2-Amino Benzamide Labeling of Oligosaccharides: How Much Sialic Acid Is Lost?

Jeffrey Rohrer,[1] Udayanath Aich,[1] Deanna Hurum,[1] Lipika Basumallick,[1] Chris Pohl,[1] and Sebastian Kandzia[2]
[1]Thermo Fisher Scientific, Sunnyvale, CA, USA; [2]GlycoThera, Hannover, Germany

Thermo SCIENTIFIC

Introduction

Determination of a glycoprotein's asparagine-linked (*N*-linked) oligosaccharide content is one of the important assays in the characterization of a glycoprotein biotherapeutic when the manufacturer needs to produce a product with a consistent state of glycosylation. Due to the recognized importance of sialylation and the terminal positions of sialic acids, the oligosaccharide sialylation state is especially important. A commonly used *N*-linked oligosaccharide assay method labels the released oligosaccharides with a fluorophore by reductive amination prior to separation by liquid chromatography (LC). The conditions used for labeling can potentially lead to oligosaccharide desialylation. 2-Amino benzamide (2-AB) is a popular label for this purpose. This presentation evaluates the extent of sialic acid loss during 2-AB labeling of *N*-linked oligosaccharides released from three common glycoproteins, as well as of sialylated oligosaccharide reference standards. High-performance LC (HPLC) with fluorescence and/or mass spectrometric detection and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) of both labeled and unlabeled oligosaccharides were used to evaluate oligosaccharide desialylation. For more highly sialylated oligosaccharides, the loss of sialic acids is greater than the <2% value commonly cited. We discuss the experimental reasons for the discrepancy between our findings and the <2% value.

Methods

Experimental details can be found in reference 1 and the experimental workflow in Figure 1. Briefly, bovine fetuin, human α1-acid glycoprotein (AGP), and human polyclonal IgG were treated with PNGase F to release *N*-glycans. A portion of these glycans were labeled with 2-AB. Native and labeled glycans were analyzed by both HPAE-PAD and HPLC with fluorescence detection (HPLC-FLD) and/or HPLC-mass spectrometry (MS). A Thermo Scientific™ Dionex™ ICS-3000 or ICS-5000+ system was used for all HPAE-PAD analysis and a Thermo Scientific™ UltiMate™ 3000 Rapid Separation LC (RSLC) system was used for HPLC-FLD and HPLC-MS analyses. A Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap Mass Spectrometer was also used for analysis.

Chromatographic Conditions: Oligosaccharides

	Method 1 (HPAE-PAD)	Method 2 (HPLC-FLD)
Column	Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard (3 × 30 mm) and Analytical (3 × 250 mm)	Thermo Scientific GlycanPac™ AXH-1, 1.9 µm (2.1 × 150 mm)
Mobile Phases	(A) DI water; (B) 100 mM NaOH; and (C)100 mM NaOH + 200 mM sodium acetate	(A) 80% MeCN; (B) 80 mM ammonium formate, pH 4.4
Gradient	0–30 min 50–100% B (50–0% A); 30.1–35 min 100% B; 35.1–50 min 0–100% C (100–0%B); 50.1–60 min (100% C); 60.1–75 min 50% B (50% A)	0–30 min 2.5–12.5% B, 30.1–35 min 12.5–25% B, 35.1–40 min 25–37.5% B, 40.1–50 min 2.5% B
Total Run Time	75 min	50 min
Flow Rate	0.5 mL/min	0.4 mL/min
Injection Volume	5.0 µL	5.0 µL
Temperature	30 ºC	30 ºC
Detection	PAD, Au working electrode, Ag/AgCl ref. electrode, 4-potential waveform[2]	Fluorescence: Ex-320 nm, Em-420 nm

Results

The *N*-glycans from each glycoprotein were separated with conditions optimized for IgG, which has 90% uncharged oligosaccharides (Figure 2). Each separation was consistent with published separations. A HPAE-PAD determination of the sialic acid content of each glycoprotein also suggested they had typical sialylation (not shown). The free Neu5Ac in the HPAE-PAD chromatograms of fetuin and AGP represented 0.004 and 0.047% of their total sialic acids, and therefore sialic acid loss during preparation is insignificant. Oligosaccharides were labeled with 2-AB per Bigge et al.[3] (65 °C 3 h) in triplicate and then separated on a Tosoh TSKgel Amide 80 column. These analyses (not shown) demonstrated that the labeling was reproducible and the chromatograms matched those in the literature, suggesting the labeling was typical. 2-AB glycans were then analyzed by HPAE-PAD under the same conditions as native glycans.

FIGURE 1: Schematic of the experimental workflow to investigate possible loss of sialic acid during 2-AB labeling of *N*-glycans.

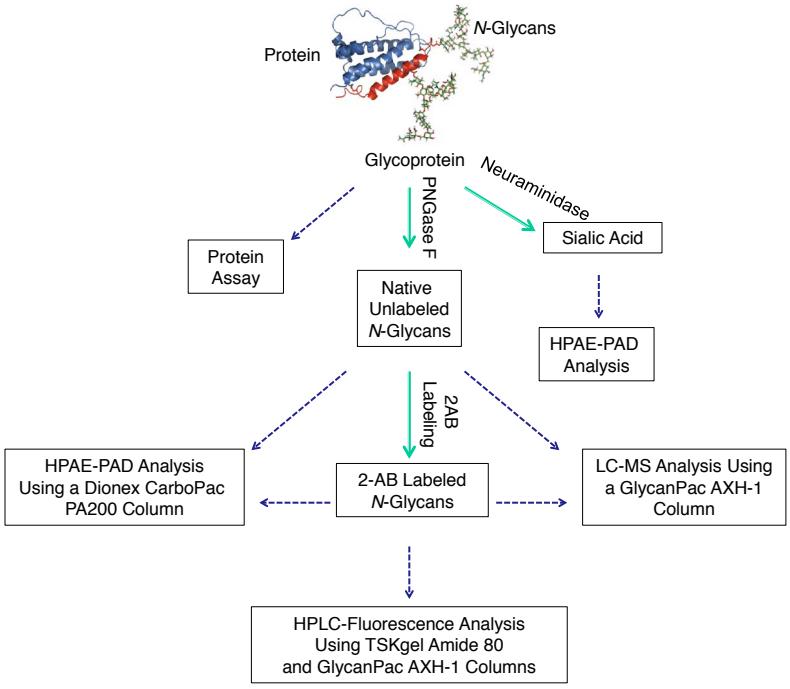


FIGURE 2: HPAE-PAD chromatograms of human IgG, human α_1-acid glycoprotein, and bovine fetuin native *N*-glycans.

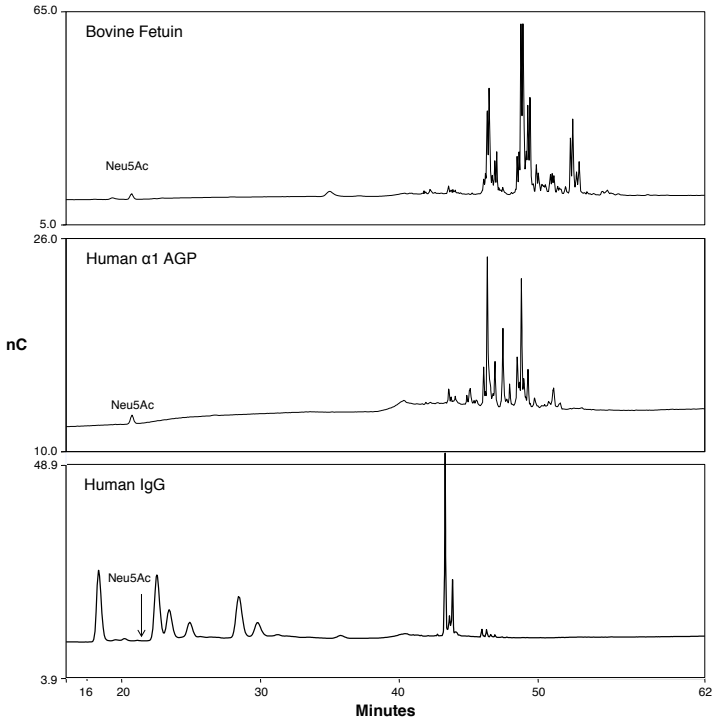
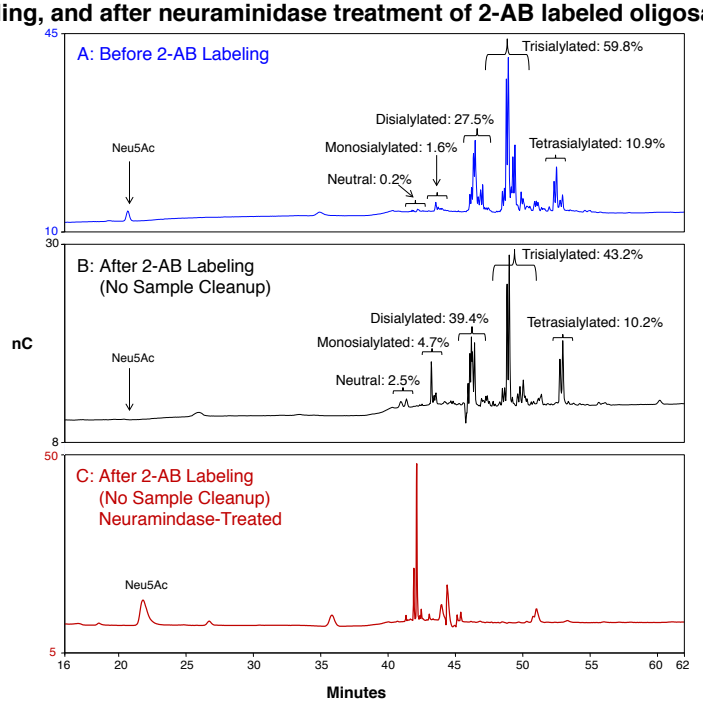


Figure 3 shows the separation of bovine fetuin *N*-glycans before and after 2-AB labeling. No free sialic acid was observed in the 2-AB labeled oligosaccharide preparation, but control experiments demonstrated that free sialic acid is destroyed under 2-AB labeling conditions (not shown). The 2-AB-oligosaccharides elute in the same order as native, but there appears to be >2% desialylation after labeling. Oligosaccharides were grouped by their degrees of sialylation and the displayed percentage values are percents of the total peak area. Sialylation was confirmed with neuraminidase treatment (Figure 3C).

FIGURE 3: Separation of bovine fetuin *N*-linked oligosaccharides before and after 2-AB labeling, and after neuraminidase treatment of 2-AB labeled oligosaccharides.



The same samples were analyzed by LC-MS (Figure 4). This analysis also suggested greater than 2% sialic acid loss for the 2-AB labeled glycans. Sialic acid loss of >2% was also observed for AGP, but not IgG (not shown). To investigate this further we labeled a series of sialylated *N*-glycan standards with 2-AB and analyzed the standards by HPAE-PAD before and after labeling (Figures 5 and 6).

FIGURE 4: LC-MS analysis of unlabeled and 2-AB-labeled bovine fetuin *N*-glycans.

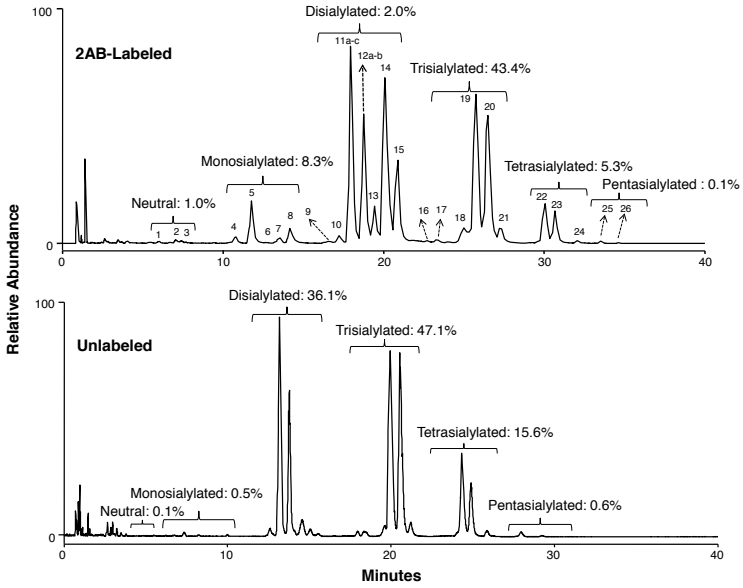


Figure 5 shows that for a tetrasialylated tetraantennary oligosaccharide there is >2% sialic acid loss during 2-AB labeling with the generation of a significant quantity of trisialylated oligosaccharide and a small quantity of disialylated oligosaccharide. Overnight labeling at 37 °C appears to eliminate disialylation, but labeling is incomplete (Figure 5C). Other multiply sialylated oligosaccharides also exhibited significant sialic acid loss during 2-AB labeling (not shown). Conversely, when a monosialylated oligosaccharide was labeled, it only lost a small amount of its sialic acid (Figure 6).

Figure 5: Representative HPAE-PAD profiles of a tetrasialylated reference *N*-linked oligosaccharide structure before and after 2-AB labeling.

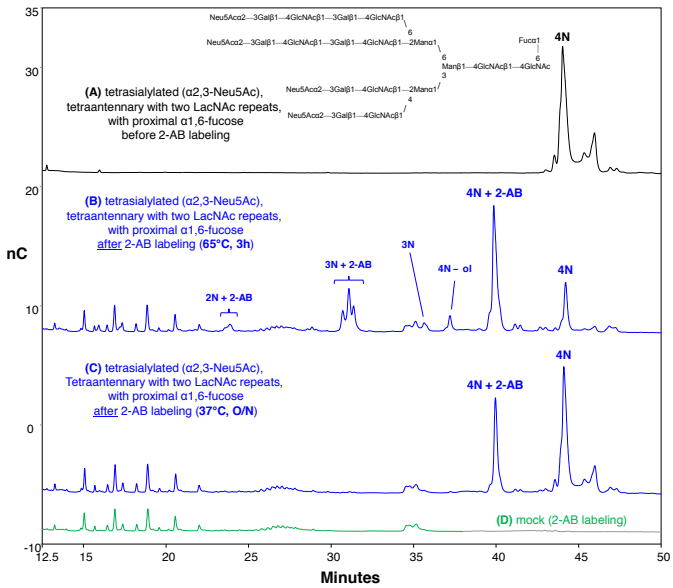
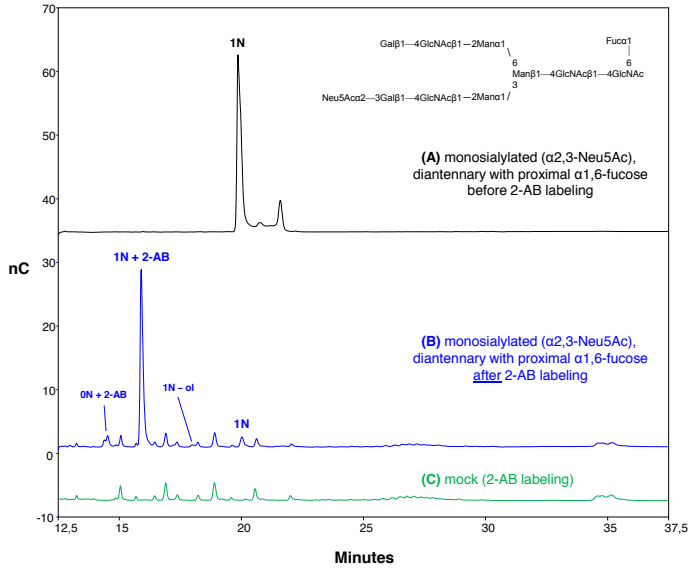


Figure 6: Representative HPAE-PAD profiles of a monosialylated reference *N*-linked oligosaccharide structure before and after 2-AB labeling.



Discussion

This series of experiments shows that there is >2% loss of sialic acid for glycans containing two or more sialic acids under the conditions typically used for 2-AB labeling. This contradicts the <2% cited in reference 3, but is consistent with Stadlmann et al. that also showed that α2,3 linked sialic acid was more readily lost that α2,6 linked sialic acid.[4] We also found that more sialic acid was lost for a disialylated glycan with all α2,3 linked sialic acid compared to the same glycan with all α2,6 linked sialic acid (not shown). A careful review of the experiments that measured sialic acid loss in reference 3 shows that our data does not contradict those results. Sialic acid loss was evaluated with three monosialylated glycans (the experimental design precluded using multiply sialylated glycans). We also found little sialic acid loss with a monosialylated glycan (Figure 6) and for IgG, which has mainly monosialylated glycans. Given that the conditions for labeling glycans by reductive amination using other labels are similar, we expect desialylation with those labeling reactions too, and have observed that with anthranilic acid labeling of glycan standards (not shown).

Conclusions

- Loss of sialic acid is >2% for *N*-glycans with >1 sialic acid during 2-AB labeling under typical conditions.
- α2,3-linked sialic acids are lost more readily than α2,6-linked sialic acids, suggesting this will be more significant for glycoproteins expressed in Chinese Hamster ovary cells.
- Characterization of a glycoprotein's *N*-glycan sialylation state should also use techniques that analyze native glycans, such as LC-MS and HPAE-PAD.

References

1. Aich, U., Hurum, D.C., Basumallick, L., Rao, S., Pohl, C., Rohrer, J.S., Kandzia, S. *Anal. Biochem.* **2014**, *458*, 27–36.
2. Thermo Scientific Technical Note 21. Sunnyvale, CA, 2013. [Online] www.thermoscientific.com/content/dam/tfs/ATG/CMD/CMD%20Documents/Application%20&%20Technical%20Notes/Chromatography/Ion%20Chromatography/IC%20and%20RFIC%20Columns/TN-21-Optimal-Settings-Pulsed-Amperometric-Detection-Carbohydrates-ED40-TN70670-EN.pdf (accessed Oct 16, 2014).
3. Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.M., Charles, S.M., Parekh, R.B. *Anal. Biochem.* **1995**, *230*, 229–238.
4. Stadlmann, J., Pabst, M., Altmann, F. *J. Clin. Immunol.* **2010**, *30(Suppl 1)*, S15–S19.

www.thermofisher.com/chromatography

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 9 3291 0200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 6 6885 1213
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC

A Thermo Fisher Scientific Brand

PN71420-EN 10/16S