Rapid Glycoprotein Sialic Acid Determination by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

Deanna Hurum and Jeffrey Rohrer, Thermo Fisher Scientific, Sunnyvale, CA, USA

Overview

Purpose: In this work, a fast (<5 min) and direct chromatographic method for sialic acid determination in therapeutic glycoproteins is developed.

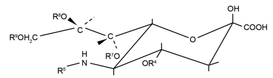
Methods: Determination of two sialic acids, *N*-acetylneuraminic (Neu5Ac) and *N*-glycolylneuraminic (Neu5Gc), is performed in two steps. Acid hydrolysis is used to release the sialic acids, which is then followed by a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) method to determine the amounts of Neu5Ac and Neu5Gc in glycoprotein samples.

Results: Five model glycoproteins were evaluated for sialic acid content: 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). The direct HPAE-PAD analyte determination makes this method appropriate for rapid glycoprotein sample screening and eliminates labeling steps common in other methods.

Introduction

Due to their critical role in protein chemistry, sialic acids (Figure 1) are commonly determined in therapeutic protein products. These carbohydrates are important in controlling glycoprotein bioavailability, function, stability, and metabolism. Because the final glycoprotein sialylation amount and identity varies by expression cell line and growth conditions for that cell line, expression experiments and production optimization have the potential to generate large numbers of samples. Supporting this process requires high-throughput analyses to allow quick evaluation and decision-making during therapeutic protein expression optimization. Although over 50 natural sialic acids have been identified, two forms, Neu5Ac and Neu5Gc, are commonly determined in glycoprotein products.

FIGURE 1. Sialic acids (neuraminic acids).



R^1	$R^{2, 3, 4, 9}$	
– C – CH_3 (O)	– H	(4, 7, 8, 9)
– C – CH_2 (O) – OH	– C – CH_3 (O)	(4, 7, 8, 9)
– C – CH_2 (O) – O – C = O – CH_3	– C – CH – CH_3 (O, OH)	(9)
	– CH_3	(8)
	– SO_3H	(8)
	– PO_3H_2	(9)

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Methods

Sample Preparation

Protein hydrolysis: calf fetuin (80 µg), h. transferrin (144 µg), b. apo-transferrin (175 µg), h. AGP (100 µg), and s. AGP (35 µg) were each added to individual 1.5 mL microcentrifuge vials along with 200 µL of 2 M acetic acid. The protein solutions were hydrolyzed by the method of Varki et al.[1]

It is strongly recommended that the hydrolysis conditions be optimized for each protein. Recommendations for developing experiments to optimize hydrolysis conditions can be found in the work of Fan et al.[2] A review of hydrolysis and sample preparation conditions suitable for HPAE-PAD sialic acid analysis has been previously published.[3]

Dilute the protein hydrolyzate 100-fold before HPAE-PAD analysis.

For greater long-term stability of the hydrolyzate or for extended storage at -40 ° C, lyophilize a 50 µL aliquot of hydrolyzate and then dissolve it in 500 µL DI water.[4]

Liquid Chromatography

Thermo Scientific Dionex ICS-3000 or 5000 ion chromatography system including:
- DP Dual Pump module
- DC Detector/Chromatography M odule
- AS Autosampler
- ED Electrochemical Detector
- Electrochemical Cell
- Disposable Gold on PTFE Working Electrode
- Reference Electrode (Ag/AgCl)

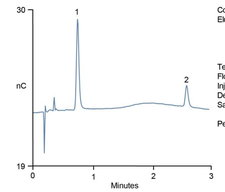
Column:	Thermo Scientific Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent A:	100 mM Sodium Hydroxide
Eluent B:	1.0 M Sodium Acetate in 100 mM Sodium Hydroxide
Gradient:	70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Flow Rate:	0.5 mL/min
Inj. Volume:	4.5 µL (full loop)
Temperature:	30 ° C
Detection:	PAD, Au on PTFE disposable electrode, 2 mil gasket, standard quad. waveform.[5]

Data Analysis

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

Results

FIGURE 2. The chromatogram below illustrates the separation of Neu5Ac and Neu5Gc within 3 min. Neu5Ac is well retained past the void, which is important when analyzing hydrolyzate samples. Neu5Gc elutes in <3 min, allowing a rapid method. This method allows sample analysis as fast as UHPLC methods that require derivatization.[6]



Column: Dionex CarboPac™ PA20 Fast Sialic Acid, 3 × 30 mm
Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (full loop)
Detection: PAD, Au on PTFE, 2 mil gasket
Samples: Neu5Ac and Neu5Gc standard

Peaks:
1. Neu5Ac 11 pmol
2. Neu5Gc 1.1

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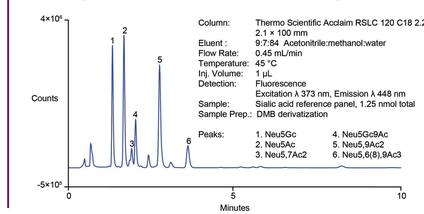
FIGURE 3. Separation of DMB-labeled sialic acid standards by a UHPLC method.[7]



Column: Thermo Scientific Acclaim RSLC 120 C18 2.2 µm, 2.1 × 100 mm
Eluent : 9:7:84 Acetonitrile:methanol:water
Flow Rate: 0.45 mL/min
Temperature: 45 °C
Inj. Volume: 1 µL
Detection: Fluorescence
Excitation λ 373 nm, Emission λ 448 nm
Sample: Sialic acid reference panel, 1.25 nmol total
Sample Prep.: DMB derivatization

Peaks:
1. Neu5Gc
2. Neu5Ac
3. Neu5,7Ac2
4. Neu5Gc9Ac
5. Neu5,9Ac2
6. Neu5,6(8),9Ac3

28028

Analyte	LOQs (pmol)	LODs (pmol)	Linear Range (pmol)	Coeff. of Determination (r^2)
Neu5Ac	0.34	0.11	0.27-68	0.9995
Neu5Gc	0.18	0.058	0.23-11	0.9997

Table 1. Limits of detection (LOD), limits of quantification (LOQs) and linearity of the proposed method. Response is linear between 0.27–68 pmol for Neu5Ac and 0.23–11 pmol for Neu5Gc with coefficients of determination ≥0.999. The correlation may vary from day to day or if different concentration ranges are selected.

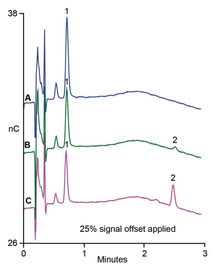
Analyte	Retention Time (min)	Retention Time RSD (%)	Retention Time σ (min)	Peak Area (nC*min)	Peak Area RSD (%)
Neu5Ac	0.745	0.88	0.007	0.3206	1.63
Neu5Gc	2.58	0.32	0.008	0.0865	1.38

Table 2. Method precision, as measured with seven sequential injections of a 11 pmol Neu5Ac and 0.11 pmol Neu5Gc standard, shows excellent peak-area and retention-time precisions. Due to the short elution times, the retention time standard deviation is shown along with the retention time RSD. As seen in Table 2, the retention times are stable within 0.008 min.

Sample Analysis

Each glycoprotein was hydrolyzed, diluted in DI water 1/100, and injected within 24 hours. As expected, Neu5Gc was not detected in human proteins. In each case Neu5Ac is well resolved from other hydrolyzate components, as illustrated in Figures 4 and 5.

FIGURE 4. Separation of fetuin, h. AGP, s. AGP hydrolyzates (1/100 dilution).



Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (full loop)
Detection: PAD, Au on PTFE, 2 mil gasket

Samples:
A) h. α_1-acid glycoprotein, 1:100 dilution (23 ng protein)
B) calf fetuin hydrolyzate, 1:100 dilution (18 ng protein)
C) s. α_1-acid glycoprotein hydrolyzate, 1:100 dilution (7.9 ng protein)

Peaks:

	A	B	C	
1. Neu5Ac	9.8	6.3	5.6	pmol
2. Neu5Gc	—	0.17	0.93	

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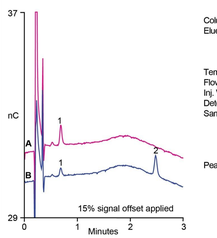
FIGURE 5. Separation of h. and b. transferrin hydrolyzates (1/100 dilution).



Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 × 30 mm
Eluent: 70–300 mM acetate in 100 mM NaOH from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min; 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (full loop)
Detection: PAD, Au on PTFE, 2 mil gasket
Samples: A) h. transferrin hydrolyzate, 1:100 dilution (32 ng protein)
B) b. apo-transferrin hydrolyzate, 1:100 dilution (39 ng protein)

Peaks:

	A	B	
1. Neu5Ac	1.4	0.50	pmol
2. Neu5Gc	—	0.71	

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Precision and Accuracy

Assay precision and accuracy were both evaluated with glycoprotein hydrolyzate samples. Retention-time precision is similar to that of standards, with peak-area precision typical for PAD detection.(<3.5%). Between-day precision for analysis of three samples of each protein was within 11%, however variability of up to 20% has been observed.

Method accuracy was tested by spiking Neu5Ac and Neu5Gc into diluted glycoprotein hydrolyzates. Spiked and unspiked samples were analyzed as soon as possible to reduce acid catalyzed sialic acid decomposition. Overall, recoveries ranged from 81–96% for Neu5Ac and 82–106% for Neu5Gc, suggesting method accuracy (Table 3).

Protein	Analyte	Average Native Amount (pmol)	Added Amount (pmol)	Recovery (%)
Hydrolyzate blank	Neu5Ac	ND	2.2	94 ± 5.8
	Neu5Gc	ND	0.22	92 ± 7.1
Fetuin	Neu5Ac	5.8	2.2	84 ± 1.0
	Neu5Gc	0.18	0.22	86 ± 2.2
h. Transferrin	Neu5Ac	1.6	2.2	95 ± 4.1
	Neu5Gc	ND	0.22	94 ± 2.4
b. apo-Transferrin	Neu5Ac	0.35	1.8	87 ± 5.0
	Neu5Gc	0.45	0.90	95 ± 3.0
h. AGP	Neu5Ac	4.5	3.6	91 ± 1.5
	Neu5Gc	ND	0.36	89 ± 3.4
s. AGP	Neu5Ac	5.8	4.5	94 ± 1.9
	Neu5Gc	1.0	0.45	98 ± 6.9

Table 3. Recoveries of Neu5Ac and Neu5Gc from glycoprotein hydrolyzate samples.

Protein	Analyte	Mol Analyte/Mol Protein, Day 1	Mol Analyte/Mol Protein, Day 2	Mol Analyte/Mol Protein, Day 3	Mol Analyte/Mol Protein, HPAE-PAD Method [4]	Mol Analyte/Mol Protein, UHPLC Method [7]
Fetuin	Neu5Gc	0.33 ± 0.02	0.30 ± 0.03	0.35 ± 0.04	0.32 ± 0.04	0.46 ± 0.05
	Neu5Ac	14 ± 1.4	16 ± 1.8	14 ± 0.77	14 ± 1.5	20 ± 2.4
h. Transferrin	Neu5Gc	ND	ND	ND	ND	ND
	Neu5Ac	3.7 ± 0.27	2.7 ± 0.80	2.9 ± 0.22	3.4 ± 0.38	4.8 ± 0.92
b. apo-Transferrin	Neu5Gc	1.4 ± 0.02	1.3 ± 0.12	1.3 ± 0.14	1.6 ± 0.18	1.9 ± 0.13
	Neu5Ac	1.0 ± 0.02	1.1 ± 0.17	1.3 ± 0.11	1.2 ± 0.13	1.9 ± 0.12
h. AGP	Neu5Gc	ND	ND	ND	ND	ND
	Neu5Ac	24 ± 3.0	30 ± 5.0	32 ± 4.0	25 ± 3.2	25 ± 4.3
s. AGP	Neu5Gc	5.1 ± 0.36	4.5 ± 0.71	4.4 ± 0.10	4.5 ± 0.54	4.0 ± 0.41
	Neu5Ac	29 ± 2.0	25 ± 3.8	24 ± 0.71	26 ± 3.5	24 ± 2.6

Table 4. The proposed method results were compared to two other methods: HPAE-PAD with a 150 mm anion-exchange column and guard column, and sample derivatization followed by UHPLC on a C18 column with fluorescence detection. Results from all three methods are very similar and largely within experimental error.

Method Step	Proposed HPAE-PAD Method	Derivatization Followed by UHPLC-Fluorescence Detection Method
Lyophilization	1.0 (Optional)	Not needed
Derivatization Reagent Preparation	N/A	0.5
Sample/Standard Derivatization and Preparation	N/A	2.5 +0.5
Chromatographic run time, one injection	0.08 (5 min)	0.17 (10 min, 5 min for very clean samples)
Total Time, post hydrolysis	0.25-1.0	3.67

Table 5. The total time necessary for analyzing a set of samples by both this high-throughput method and by derivatization followed by UHPLC with fluorescence detection. Sample analysis time is similar, with drastically reduced sample preparation time for the HPAE-PAD method, saving both time and reagent costs.

Conclusion

- This <5 minute assay allows rapid, direct, and accurate quantification of sialic acids in glycoprotein acid hydrolyzates, providing a convenient screening method.
- The described method is stable with good recoveries, good precision, and linear detection for Neu5Ac and Neu5Gc in the ranges specified.
- This method allows high-throughput results without costly and time-consuming derivatization steps required for UHPLC fluorescence detection methods.

References

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LPN2999-02