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⟨210⟩ MONOSACCHARIDE ANALYSIS

INTRODUCTION

The glycan composition of therapeutic glycoproteins may impact the biological activity, stability, pharmacokinetics, pharmacodynamics, efficacy, and immunogenicity of the molecule. The glycosylation pattern, or specific aspects of it such as the degree of sialylation, may form part of the product characterization and release specifications. One approach to ensuring the consistency of glycosylation and compliance of the product with specifications is to quantify specific monosaccharides present in the product. Monosaccharides are released by degradation of the product and quantified by chromatographic approaches. The most commonly determined sugars are *N*-acetylneuraminic acid (Neu5Ac) and its variant *N*-glycolylneuraminic acid (Neu5Gc). These monosaccharides can be selectively released from the protein either by dilute acid hydrolysis or by enzymatic methods, whereas increasingly aggressive hydrolysis conditions release neutral monosaccharides including mannose, galactose, and fucose, and then glucosamine and galactosamine. Separation and quantification of released monosaccharides by high-performance liquid chromatography (HPLC) can be achieved with or without fluorophore labeling.

SAMPLE HANDLING PRIOR TO MONOSACCHARIDE RELEASE

The glycoprotein sample should be as free of salts, excipients, and other carrier materials as possible. This is typically achieved by: 1) dialysis against water or a volatile buffer using an appropriate membrane, or ultrafiltration through an appropriate membrane; 2) HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index; 3) sample trapping on a solid-phase extraction (SPE) cartridge, followed by washing away salts and excipients and elution of the required sample. Where residual components are known to be present, their lack of interference in saccharide analysis should be confirmed during validation to verify either no bias or an acceptable level of bias due to matrix components. Sample concentration may be required.

Change to read:

SIALIC ACID QUANTIFICATION

Sialic acids can be analyzed and quantified through the use of high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) without modification, or by the use of 1,2-diamino-4,5-methylenoxybenzene (DMB) labeling and reversed-phase HPLC (RP-HPLC) analysis equipped with a fluorescence detector. The combination of DMB labeling and RP-HPLC with fluorescence detection is significantly more sensitive than HPAEC-PAD analysis.

[NOTE—Under some circumstances, the presence of large amounts of protein may diminish the PAD response. For analysis by HPAEC-PAD, the use of 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), as an internal standard, can be an option to compensate for this effect. An example is to prepare an internal standard solution: 0.1 mM of ▲KDN▲ (USP 1-May-2021) in water. In the final dilution step, add 15 µL of the internal standard solution into each standard or sample solution, and reduce the amount of water added by 15 µL to keep the final volume of solution unchanged. Integrate the Neu5Ac, Neu5Gc, and KDN peaks in each chromatogram, and use the ratio of the Neu5Ac or Neu5Gc peak areas relative to the peak area of KDN for system suitability and sample analysis calculation.]

Sialic acids may be *O*-acetylated, and depending on the protein sample and the analytical procedure selected, it may be necessary to de-*O*-acetylate before sialic acid is released. Normally, dilute acid hydrolysis and anion exchange chromatography analysis lead to de-*O*-acetylation. Enzymatic methods of release can preserve *O*-acetyl groups, and the combined use of DMB labeling and RP-HPLC analysis (as in *Procedure 3*) retains the *O*-acetyl groups during the analytical steps.

A typical de-*O*-acetylation can be achieved by adding 10% of the sample's volume of 1 M sodium hydroxide to the sample, incubating at room temperature for 30 min, and then adding 10% of the sample's volume of 1 M hydrochloric acid to neutralize the reaction (e.g., to 20 µL of sample add 2 µL of 1 M sodium hydroxide, incubate at room temperature for 30 min, and then add 2 µL of 1 M hydrochloric acid to neutralize).

PROCEDURES

Change to read:

• PROCEDURE 1: ENZYMATIC RELEASE AND ANALYSIS BY HPAEC-PAD OF UNLABELED SIALIC ACIDS

Sample buffer: 20 mM sodium acetate prepared as follows. Dissolve 164 mg of sodium acetate anhydrous in 80 mL of water. Adjust with glacial acetic acid to a pH of 5.2, and dilute with water to 100 mL.

Standard stock solution: 50 µmol/L (µM) of [USP N-Acetylneuraminic Acid RS](#) and 2 µM of [USP N-Glycolylneuraminic Acid RS](#) in water

Standard solutions: See [Table 1](#) for typical five-level calibration *Standard solutions*.

Table 1

Standard Solutions	Standard Stock Solution (µL)	Sample Buffer (µL)	Water (µL)	Concentration of Neu5Ac (µM)	Concentration of Neu5Gc (µM)
Blank	0	200	300	0	0
1	10	200	290	1	0.04
2	20	200	280	2	0.08
3	30	200	270	3	0.12
4	40	200	260	4	0.16
5	50	200	250	5	0.2

[NOTE—1 µM = 1 nmol/mL = 1 × 10⁻³ nmol/µL = 1 pmol/µL.]

Sample solution: To 50 µL of 6–60 µg/mL of protein sample (equivalent to about 1.5 nmol of sialic acid) in *Sample buffer* add 4 µL of 0.5 milliunit/µL (2 milliunits) of neuraminidase¹ in water and make up to a total volume of 200 µL with *Sample buffer*. Incubate for 5 h at 37°.

[NOTE—Or use validated sample preparation and time/temperature ranges.] Add 300 µL of water, and mix.

HPAEC analysis

[NOTE—Use either *Chromatographic system 1* or *Chromatographic system 2*.]

Solution A: 0.1 M sodium hydroxide prepared as follows. Dilute 5.28 mL of 50%–52% sodium hydroxide solution with water to 1000 mL.

[NOTE—The solution must be sealed well to avoid absorbing carbon dioxide.]

Solution B: 1 M sodium acetate in 0.1 M sodium hydroxide prepared as follows. Dissolve 82.0 g of anhydrous sodium acetate in 800 mL of water. Pass through a nylon filter of 0.2-µm pore size. Add 5.28 mL of 50%–52% sodium hydroxide solution, and dilute with water to 1000 mL.

Chromatographic system 1

(See [Chromatography \(621\), System Suitability](#).)

Mobile phase: See [Table 2](#).

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	98	2
2	98	2
9.5	82	18
11	82	18
11.5	98	2
17	98	2

Mode: LC

Detector: Integrated amperometric detector with gold electrode (for an example of a waveform, see [Table 4](#))

Columns

Guard: 3-mm × 3-cm; packing [L69](#)

Analytical: 3-mm × 15-cm; packing [L69](#)

Flow rate: 0.5 mL/min

Injection volume: 50 µL

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 8 and 12 min, respectively.]

Chromatographic system 2

(See [Chromatography \(621\), System Suitability](#).)

Mobile phase: See [Table 3](#).

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	93	7
10	70	30
11	70	30
12	93	7
15	93	7

Mode: LC

Detector: Integrated amperometric detector with gold electrode (for an example of a waveform, see [Table 4](#))

Columns

Guard: 4-mm × 5-cm; 10-μm packing [L46²](#)

Analytical: 4-mm × 25-cm; 10-μm packing [L46²](#)

Flow rate: 0.8 mL/min

Injection volume: 50 μL

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 6 and 10 min, respectively.]

Table 4

Time (s)	Potential (V)	Gain Region	Ramp	Integration
0	0.1	Off	Ramp	Off
0.2	0.1	On	Ramp	On
0.4	0.1	Off	Ramp	Off
0.41	-2	Off	Ramp	Off
0.42	-2	Off	Ramp	Off
0.43	0.6	Off	Ramp	Off
0.44	-0.1	Off	Ramp	Off
0.5	-0.1	Off	Ramp	Off

System suitability

Samples: Blank and Standard solutions

Suitability requirements

Chromatogram of the blank: No peaks at the same retention time as Neu5Ac or Neu5Gc, Blank

▲ Coefficient of determination (R^2)▲ (USP 1-May-2021) **calculated for the standard curve:** NLT ▲0.98▲ (USP 1-May-2021), Standard solutions

Relative standard deviation: NMT 5%, Standard solution 3

Analysis

Samples: Standard solutions and Sample solution

For the Standard solutions, generate a Neu5Ac and Neu5Gc standard curve of peak response versus concentrations using least square regression. From the standard curve, determine the concentration of Neu5Ac and Neu5Gc in the Sample solution. Divide the determined Neu5Ac and Neu5Gc concentrations by the protein concentration in the Sample solution, and report as molar ratio (nmol:nmol). [NOTE—Convert the protein concentration from mg/mL to μM (1 μM = 1 nmol/mL).]

• PROCEDURE 2: ACID HYDROLYSIS AND ANALYSIS BY HPAEC-PAD OF UNLABELED SIALIC ACIDS

Sample buffer, HPAEC analysis, System suitability, and Analysis: Proceed as directed in Procedure 1.

[NOTE—*Standard solutions* and *Sample solution* are to be prepared at the same time and subjected to the same hydrolysis and drying procedures. Using vacuum centrifuge for drying is described in the procedures; other suitable drying methods may also be used.]

[NOTE—Sialic standards subjected to the hydrolysis procedure may degrade to a different extent than sialic acids released from a glycoprotein during the procedure. A validated method in which the standard is not subjected to the hydrolysis conditions may be used.]

Standard stock solution: 50 µM of [USP N-Acetylneuraminic Acid RS](#) and 2 µM of [USP N-Glycolylneuraminic Acid RS](#) in water

Standard solutions: Add the amount of *Standard stock solution* to each microcentrifuge tube as indicated in [Table 5](#).

Table 5

Standard Solutions	Standard Stock Solution (µL)	Neu5Ac (nmol)	Neu5Gc (nmol)
Blank	0	0	0
1	10	0.5	0.02
2	20	1	0.04
3	30	1.5	0.06
4	40	2	0.08
5	50	2.5	0.1

Dry each standard in a vacuum centrifuge without heat. Add 100 µL of 2 M acetic acid to each microcentrifuge tube, and hydrolyze all the standards series at approximately 80° for 2 h ± 15 min in a heating block. [NOTE—Or use validated time/temperature ranges.] Allow the tubes to cool to room temperature for approximately 10 min, and briefly centrifuge to ensure the entire sample is in the well of the tube. Dry the solutions in a vacuum centrifuge without heat, and resuspend in 300 µL of water. Before analysis, add 200 µL of *Sample buffer*. The final concentrations are summarized in [Table 6](#).

Table 6

Standard Solutions	Concentration of Neu5Ac (µM)	Concentration of Neu5Gc (µM)
Blank	0	0
1	1	0.04
2	2	0.08
3	3	0.12
4	4	0.16
5	5	0.2

Sample solution: Add desalted protein, approximately 6–60 µg (equivalent to about 1.5 nmol of sialic acid) into a microcentrifuge tube. Dry in a vacuum centrifuge without heat. Add 100 µL of 2 M acetic acid, and hydrolyze at approximately 80° for 2 h ± 15 min in a heating block. [NOTE—Or use validated sample preparation and time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min and briefly centrifuge to ensure the entire sample is in the well of the tube. Dry the sample in a vacuum centrifuge without heat, and resuspend the sample in 300 µL of water. Before analysis add 200 µL of *Sample buffer*.

Change to read:

• **PROCEDURE 3: ENZYMATIC HYDROLYSIS AND ANALYSIS BY RP-HPLC OF DMB-LABELED SIALIC ACIDS**

Labeling reagent: Aqueous solution of DMB, acetic acid, 2-mercaptoethanol, and sodium hydrosulfite³

Standard stock solution: 200 µM of [USP N-Acetylneuraminic Acid RS](#) and 10 µM of [USP N-Glycolylneuraminic Acid RS](#) in water

Standard solutions: See [Table 7](#) for the typical dilutions. Then label each solution as directed by *Labeling*.

Table 7

Standard Solutions	Standard Stock Solution (μL)	Water (μL)	Concentration of Neu5Ac before Labeling (μM)	Concentration of Neu5Gc before Labeling (μM)
Blank	0	400	0	0
1	4	396	2	0.1
2	8	392	4	0.2
3	20	380	10	0.5
4	40	360	20	1
5	80	320	40	2

The concentrations of the resulting labeled *Standard solutions* are listed in [Table 8](#).

Table 8

Standard Solutions	Concentration of Neu5Ac after Labeling (μM)	Concentration of Neu5Gc after Labeling (μM)
Blank	0	0
1	0.02	0.001
2	0.04	0.002
3	0.1	0.005
4	0.2	0.01
5	0.4	0.02

Labeling: Transfer 5 μL of the to-be-labeled *Standard solution* or *Sample solution* into a microcentrifuge tube, add 20 μL of *Labeling reagent*, cap the tube, mix by vortexing, and incubate for 3 h at 50° in the dark. Terminate the reaction by adding 475 μL of water, cap, and vortex thoroughly. This solution should be kept at 2°–8° and analyzed within 24 h. [NOTE—The *Standard solution* and *Sample solution* are to be labeled simultaneously.]

Sample solution: Transfer approximately 0.5–50 μg of protein (equivalent to about 5 pmol of sialic acid) into a 1.5-mL microcentrifuge tube. Perform de-*O*-acetylation if needed. Adjust to a pH of 6.0 ± 0.3. [NOTE—The pH needs to be optimized based on the enzyme used.] Add 2 μL of 5 units/mL (10 milliunits) of neuraminidase,¹ adjust to a total volume of 50 μL, and incubate for 16–24 h at 37°. [NOTE—Or use validated sample preparation and time/temperature ranges.] Label the solution as directed in *Labeling* before analysis.

RP-HPLC analysis

[NOTE—Use either *Chromatographic system 3* or *Chromatographic system 4*.]

Mobile phase: Acetonitrile, methanol, and water (9:7:84)

Chromatographic system 3

(See [Chromatography \(621\)](#), [System Suitability](#).)

Mode: LC

Detector: Fluorescence detector with an excitation wavelength of 373 nm and an emission wavelength of 448 nm

Column: 2.1-mm × 10-cm; 1.9- to 2.2-μm packing [L1](#)

Flow rate: 0.25 mL/min

Injection volume: 5 μL

Temperatures

Column: 30°

Autosampler: 2°–8°

Run time: 10 min (isocratic)

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 2.5 and 2 min, respectively.]

Chromatographic system 4

(See [Chromatography \(621\)](#), [System Suitability](#).)

Mode: LC

Detector: Fluorescence detector with an excitation wavelength of 373 nm and an emission wavelength of 448 nm

Column: 4.6-mm × 15-cm; 3-µm packing [L1](#)

Flow rate: 0.5 mL/min

Injection volume: 5 µL

Temperatures

Column: 30°

Autosampler: 2°–8°

Run time: 30 min (isocratic)

[NOTE—For the system described, typical retention times for Neu5Ac and Neu5Gc are about 10 and 8 min, respectively.]

System suitability

Samples: *Blank* and *Standard solutions*

Suitability requirements

Chromatogram of the blank: No peaks at the same retention time as Neu5Ac or Neu5Gc, *Blank*

▲Coefficient of determination (R^2)▲ (USP 1-May-2021) **calculated for the standard curve:** NLT ▲0.98▲ (USP 1-May-2021), *Standard solutions*

Relative standard deviation: NMT 5%, *Standard solution 3*

Analysis

Samples: *Standard solutions* and *Sample solution*

For the *Standard solutions*, generate a Neu5Ac and Neu5Gc standard curve of peak response versus concentrations using least square regression. From the standard curve, determine the concentration of Neu5Ac and Neu5Gc in the *Sample solution*. Divide the determined Neu5Ac and Neu5Gc concentrations by the protein concentration in the *Sample solution*, and report as molar ratio (nmol:nmol). [NOTE—Convert the protein concentration from mg/mL to µM (1 µM = 1 nmol/mL).]

• **PROCEDURE 4: ACID HYDROLYSIS AND ANALYSIS BY RP-HPLC OF DMB-LABELED SIALIC ACIDS**

Labeling reagent, Blank, Labeling, RP-HPLC analysis, System suitability, and Analysis: Proceed as directed in *Procedure 3*.

[NOTE—*Standard solutions* and *Sample solution* are to be prepared at the same time and subjected to the same hydrolysis and drying procedures. Using vacuum centrifuge for drying is described in the procedures; other suitable drying methods may be used.]

[NOTE—Sialic standards subjected to the hydrolysis procedure may degrade to a different extent than sialic acids released from a glycoprotein during the procedure. A validated method in which the standard is not subjected to the hydrolysis conditions may be used.]

Standard stock solution: 50 µM of [USP N-Acetylneuraminic Acid RS](#) and 2.5 µM of [USP N-Glycolylneuraminic Acid RS](#) in water

Standard solutions: Add the amount of *Standard stock solution* as indicated in [Table 9](#) to each microcentrifuge tube. Dry in a vacuum centrifuge without heating.

Table 9

Standard Solution	Standard Stock Solution (µL)	Neu5Ac (pmol)	Neu5Gc (pmol)
Blank	0	0	0
1	1	50	2.5
2	2	100	5
3	5	250	12.5
4	10	500	25
5	20	1000	50

Add 25 µL of 2 M acetic acid into the tube and briefly centrifuge to ensure the sample is entirely in the well of the tube. Incubate at 80° for 2 h ± 15 min. [NOTE—Or use validated time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min, and then vortex and centrifuge. Label the solution as directed in *Labeling* before analysis. The concentrations of the resulting labeled *Standard solutions* are listed in [Table 10](#).

Table 10

Standard Solution	Concentration of Neu5Ac after Labeling (µM)	Concentration of Neu5Gc after Labeling (µM)
Blank	0	0

Standard Solution	Concentration of Neu5Ac after Labeling (μM)	Concentration of Neu5Gc after Labeling (μM)
1	0.02	0.001
2	0.04	0.002
3	0.1	0.005
4	0.2	0.01
5	0.4	0.02

Sample solution: Transfer a desalted sample containing approximately 0.5–50 μg of protein (equivalent to about 5 pmol of sialic acid) into a 0.5-mL microcentrifuge tube. Dry in a vacuum centrifuge without heating. Add 25 μL of 2 M acetic acid into the tube and briefly centrifuge to ensure all of the sample is in the well of the tube. Incubate at 80° for 2 h ± 15 min. [NOTE—Or use validated sample preparation and time/temperature ranges.] Allow the tube to cool to room temperature for approximately 10 min. Then vortex and centrifuge. Label the solution as directed in *Labeling* before analysis.

Change to read:

- [USP REFERENCE STANDARDS \(11\)](#)
[USP N-Acetylneuraminic Acid RS](#)
[USP N-Glycolylneuraminic Acid RS](#)

▲ (USP 1-May-2021)

- 1 Neuraminidase: recombinant *Arthrobacter ureafaciens* neuraminidase produced in *Escherichia coli* (*E. coli*), or similar enzyme, that releases both α2-3 and α2-6-linked sialic acid. One unit is defined as the amount of enzyme that will hydrolyze 1 μmol of 2'-O-substituted α-D-N-acetylneuraminic acid/min at the specified pH at 37°.
- 2 Ethylvinylbenzene/divinylbenzene substrate available from Thermo as Carbopac PA10, or a suitable equivalent.
- 3 Available from Ludger as LudgerTag Sialic Acid DMB Labeling Kit, catalog #LT-KDMB-A1, or a suitable equivalent. [Note—For example, prepare the DMB reagent in the order that follows. Add 1.5 mL of water to a glass vial. To this solution add 172 μL of glacial acetic acid, and mix well. Add 112 μL of 2-mercaptoethanol, and mix well. Add 4.9 mg of sodium hydrosulfite to the solution, and mix well. The solution may then become cloudy in appearance. Lastly, add 3.5 mg of DMB hydrochloride and 200 μL of water, and mix the solution well. This solution is aliquoted into 200-μL aliquots and can be stored in the dark at –20° for up to 12 months.]

Auxiliary Information - Please [check for your question in the FAQs](#) before contacting USP.

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