Sialic Acid Determination in Glycoproteins: Comparison of Two Liquid Chromatography Methods

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Introduction

Sialic acids are critical in determining glycoprotein bioavailability, function, stability, and eventual catabolism. Although over 50 natural sialic acids have been identified, two forms are commonly determined in glycoprotein products: *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Because humans do not generally synthesize Neu5Gc and have been shown to possess antibodies against Neu5Gc, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response. Consequently, glycoprotein sialylation and the identity of the sialic acids play important roles in therapeutic protein efficacy, pharmacokinetics, and potential immunogenicity.

For purposes of illustration, this work compares two independent chromatographic assays developed for sialic acids in five model glycoproteins: calf fetuin, bovine apo-transferrin (b. apo-transferrin), human transferrin (h. transferrin), sheep α_1-acid glycoprotein (s. AGP), and human α_1-acid glycoprotein (h. AGP). Acid hydrolysates of these proteins were used to evaluate analyses by both high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and UHPLC with fluorescence detection (UHPLC-FLD).

Experimental

Method 1

Thermo Scientific Dionex ICS-3000/5000 Ion Chromatography system consisting of:

- SP Single Pump or DP Dual Pump Module
- DC Detector/Chromatography Module (single or dual temperature zone configuration)
- AS Autosampler
- ICS-3000 ED Electrochemical Detector
- Electrochemical Cell
- Disposable Gold Electrode, Au on PTFE
- Reference Electrode, Ag/AgCl
- 10 µL PEEK™ Sample Injection Loop

Method 2

Thermo Scientific Dionex UltiMate™ 3000 RSLC system consisting of:

- SRD-3600 Solvent Rack
- HPG-3400RS Pump with a 350 µL Mixer
- WPS-3000TRS Autosampler
- TCC-3000RS Column Compartment
- Precolumn Heater
- Viper™ Capillary Kit, RS System
- FLD-3400RS Fluorescence Detector with Dual PMT

The Thermo Scientific Dionex Chromeleon™ Chromatography Data System was used for system control and data processing for both methods.

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Sample Preparation

Protein hydrolysis: Add 14 µg, 20 µg, 25 µg, 13 µg, and 7 µg of fetuin, h. transferrin, b. apo-transferrin, h. AGP, and s. APG, respectively, to 1.5 mL microcentrifuge vials with 200 µL of 2 M acetic acid. Hydrolyze the protein solutions by the method of Varki, et al. to preserve O-acetylated sialic acids.[1]

It is strongly recommended that the hydrolysis conditions be optimized for a particular protein of interest. Recommendations for developing experiments to optimize the hydrolysis conditions can be found in the work of Fan, et al.[2] An overview of hydrolysis conditions suitable for HPAE-PAD has been published elsewhere.[3]

Neuraminidase digestion: Add the protein amounts above to 200 µL of 100 mM acetate buffer, pH 5, containing 1 mU of neuraminidase, from *Arthrobacter ureafaciens*. Incubate at 37 °C for 18 h. Prior to injection onto the HPAE-PAD system, dilute the digestion to a total of 500 µL with DI water.

Derivatization conditions: Use a modified neuraminic acids derivatization method (Hara, et al.)[4] with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB). As with the acid hydrolysis, optimization of derivatization conditions for the protein of interest is highly recommended.

Hydrolysate preparation for HPAE-PAD analysis:

Option 1: Lyophilize 50 µL of the hydrolysate described above. Redissolve hydrolysates in 500 µL of DI water.

Option 2: Hydrolyze 150 µg of protein. Dilute the protein hydrolysate 80-fold.

Results

Sialic acids determination is possible by both HPAE-PAD and UHPLC-FLD methods. Figure 1 shows the separation of a sialic acids standard by HPAE-PAD. Both Neu5Ac and Neu5Gc are easily determined within 10 min.[5]

FIGURE 1. Separation of sialic acid standards using a Thermo Scientific Dionex CarboPac PA20 column.

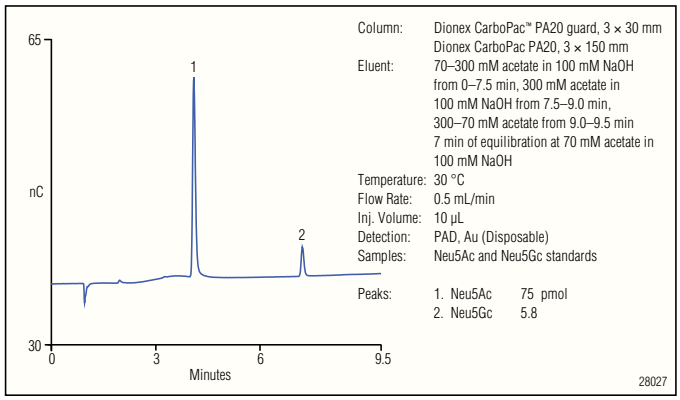

Figure 2 shows the separation of DMB derivatized *N*-acetyl- and *O*-acetylneuraminic acids. Neu5Ac and Neu5Gc are easily determined in 5 min, although the runtime is extended to 10 min to avoid interferences from other components.

FIGURE 2. Separation of DMB derivatized mixed sialic acid standards using a Thermo Scientific Acclaim RSLC 120 C18 column.

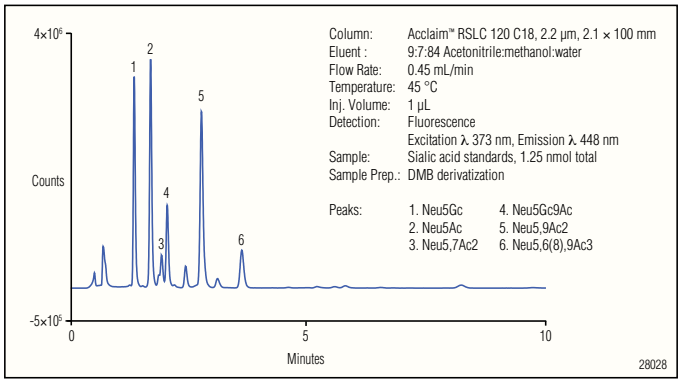

Figure 3 shows representative chromatograms from lyophilized protein acid hydrolysates by HPAE-PAD. The peak for Neu5Ac is well resolved from the void, avoiding interferences from other components in the hydrolysates. As expected, Neu5Gc is not detected in the human glycoproteins.

DMB derivatized acid hydrolysates were separated isocratically using the Acclaim RSLC 120 C18 column. Neu5Ac and Neu5Gc are easily determined, although a reagent peak is present that can interfere with determining Neu5,9Ac2. Figure 4 illustrates the separation.

Triplicate samples of each protein were hydrolyzed and 50 µL portions were analyzed by both HPAE-PAD and derivatization followed by UHPLC-FLD. Table 2 shows the average determined amount by both methods, as well as by HPAE-PAD following neuraminidase digestion.

FIGURE 3. Separation of protein hydrolysates using a Dionex CarboPac PA20 column.

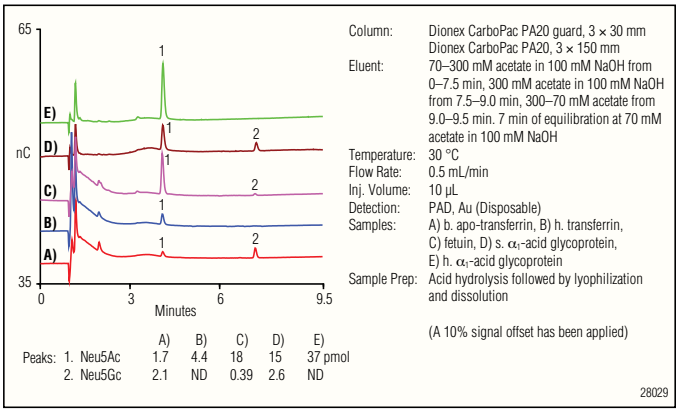

FIGURE 4. Separation of DMB derivatized protein hydrolysates using an Acclaim RSLC 120 C18 column.

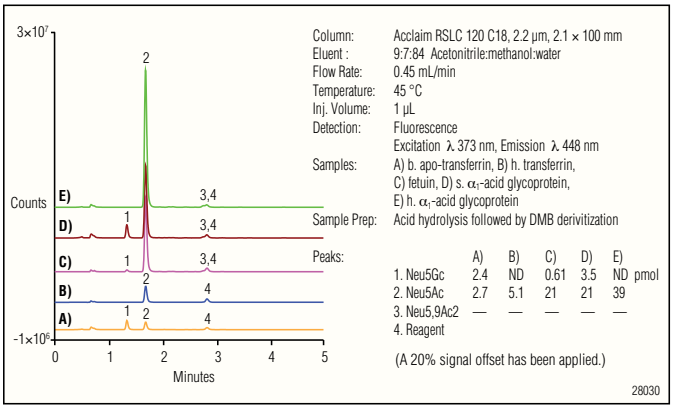

Table 1. Precision, Linearity, LOD, and LOQ of Sialic Acid Determination

Analyte	Method	Ret Time (min)	Ret Time Precision (RSD)	Peak Area Precision* (RSD)	Range (pmol)	Coeff. of Deter. (r^2)	LOQ (pmol)	LOD (pmol)
Neu5Ac	HPAE-PAD	4.13	0.12	1.56	1–100	0.9997	0.50	0.17
	UHPLC-FLD	1.70	0.08	2.00	2.1–50	0.9951	0.05	0.017
Neu5Gc	HPAE-PAD	7.33	0.07	2.62	0.39–7.8	0.9995	0.30	0.080
	UHPLC-FLD	1.35	0.06	0.81	0.16–3.9	0.9951	0.05	0.018

*Peak area precision is measured with a standard of 25 pmol Neu5Ac and 2.0 pmol Neu5Gc.

Table 2. Comparative Analysis, n=3

Sample	Analyte	UHPLC-FLD Average (mol analyte/ mol protein)	HPAE-PAD Average (mol analyte/ mol protein)	Neuraminidase Digestion* HPAE-PAD Average (mol analyte/mol protein)
Fetuin	Neu5Gc	0.46	0.32	0.30
	Neu5Ac	20	14	19
h. Transferrin	Neu5Gc	ND	ND	ND
	Neu5Ac	4.8	3.4	4.8
b. apo-Transferrin	Neu5Gc	1.9	1.6	1.4
	Neu5Ac	1.9	1.2	1.9
h. AGP	Neu5Gc	ND	ND	ND
	Neu5Ac	25	25	30
s. AGP	Neu5Gc	4.0	4.5	3.1
	Neu5Ac	24	26	25

*Amounts determined after neuraminidase digestion have been corrected for dilution of the digest for simple comparison with other data.

Analysis precision within one day and across several days can be highly variable by either method, although between-day precision is slightly better by HPAE-PAD (Table 3).

Table 3. Triplicate Sample Analysis Precision and Between-Day Precision Over 3 Days

Sample	Analyte	Intraday Precision UHPLC-FLD (RSD)	Intraday Precision HPAE-PAD (RSD)	Between-day Precision UHPLC-FLD (RSD)	Between-day Precision HPAE-PAD (RSD)
Fetuin	Neu5Gc	19	22	18	14
	Neu5Ac	18	21	19	13
h. Transferrin	Neu5Gc	—	—	—	—
	Neu5Ac	2.7	7.8	12	8.6
b. apo-Transferrin	Neu5Gc	2.4	8.6	13	7.9
	Neu5Ac	2.4	9.5	9.5	9.4
h. AGP	Neu5Gc	—	—	—	—
	Neu5Ac	6.8	1.7	1.3	8.9
s. AGP	Neu5Gc	6.1	4.6	11	12
	Neu5Ac	6.0	4.6	11	13

Method accuracy was evaluated by recovery studies. Recoveries for the UHPLC-FLD assay ranged from 81.6–108%.In comparison, recoveries for the HPAE-PAD assay were similar, ranging from 76.3–102% when samples were prepared by dilution.

Table 4. Recoveries, Triplicate Hydrolyses

Sample	Analyte	Average Recovery, Derivatization (UHPLC-FLD) (%)	Average Recovery, Dilution (HPAE-PAD) (%)	Average Recovery, Lyophilization (HPAE-PAD) (%)
Fetuin	Neu5Gc	87.5	92.7	75.9
	Neu5Ac	90.7	91.3	78.7
h. Transferrin	Neu5Gc	86.4	99.0	86.4
	Neu5Ac	83.5	99.8	74.9
b. apo-Transferrin	Neu5Gc	102	77.4	74.6
	Neu5Ac	97.9	76.3	84.9
h. AGP	Neu5Gc	86.7	98.8	74.9
	Neu5Ac	86.6	102	74.6
s. AGP	Neu5Gc	101	88.9	84.9
	Neu5Ac	108	87.2	78.9

Conclusions

- Both HPAE-PAD and UHPLC-FLD can determine sialic acids in proteins with similar results, precision, and accuracy.
- UHPLC-FLD requires sample derivatization, which is an additional step that must be optimized for the matrix of the sample, and standards must be prepared in the same matrix. This step can take up to an additional 3 h of sample preparation after hydrolysis.
- The derivatization method for UHPLC-FLD requires strongly acidic reaction conditions, making derivatization of neuramidase digestion samples—which are maintained in a pH 5 buffer—inefficient.
- HPAE-PAD determination detection is direct, requiring no sample derivatization and can be used with neuramidase digestion.

References

1. Varki, A.; Diaz, S. *Anal. Biochem.* **1984,** *137,* 236–247.
2. Fan, J-Q.; Namiki, Y.; Matsuoka, K.; Lee, Y.C. *Anal. Biochem.* **1994**, *219,* 375–378.
3. Rohrer, J.S.; *Anal. Biochem*., **2000**, *283,* 3.
4. Hara, S.; Yamaguchi, M.; Takemori, Y.; Furuhata, K.; Ogura, H.; Nakamura, M.; *Anal. Biochem.*, **1989**, *179,* 162.
5. Dionex Corporation. Direct Determination of Sialic Acids in Glycoprotein Hydrolyzates by HPAE-PAD. Application Update 180, LPN 2831, Sunnyvale, CA, 2011.
6. Dionex Corporation, UHPLC Determination of Sialic Acids with Fluorescence Detection. Application Note 278, LPN 2817, Sunnyvale, CA, 2011.

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