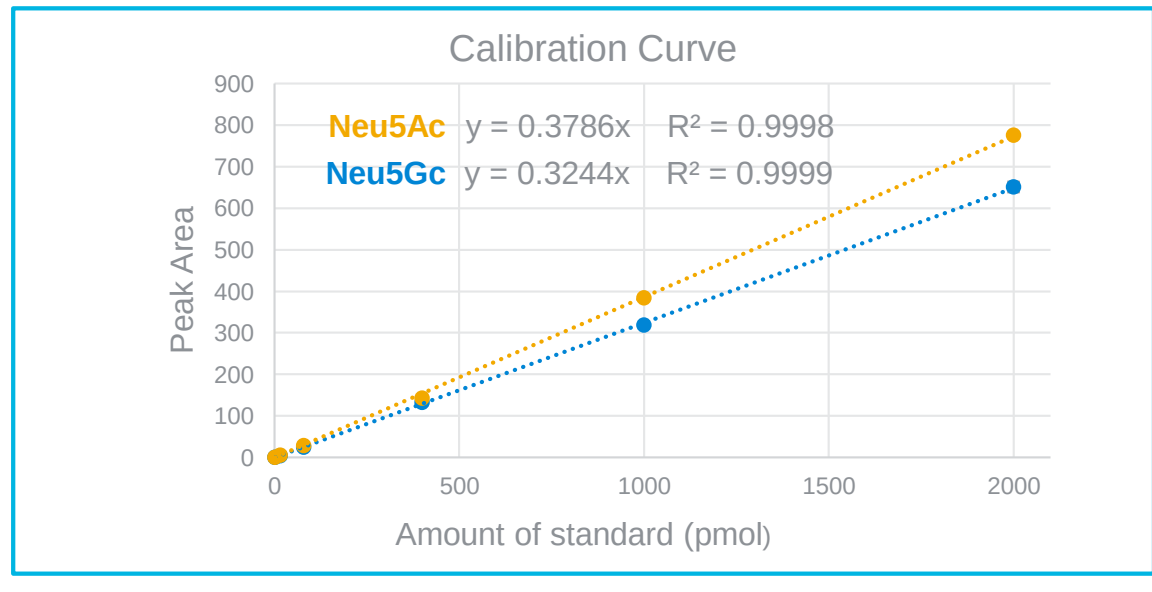

Figure 5. Neu5Gc and Neu5Ac calibration curves, n=2.

Results and Discussion

Sialic Acid	LOD (pmol)	LOQ (pmol)
Neu5Gc	0.012	0.040
Neu5Ac	0.016	0.053

Table 3. LOD and LOQ for Neu5G and Neu5Ac are shown in the table.

	Concentration (mg/ml)	Sample Mass (µg)	Neu5Gc (pmol/µg)	%CV	Neu5Ac (pmol/µg)	%CV
Rituxan	10	200	0.02	1.8%	0.60	4.2%
Enbrel	0.25	5	n.d.	-	228	6.9%
NIST mAb	10	200	0.36	1.8%	n.d.	-
Cetuximab	2	40	3.72	7.1%	0.12	10.9%

Table 4. Compiled table of Table of calculated mol/sialic acid for Rituxan, Enbrel, cetuximab, and NISTmAb. N=3, n.d.=not detectable

Conclusions

•DMB labeled sialic acids shows improved sensitivity for proteins with low levels of sialylation such as monoclonal antibodies with a single N-glycosylation site in the Fc region.

•The updated DMB labeling workflow eliminates the dry down step of samples, decreasing sample preparation time.

•This workflow provides a method to determine both absolute molar quantities and relative % area of Neu5Gc and Neu5Ac in biotherapeutics.

•Sample preparation uses a high throughput 96 well plate format, and is highly reproducible.

•Quantitative data is comparable to older DMB labeling workflows (GKK-407) and AdvanceBio total sialic acid quantitation kit (GS48-SAQ) results (data not shown).

References

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