

Carbohydrate Composition Analysis of Glycoproteins Using Highly Sensitive Fluorescence Detection Methods

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1. Introduction

Proteins with covalently bound sugars are known as glycoproteins and are widely distributed in nature, e.g., plants, animals, bacteria, and viruses. Hormones, enzymes, and toxins are examples of proteins that are both biologically active and glycosylated. In addition, a number of proteins on the cell's surface, in cytosol, and in nucleus are glycosylated. During the last few years, enormous advances have been made in the understanding of glycoproteins, specifically their structure and biochemistry. This increased understanding is primarily due to the advent of new tools for the study of complex carbohydrates. These new analytical methods have allowed for the reporting of a large number of well-characterized glycoproteins. The complete analysis of a glycoprotein provides information on the primary structure of the oligosaccharides as well as their variation at individual glycosylation sites. Such analysis requires a multi-pronged approach involving mapping and characterization of oligosaccharides, which is described in Chapter 9 of this book, and determination of carbohydrate composition.

Carbohydrate composition analysis of glycoproteins is similar to amino acid analysis of proteins. Just as an accurate amino acid composition is critical to protein-structure determination and identification by database searching, the accurate determination of the carbohydrate composition of a glycoprotein provides information as to the type and extent of glycosylation. In order

to determine and understand the structure of a glycoprotein, the individual monosaccharides present must be identified and quantitated. This is critical since as a class, hexoses and hexosamines have the same molecular weights, and therefore, can not be determined by mass spectrometry.

Another aspect to carbohydrate analysis became apparent with the advent of glycoprotein biopharmaceuticals. With respect to biopharmaceuticals, there is a need to demonstrate consistency of glycosylation in production lots that are intended for human therapy. In addition, there is an increasing demand to provide a well-characterized product description for regulatory submissions. One aspect, which can be used to determine the consistency of glycoprotein production lots, is the amount of carbohydrate it contains (expressed in % carbohydrate). The other aspect is monitoring of the sialic acid content in glycoprotein drugs, which is important since sialic acid may be critical for biological function (1,2).

For the last decade, high performance anion exchange chromatography with pulsed amperometric detection method (HPAE-PAD) was used for determining carbohydrate composition (3,4). Today, newer highly sensitive and reproducible methods using reversed phase high-performance chromatography (RP-HPLC) with fluorescence detection are available and described in this report. The HPLC methods for carbohydrate composition, namely of monosaccharides and sialic acids, are based on pre-column derivatization with fluorescent tags.

Carbohydrate composition is determined by acid hydrolysis of a glycoprotein sample to release the individual monosaccharides. After hydrolysis, the monosaccharides (neutral and amino sugars) are derivatized with anthranilic acid (AA, 2-aminobenzoic acid), and then separated from each other and from excess reagent using RP-HPLC (5,6). The resulting peak areas are compared to those of concomitantly derivatized and analyzed monosaccharide standards to determine the amount of each monosaccharide in the sample.

For sialic acid determination, the sialic acids are initially released from the glycoprotein by mild acid hydrolysis followed by derivatization with o-phenylenediamine (OPD) to yield a fluorescent quinoxaline derivative. The derivative is separated from excess reagent by RP-HPLC for quantitation using fluorescence detection (6,7). The resulting peak areas are compared to those of concomitantly derivatized sialic acid standards to determine the amount of the sialic acids (*N*-acetyl and *N*-glycolylneuraminic acids).

The methods described here are based on the use of highly fluorescent tags. They offer the most sensitive approach to analyze glycoproteins at this time, and therefore, these methods are suitable for analyzing samples available in limited amounts.

2. Materials (see Notes 1 and 2)

2.1. Excipient Removal and Protein Concentration Determination

1. 2% (w/v) Ammonium bicarbonate.
2. Ethanol:ethyl acetate mixture 1:1 (v:v) with 0.5% acetic acid (see **Note 3**).
3. 5% (v/v) Acetic acid solution (used with basic glycoproteins only).
4. 50 mM Sodium hydroxide (used with acidic glycoproteins only). Store in a plastic container.
5. Glass tubes 13 × 100 mm with Teflon-lined screw caps (Reactor Vessels Oxford GlycoSciences, cat no. I-4022).
6. Vacuum centrifuge.

2.2. Monosaccharide Analysis

1. Neat trifluoroacetic acid (TFA).
2. 1% (w/v) Aqueous sodium acetate solution.
3. Polypropylene vials (1.6 mL) with O-ring seal screw caps (Fisher, 118448 or 2.5.15. National Scientific, BC16NA-BP) (see **Note 4**).
4. 4% (w/v) Sodium acetate (trihydrate)-2% (w/v) boric acid (granular) in methanol (see **Note 3**).
5. Anthranilic acid (AA) solution: Weigh approx 45 mg of anthranilic acid (2-amino benzoic acid) into a polypropylene vial. Add approx 30 mg of sodium cyanoborohydride. Dissolve the solids in 1.5 mL of the sodium acetate-boric acid-methanol solution. (*Note*: Sodium cyanoborohydride is a poison and tends to absorb moisture readily from the air, which may affect the derivatization reaction. Limit the exposure of this chemical to air when weighing.)
6. Monosaccharide Standard Solution (1.0 mM): Weigh exactly 43.0 mg each of glucosamine hydrochloride and galactosamine hydrochloride and 36.0 mg each of galactose, mannose, and glucose into a 200-mL volumetric flask. Add 32.8 mg of fucose into the same volumetric flask and bring to volume with Milli-Q water. Mix well and aliquot small volumes for storage. All the monosaccharides were from either Sigma or Pfansteihl Labs. Expiration: 1 yr at -20°C if used more than once (see **Note 5**). Monosaccharide Working Standard Solution: Dilute the 1.0 mM monosaccharide standard solution 1:100 with Milli-Q water (see **Note 6**).
7. Chromatographic solvents: see **Subheading 2.4**.
8. Temperature controlled oven and/or heating block

2.3. Sialic Acid Analysis

1. 0.5 M Sodium bisulfate (NaHSO₄).
2. 0.25 M NaHSO₄: dilute the 0.5 M sodium bisulfate solution with an equal volume of Milli-Q water.
3. OPD derivatization solution: prepare a 20 mg/mL OPD solution in 0.25 M sodium bisulfate.

4. 0.05% (v/v) Acetic acid-water solution for preparing the sialic acid stock standard.
5. 1.0 mM Sialic acid standard stock solution made in 0.05% acetic acid-water (*see Note 7*). Sialic acid working standard solution: dilute the sialic acid standard solution 1 : 100 to 500 with 0.25 M NaHSO₄ (*see Note 8*).
5. Chromatographic solvents: *see Subheading 2.4.* below.
6. Temperature controlled oven and/or heating block.

2.4. Chromatography System

1. HPLC with a fluorescence detector (highly sensitive fluorescence detectors such as Jasco FP 920, Waters 474, and HP 1100 were used in these studies.
2. Thermostatted column compartment.
3. C18 Column: Waters Symmetry (3.9 × 150 mm) for monosaccharide analysis.
4. C18 Column: Ultrasphere ODS (4.6 × 150 mm, 5 μm, Beckman, cat no. 235330) for sialic acid analysis.
5. Column prefilter (Upchurch Scientific, A-315) and 0.2 μm insert (Upchurch Scientific, A-101X).
6. The following solvents were used for the analysis of both monosaccharides and sialic acids: Solvent A is composed of 0.2% (v/v) 1-butylamine (Aldrich), 0.5% (v/v) phosphoric acid, and 1.0% (v/v) tetrahydrofuran (inhibited, Aldrich) in Milli-Q water (*see Notes 9 and 10*). Solvent B: Dilute solvent A with an equal volume of acetonitrile (HPLC grade) (*see Note 10*).

3. Methods

3.1. Removal of Formulation Ingredients and Protein Concentration Determination

Sugars (sucrose, mannitol), detergents, buffers, and so on, may also be present as excipients in the glycoprotein or biopharmaceutical samples. These excipients are likely to interfere in the analysis, and therefore, these substances must be removed prior to analysis of the sample. If the sample quantity is limited, a variety of desalting methods including dialysis and desalting using non-Sephadex resins, new types of centrifuge filters and mini-columns, and so on, could be used to remove the excipients. Samples can also be desalted by drying directly onto polyvinylidene difluoride (PVDF) membranes. In addition, glycoproteins that have been electroblotted onto a PVDF membrane following gel electrophoresis can be analyzed. This line of approach is useful when small amounts of the sample are available or several proteins exist as a mixture. Any gel technique may be used to perform this step. Once the band of interest has been identified (preferably on PVDF), it is excised and cut into small pieces. The pieces are placed in the bottom of the vial before proceeding with the hydrolysis step in **Subheading 4.3.1.** (*see Note 11*).

The following procedure is routinely used for removal of excipients from formulated biopharmaceuticals. If the amount of glycoprotein sample is limited, the procedure may be followed by reducing the reagents proportionally or by selecting another method for desalting as mentioned previously.

3.1.1. Removal of Formulation Ingredients (Excipients) (see **Note 12**)

1. Place about 1.0–2.0 mg of each sample into each of two or four separate glass tubes (13 × 100 mm) with Teflon-lined screw caps.
2. Add 0.5 mL of 2% ammonium bicarbonate solution to each and vortex briefly.
3. Add 2 mL of the ethyl acetate/alcohol mixture to each vessel. Cap the vials and mix on a Vortex for about 20 s.
4. Allow the samples to stand for at least 10 min after vortexing.
5. Centrifuge the samples for about 5 min at the maximum setting in a suitable centrifuge.
6. Remove the samples from the centrifuge without disturbing the pellet.
7. Decant the supernatant from the pellet into a suitable waste container.
8. Invert the vial on a clean tissue paper (Kimwipe) placed at the bottom of a test tube rack for a minute to allow the residual solvent to drain (*see Notes 13–15*).
9. Repeat **steps 2–8** for a total of 4 precipitations.
10. If the glycoprotein is basic: After the fourth extraction, invert the vial on a tissue paper (Kimwipe) and allow the protein pellet to dry to insure that all the organic solvent has been removed (approx 5 min). Add about 300 µL of 5% acetic acid solution to each vial and mix well. Approximate protein concentration should be 5 mg/mL. Skip to **step 14**.
11. If the glycoprotein is acidic: After draining the supernatant from the fourth extraction, cover the vials with several layers of parafilm and puncture the parafilm 5–7 times with a clean needle.
12. Dry the pellets in a vacuum centrifuge for 5–10 min (*see Note 16*).
13. Add about 300 µL of 50 mM sodium hydroxide to each vial and mix well. Approximate protein concentration should be 5 mg/mL.
14. Vortex the samples for about 30 s and allow them to stand for at least 48 h to clarify. Intermittent vortexing is recommended throughout the 48 h period (*see Note 17*).

3.1.2. Protein Concentration Determination

Each sample should be clear at this point (*see Note 18*). Pool the contents of the clarified samples if additional amount of protein is required (*see Note 19*).

1. Prepare a 10-fold dilution of the pooled material using Milli-Q water (acidic samples) or 5% acetic acid solution (basic samples). This diluted purified sample will be used in the monosaccharide analysis.

2. Read the absorbance of the diluted material at 280 nm against a blank, which has been diluted in similar manner to the sample (*see Note 20*).
3. Divide the observed absorbance by the extinction coefficient of that protein to obtain the protein concentration (*see Note 21*).

3.2. Analysis of Monosaccharides

This section describes a method for the determination of carbohydrate composition of glycoproteins (5,8). Carbohydrate composition is determined by acid hydrolysis of a glycoprotein sample to release monosaccharides. After hydrolysis, the monosaccharides are derivatized with anthranilic acid (a highly sensitive fluorescent tag) and separated from excess reagent using RP-HPLC. Monosaccharide standards are concomitantly derivatized with the samples and analyzed. The resulting peak areas are compared to determine the amount of each monosaccharide present.

3.2.1. Hydrolysis

1. Place 0.1 mL aliquots of the sample into three separately labeled sample vials or an excised and minced PDVF band into a vial for monosaccharide analysis. Place 0.1 mL of the appropriate blank into the fourth vial (*see Note 22*).
2. Add 0.3 mL of Milli-Q water and mix gently.
3. Add 0.1 mL of neat TFA to each vial using a positive-displacement pipet, cap tightly, seal with 4–6 layers of Teflon tape and vortex gently on a low setting.
4. Hydrolyze the samples by placing them in a temperature-controlled oven at $100 \pm 2^\circ\text{C}$ for $6 \text{ h} \pm 5 \text{ min}$ (*see Note 23* and **Fig. 1**).
5. Remove the samples from the oven and allow them to cool to room temperature. Centrifuge these samples briefly to collect the solution at the bottom of the tube.
6. Remove the Teflon tape and open the caps half way. Dry the samples overnight without heat in a vacuum centrifuge.
7. Similarly, excised and minced PDVF bands containing glycoproteins and appropriate blanks are prepared for monosaccharide analysis. However, 0.3 mL glass crimp-top micro vials are used with a 75 μL hydrolysis volume (8).

3.2.2. Derivatization of Samples and Monosaccharide Standards

1. Dissolve the dried hydrolysis sample replicates in 100 μL each of 1% sodium acetate solution and vortex vigorously for at least 2 min at the highest possible setting (*see Note 24*). Allow the sample to sit at room temperature for at least 30 min, vortex intermittently (approx every 10 min) to ensure the pellet is completely dissolved (*see Note 25*).
2. Centrifuge the tubes briefly to spin the solution to the bottom of the tube and transfer 50 μL of each hydrolyzed sample to separately labeled 1.6 mL polypropylene vials (*see Note 26*). Appropriately label additional 1.6 mL polypropylene vials as the standards and aliquot 50 μL of the monosaccharide working solution into each vial.

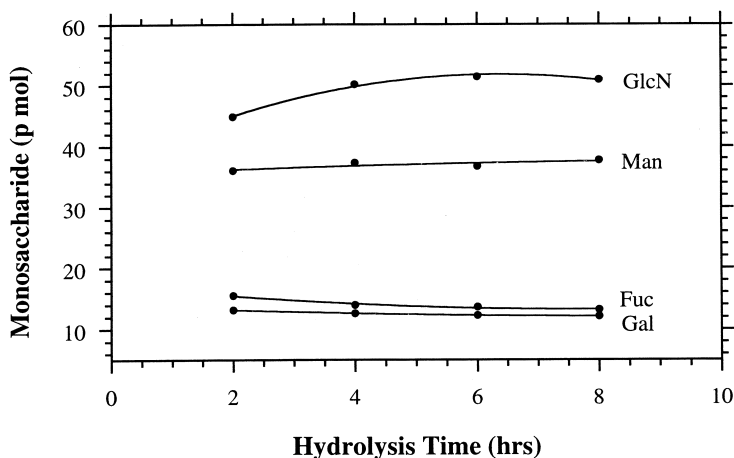


Fig. 1. Effect of hydrolysis time on the recovery of monosaccharides.

3. Add 100 μL of anthranilic acid reagent to each sample and monosaccharide standard, cap the vials tightly, vortex, and centrifuge briefly to collect the solution to the bottom of the tube.
4. Heat the tubes for 45 min at $80 \pm 2^\circ\text{C}$ in a thermal heating block (*see Note 27*).
5. After the incubation, remove all tubes from the heating block and allow them to cool to room temperature.
6. Add 850 μL of mobile phase A to each tube, cap, and vortex vigorously.
7. Centrifuge all tubes for 5 min at maximum speed in the centrifuge to obtain a solution that is free of particulates.
8. Transfer each solution to an autosampler vial (*see Note 28*). Install the vials on the HPLC system autosampler and analyze using appropriate injection volumes (typically 50 μL).

3.2.3. Chromatography

1. Equilibrate a Waters Symmetry column (C18, 3.9×150 mm) in 5% Solvent B at a flow rate of 1 mL/min.
2. Make duplicate injections of 50 μL from samples and standards.
3. Separate the monosaccharides as follows: 5% solvent B isocratic for 7 min followed by a linear gradient from 5 to 8% B over 18 min. This is followed by a 5 min wash using 100% B and an 8 minute equilibration at initial condition prior to next injection. The column temperature is maintained at 17°C (*see Note 29*). Total run time is 40 min with 28 min of data collection. The fluorescence detector settings are excitation $\lambda = 360$ nm and emission $\lambda = 425$ nm (*see Note 30*). *See Fig. 2* for representative standard and sample chromatograms (*see Note 31*). *See Fig. 3* for a chromatogram of fetuin using a PVDF membrane.

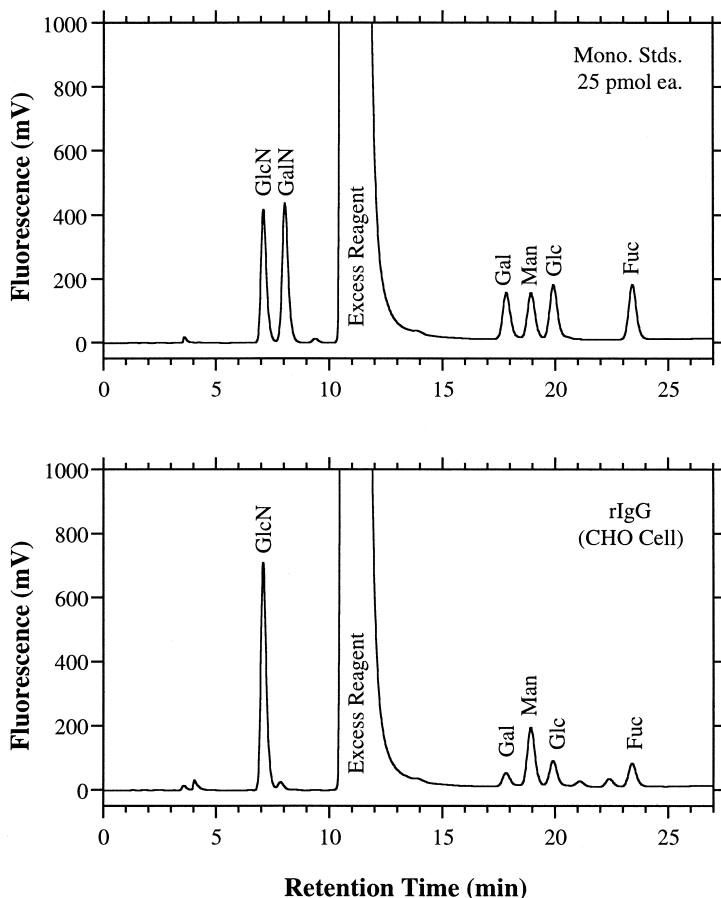


Fig. 2. Representative monosaccharide chromatograms obtained with a standard (top) and a rIgG produced in Chinese hamster ovary cells (bottom).

3.2.4. Calculations

1. Program the computer data system to calculate the peak area response for each sample (in pmol) from the average peak area response of the monosaccharide standard replicates (total six data sets, i.e., duplicate injections from three vials).
2. Calculate the amount of each monosaccharide in pmol per mg of protein taking into account dilution factors, sample volumes, and injection volumes used.
3. Calculate the moles of monosaccharide per mole of protein by the following formula:

$$\text{moles of each monosaccharide per mole of protein} = \frac{(\text{pmol/mg of protein} \times \text{MW})}{10^9}$$

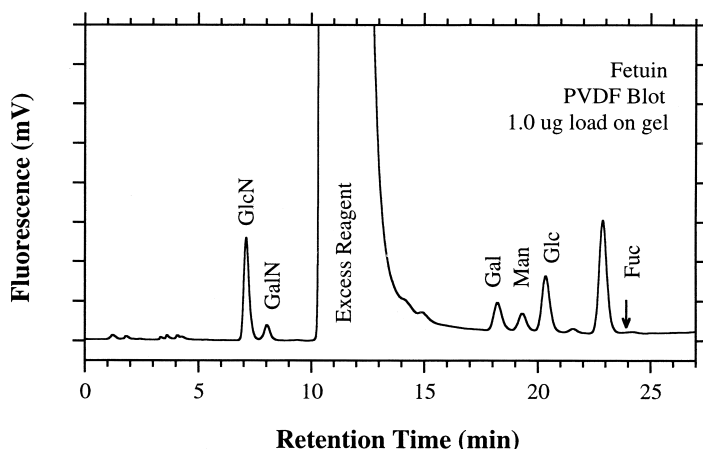


Fig. 3. An example of monosaccharides obtained from one of the three bands of fetuin electroblotted onto PVDF following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Glucose is a contaminant in this analysis (9).

where:

10^9 is the conversion factor from pmol to moles

MW is the molecular weight as determined from the amino acid sequence of the protein

4. Calculate the percent carbohydrate (w/w):

Percent carbohydrate (w/w) =

$$\frac{\text{all monosaccharides per mole of protein} \times \text{mol. wt. of that monosaccharide} \times 100}{\text{all monosaccharides per mole of protein} \times \text{mol. wt. of that monosaccharide} + \text{MW}}$$

Use the following molecular weights for the monosaccharides: Glucosamine; 221.2; Galactose; 180.2; Mannose; 180.2; Fucose; 164.2 and Sialic Acid; 309.3 (see Note 32).

3.2.5. Acceptance Criteria for Test Results

The relative standard deviation between six standard injections is less than 3.0% for each monosaccharide (usually 1.0%). The relative standard deviation between sample injections is less than 5.0% for each monosaccharide (usually 2.0%) (see Note 33).

3.3. Analysis of Sialic Acids

This section describes the method of quantitation of sialic acid (*N*-acetyl and *N*-glycolylneuraminic acids) in glycoproteins (6). The sialic acids are released

from the glycoproteins by mild acid hydrolysis followed by derivatization with OPD to yield a fluorescent quinoxaline derivative. The derivative is separated from excess reagent by RP-HPLC for quantitation using fluorescence detection.

3.3.1. Mild Acid Hydrolysis

1. Place 50 μL of the excipient free undiluted sample into labeled sample vials.
2. Prepare a vial with the appropriate blank (*see Note 34*).
3. Add 50 μL of 0.5 *M* sodium bisulfate to the blank and each sample, cap each vial tightly, and vortex each slowly for several seconds.
4. Hydrolyze the samples by placing them in a temperature controlled oven or heating block at $80 \pm 2^\circ\text{C}$ for 20 min.
5. Remove the vials from the heating block and allow them to cool to room temperature.

3.3.2. Derivatization of the Samples and Sialic Acid Standards

1. Aliquot 100 μL of the sialic acid working solution to labeled vials.
2. Add 100 μL of OPD solution to the hydrolyzed samples and standard vials. Cap the tubes tightly and vortex.
3. Heat the tubes for 40 min at $80 \pm 2^\circ\text{C}$ in a thermal heating block.
4. Remove all vials from the heating block and allow them to come to room temperature.
5. Add 800 μL of solvent A to each sample and standard. Cap the tubes and vortex them vigorously.
6. Centrifuge all the vials for 5 min at the maximum setting to obtain a particulate-free solution.
7. Transfer each solution to an autosampler vial (*see Note 26*). Install the vials on the autosampler and analyze.

3.3.3. Chromatography

1. Equilibrate a Beckman ODS column (4.6×150 mm, 5 μm) in 8–12% solvent B at a flow rate of 1 mL/min (*see Note 35*).
2. Inject 100 μL of each sample or standard in duplicate.
3. Separate the sialic acids as follows: isocratic at initial solvent B for 15 min followed by a 10-min wash at 95% solvent B and 10-min equilibration at initial conditions. The column temperature is maintained at 17°C (*see Note 27*). Total run time is 35 min with 20 min of data collection. The fluorescence detector settings are excitation $\lambda = 230$ nm and emission $\lambda = 425$ nm. *See Fig. 4* for representative standard and sample chromatograms (*see Note 36*).

3.3.4. Calculations

1. Calculate sialic acid amount (in pmol) per injection in samples using the average peak area response of the 100 pmol sialic acid standard.

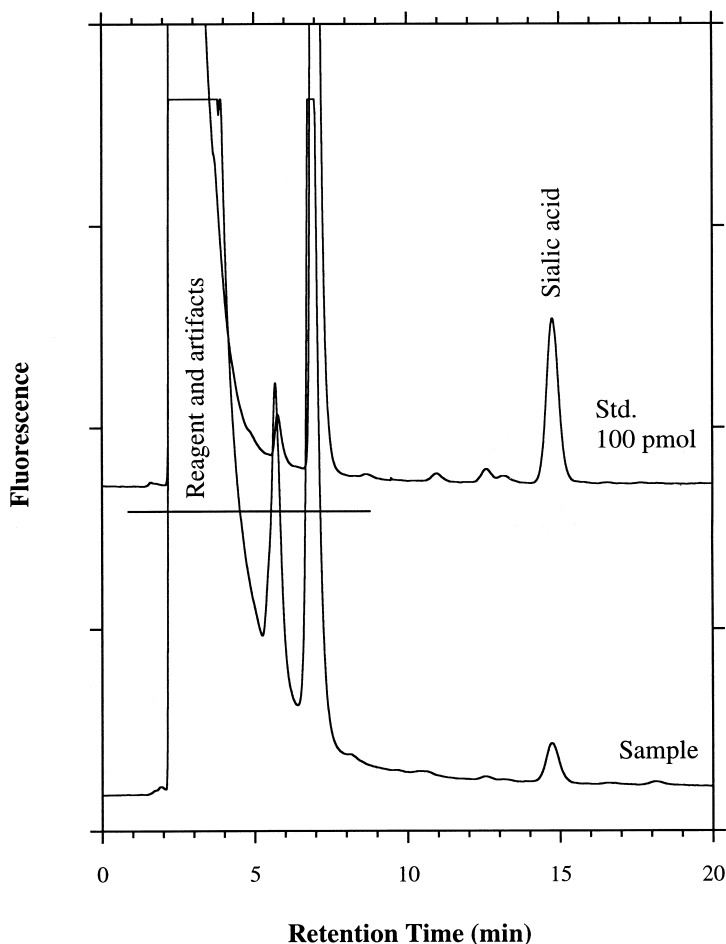


Fig. 4. Representative sialic acid (*N*-acetylneuraminic acid) chromatograms obtained with a standard (top) and a rIgG produced in Chinese hamster ovary cells (bottom). The rIgG typically contains about 2% (w/w) carbohydrate and less than 5% of the oligosaccharides are sialylated.

2. Calculate the quantity of sialic acid (in pmol) per mg of protein taking into account dilution factors, sample volumes, and injection volumes used.
3. The moles of sialic acid per mole of protein is determined by the following formula:

$$\text{moles of sialic acid per mole of protein} = \frac{(\text{pmol/mg of protein} \times \text{MW})}{10^9}$$

where:

10^9 is the conversion factor from pmol to moles

MW is the molecular weight of the protein as determined from the amino acid sequence.

3.3.5. Acceptance Criteria for Test Results

The relative standard deviation for six sialic acid standard injections is less than 3.0% as determined by peak area. The relative standard deviation between sample injections is less than 5.0% for sialic acid as determined by peak areas (usually 2.0%) (*see Note 37*).

4. Notes

1. CAUTION: Cyanoborohydride, trifluoroacetic acid, 1-butylamine, THF, acetonitrile, and OPD are toxic and/or flammable. Avoid contact with skin or inhalation. Wear gloves when preparing all solutions. When acetonitrile, phosphoric acid, THF, TFA, 1-butylamine, and cyanoborohydride are not in use they should be stored in an appropriate place. Dispose these chemicals appropriately.
2. The manufacturer's part numbers for the equipment and reagents are meant as a guide. Equivalent substitutions may be made.
3. Expiration: 6 mo at room temperature.
4. This type of vial and cap are critical and should not be substituted.
5. An unfrozen stock standard stored at -20°C is accurate for 5 yr.
6. The working standard contains 10 nmol of each monosaccharide/mL.
7. Aliquot the solution into 500 μL portions. Expiration: 1 yr at -15 to -25°C .
8. Typical working standard contains 2–10 nmol/mL of sialic acid.
9. Make sure the butylamine has completely dissolved before proceeding to the next step. For convenience, use graduated glass pipet to add the THF.
10. Expiration: 1 mo at room temperature if containers are sealed.
11. Make sure to prepare simultaneously a blank piece of PVDF as a control.
12. If the glycoprotein is basic then follow the procedure using acetic acid as the diluent. If the glycoprotein is acidic then follow the procedure using sodium hydroxide as the diluent.
13. It is important to mix the sample vigorously to disperse the pellet before proceeding to the next step.
14. The solution should become cloudy. If the protein settles at the bottom then intermittent vortexing may be appropriate during this time.
15. Be careful: the pellet may slide out of the inverted vial. Before proceeding, it is helpful to gently tap the opening of the inverted vial on a clean tissue paper (Kimwipe) to remove any residual solvent remaining in the treads of the vial.
16. Do not over-dry. The pellet may not go into solution.
17. Most proteins will clarify in less time but the actual time may vary depending on the protein.

18. Additional dilution and/or clarification time may be necessary if the samples are not completely clear. Inspect the bottom of the tube for undissolved protein.
19. It is recommended that the pooled samples be allowed to stand overnight, however this is not a necessity.
20. This diluted blank would be either a 1:10 dilution of the 50 mM sodium hydroxide solution in water or the 5% acetic acid solution depending upon which diluent was used in **Subheading 3.2**.
21. Multiply the calculated protein concentration by the dilution factor (i.e., 10 \times). It is best if the protein concentration is between 3–5 mg/mL after correcting for the dilutions (e.g., rIgGs with approx 2% (w/w) carbohydrate). Lower concentrations may make the accurate quantitation of residual sialic acid difficult for proteins with very low sialic acid content (e.g., rIgGs). Higher concentrations may produce off-scale peaks for glucosamine and galactosamine, which would require an additional dilution prior to analysis. The recommended ranges are dependent on the degree of glycosylation of the protein.
22. For the blank, use either the 5% acetic acid solution or a 10-fold dilution of the 50 mM sodium hydroxide solution prepared in **Subheading 3.3.2**.
23. It may be necessary to determine the optimum hydrolysis time through the use of a time-course study over 4–8 h.
24. It is crucial to completely dissolve the pellet before proceeding. It is recommended that the end of a pipet tip be used to physically crush/scrape the pellet from the bottom of the tube. Rinse the tip with the sample's sodium acetate diluent several times to ensure that any residual pellet is cleared from the tip.
25. If a flat-top Vortex mixer is available, samples can be bound together by a rubberband or placed in a small box and placed on the vortex for 15 min.
26. Transfer only the supernatant and none of the pellet.
27. Cover the top of the heating block with insulating material (e.g., foam, paper towels, and/or wool pads, etc.) to maintain the temperature.
28. Some samples may contain particulates at the bottom of the tube. These particulates should not be transferred and injected into the HPLC. It is best to use amber autosampler vials to minimize light exposure to the derivatized samples.
29. The actual temperature is not critical, however, the column should be maintained at a constant temperature in order to obtain reproducible retention times. A column cooler (Cool Pocket, Keystone Scientific) can be used for this purpose.
30. For less sensitive fluorescence detectors, an excitation of 230 nm may be used.
31. Sample chromatograms may have some additional peaks but the monosaccharides peaks should be resolved from any artifact peaks. It is common to observe varying levels of glucose in the samples and blank. Due to the abundance of glucose-containing polymers (e.g., lint) in the environment, complete elimination is nearly impossible. Glucose levels, therefore, cannot be accurately determined.
32. The value for the number of moles of sialic acid is determined by the procedure described in the sialic acid method.
33. This value may be greater with very low amount of a particular monosaccharide in the glycoprotein. The tailing factor for each of the peaks in the standard

chromatogram must be less than 1.2. The resolution of the mannose in the standard chromatogram peak must be greater than 1.2.

34. The blank would either be a similarly diluted blank of the 50 mM sodium hydroxide solution or the 5% acetic acid solution, depending on which solution was used in **Subheading 3.2**.
35. It is usually necessary to optimize the chromatography on a particular lot of columns by adjusting the initial percentage of solvent B to between 8 and 12%. Sialic acid peak must separate from the artifacts.
36. Sialic acid should elute within 15 min.
37. This value may be greater with very low abundance of sialic acid in the glycoprotein. The tailing factor for the sialic acid peaks in the standard chromatogram must be less than 1.4. The standard theoretical plate count should be greater than 4000.

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Kannicht, C. (Ed.)

2002, XI, 322 p. 19 illus., Hardcover

ISBN: 978-0-89603-678-9

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