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Screening Sialic Acid Content to Optimize Pharmacokinetics and Pharmacodynamics of Glycoprotein Therapeutics

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Abstract

Sialic acid serves a critical role in mediating the effectiveness of recombinant therapeutic proteins, especially those intended for intravascular administration. The presence or absence of this terminal sugar on the glycan side chains can dramatically alter the residence time in the plasma (pharmacokinetics) and can also affect the pharmacodynamics of the protein. The absence of sialic acid is a useful indicator that other carbohydrate residues, such as mannose, galactose or N-acetylgalactosamine, occupy terminal positions on the glycan, which can interact with cell surface receptors and drive clearance of the therapeutic from circulation (Hara *et al.* 1989; Hudson *et al.* 1973). In addition, the level of sialic acid and type of linkage to the penultimate galactose residue on the Fc-glycan chains of therapeutic antibodies elicit either an inflammatory or an anti-inflammatory response (Kaneko *et al.* 2006; Scallon *et al.* 2007).

It has been well established that sialic acid content and species distribution can be altered by cell culture conditions; the cell type used for the expression host; and cell culture media components. This mandates the evaluation of both sialic acid content and its various molecular species over the course of any therapeutic protein process development effort, such as cell-line selection, scale-up and cell-culture optimization.

In order to meet the need for a rapid, high-throughput means for screening a large number of samples, we have developed a fluorometric, enzyme-coupled method for sialic acid quantitation, which utilizes low levels (2 – 60 μg) of protein in a 96-well plate format. We have qualified the assay using two instrument platforms, a filter-based (PerkinElmer Victor²™ 1420 Multilabel Counter) and a monochrometer-based (BioTek® Synergy™ 4) fluorometric plate reader. Optimization of the optical parameters for both assay platforms was performed. Both instruments gave similar results with a wide variety of sialylated glycoproteins, including immunoglobulins. The results are consistent with previously reported values for sialic acid content using well-established, but significantly more complex and time consuming assays. In the high-throughput version of the assay, detection as low as 200 pmol of sialic acid can be made with an inter-assay relative error of 2 – 3%. At the 1,000 pmol level, the signal-to-background ratio was >30:1. The assay is designed for maximal flexibility; assays can be carried out on a limited number of analytical samples or high volume analysis. Detection using fluorescence intensity can be made at 50 – 1000 pmol of sialic acid and absorbance detection can be made at 1000 – 5000 pmol.

Introduction

Although a number of methods for quantitation of sialic acid have been developed, one of the most frequently employed methods is analysis by high-performance liquid chromatography (HPLC) of pre-column derivatized fluorescent-labeled samples. HPLC analysis is advantageous because it offers an extremely high level of sensitivity (femtomole range) and allows the identification of individual sialic acid species. However, HPLC analysis is cumbersome and time consuming, particularly when analysis of multiple samples is required. Moreover, the method is not readily adaptable to a high-throughput modality.

We have developed a sensitive, enzyme-based method for high-throughput sialic acid quantitation. This method utilizes neuraminyl aldolase coupled with pyruvate oxidase to convert released sialic acid to hydrogen peroxide. The resulting peroxide reacts stoichiometrically with a dye that is intensely fluorescent (or absorbant) (Figure 1). This method allows sample digestion, conversion, detection, and quantitation to be performed in a single well of a 96-well microtiter plate for fast and simple processing. Thus allowing sialic acid quantitation to be a suitable assay for drug discovery and other screening applications.

This simple assay may be performed on purified whole protein or the glycan alone, and requires only three manipulations by the operator after the samples have been dispensed into the wells: enzymatic digestion to release sialic acid; reconstitution of the lyophilized assay mixture in cold water; and addition of the resulting mixture to the sialic acid standards and protein samples. The progress of the assay can be monitored directly by following the formation of the fluorescence or absorbance of the reporter dye and has been tested on multiple instruments. In most cases, the reaction is complete after 20 minutes at 37°C or 40 minutes at room temperature making this process readily adaptable for high-throughput sialic acid quantitation.

Materials and Methods

Release Enzyme: Glyko Sialidase A™–51 (recombinant gene from *Arthrobacter ureafaciens*, expressed in *E. coli*; ProZyme product code GK80045). Assay mixture: pyruvate oxidase (*Aerococcus sp.*); N-acetylneuraminic acid aldolase (*E. coli*); horseradish peroxidase; and dye. Sialic Acid Standard: Sialic acid (*E. coli*, 98% pure) was thoroughly dried over P_2O_5 for 1 week under vacuum. A stock solution was prepared at a concentration at 100 m*M* in water.

Instruments: Absorbance determinations were made on a Multiskan® microtiter plate reader (Thermo Fisher Scientific). Fluorescence intensity determinations were made on a Victor² 1420 Multilabel Counter (PerkinElmer) using an optimized filter set, 530DF30 and 590DF35 (Omega® Optical) (Figure 1), and a monochromator-based Synergy 4 Multi-Detection Microplate Reader (BioTek Instruments). Thermomixer® microtiter plate shaker with temperature control (Eppendorf) was used for plate incubation before measurements. Microtiter Plates: Black, flat bottom plates, Non-Treated (high protein binding) and Non-Binding Surface, polystyrene (Corning® Costar®) were used for analysis.

The Method: Sialic acid was released from the target proteins (2 – 60 μg) by enzyme digestion using 20 – 40 mU of Sialidase A-51. Digestions were performed in a 96-well microtiter plate in 50 m*M* phosphate buffer (pH 6.0) in a total volume of 50 μl for 0.5 – 1 hour at 37°C. Solutions of the Sialic Acid Standard were added to empty wells at various levels and water was added to bring them to a total volume of 50 μl. The assay mixture, once reconstituted, was added immediately to the wells with the sialic acid standards and the protein samples. The plate was mixed for ~30 seconds on a shaker and then incubated, with or without shaking, at room temperature or at 37°C for varying periods of time, with a total volume of 120 μl in each well prior to analyzing.

Figure 1 Optimization of Optical Parameters for a Filter-Based Instrument

Dye absorbance (A) and emission spectra (E) overlaid with the optimal excitation filter (530 ± 15 nm) and the optimal emission filter (590 ± 17 nm)

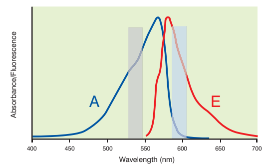

Figure 2 Enzyme-Coupled Assay for Sialic Acid Quantitation on Glycoproteins Using Sialidase Release

Step 1: Release of Sialic Acid

Glycoprotein + Sialidase ⟶ Sialic Acid + Glycoprotein

Digestion times for optimal release of sialic acid can be determined independent of the detection step. This allows for variations in the accessibility of the sialidase to the sialic acids residues on different types or classes of proteins.

Step 2: Detection of the Released Sialic Acid Using Coupled-Enzymes

N-acetylneuraminic acid aldolase catalyzes the reversible reaction:

Sialic Acid ⟷ Mannosamine + Pyruvic Acid

Variants of sialic acid, such as N-glycolyl- and some O-acetylneuraminic acids, are also converted to pyruvic acid and the corresponding mannosamine. Then pyruvate oxidase catalyzes the reaction:

Pyruvic Acid ⟶ Acetylphosphate + H_2O_2

Under the proper conditions, the forward aldolase reaction predominates; and when coupled with H_2O_2 generation, the reaction will go to completion.

Hydrogen peroxide forms a 1:1 molecular complex with the selected dye when catalyzed by horseradish peroxidase; the complex is intensely fluorescent (or absorbant) and can be readily quantitated.

Dye + H_2O_2 **⟶ Reporter Dye**

Figure 3 Determination of Optimal Excitation and Emission Settings Using a Monochromator-based Instrument

a. Emission is scanned reading every 5 nm, while excitation is fixed at 560 nm.

b. Excitation is scanned with emission fixed at 590 nm. Inserts show the signal-to-background ratio at the various wavelengths.

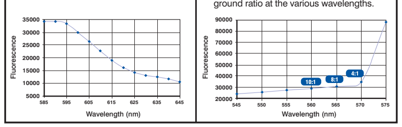

Figure 4 Sialic Acid Standard Curve at the 200 – 1000 pmol Scale Using Fluorescence Detection
(y = 7785.2 x + 13.85, R^2 = 0.9991)

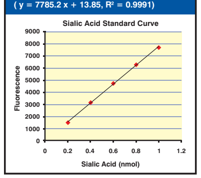

Figure 5 Sialic Acid Standard Curve at the 1 – 5 nmol Scale Using Absorbance Detection
(y = 0.0991 x – 0.007, R^2 = 0.9990)

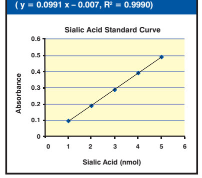

Figure 6 Change in Slope of Sialic Acid Standard Curves Over Time: Comparing Various Plate Incubation Methods

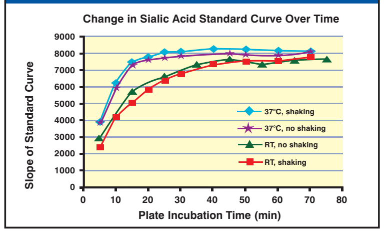

Figure 7 Sialic Acid Standard Curves Using Fluorescence Detection
Filter-based vs. Monochromator-based Instruments

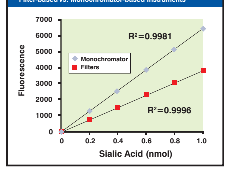

Table 1 Sialic Acid Content of Typical Glycoproteins: Enzyme-coupled Assay Using Fluorescence Intensity

Glycoprotein	MW (Da)	Amount of Protein Analyzed	Sialic Acid (mol/mol protein) Reported	Assay
Fetuin (bovine)	48,000	0.037 nmol /1.8 μg	13 - 17[a-c]	14.8 ± 0.40
Transferrin (human)	78,000	0.119 nmol / 9.3 μg	3.8[d]	4.6 ± 0.05
IgG (monkey)[1]	150,000	0.260 nmol / 39 μg	1.1[e]	0.9 ± 0.02

[1] Sialic acid is almost exclusively NGNA.
[a] (Townsend *et al.*, 1997); [b] (Spiro, 1960); [c] (Rohrer *et al.*, 1997); [d] (Regoeczi *et al.*, 1979); and [e] (Raju *et al.*, 2000).

Figure 8 Assay Performance Using High Protein Binding (■) *vs.* Non-Binding Surface (■) Microtiter Plates

a. Filter-based instrument

b. Monochromator-based instrument

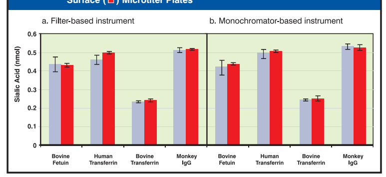

Table 2 Assay Performance Using High Protein Binding *vs.* Non-Binding Surface Microtiter Plates

Glycoprotein	% Relative Error High Protein Binding	Non-Binding Surface
Fetuin (bovine)	6.33	1.41
Transferrin (human)	3.62	1.00
Transferrin (bovine)	1.25	3.05
IgG (monkey)	2.03	1.36

Results

The overall scheme for the formation of free sialic acid and its subsequent conversion to hydrogen peroxide is shown in Figure 2. Sialidase A-51 (a 51 kDa isoform of Sialidase A) was selected as a means to release the sialic acid from the protein substrates because its smaller size allows access to sterically hindered sialic acid residues and its broad substrate specificity allows cleavage of all molecular species of sialic acids, including most O-acetyl and N-glycolyl forms (**Step 1**). After the complete release of sialic acid, the sample was treated with sialic acid aldolase, pyruvate oxidase, and horseradish peroxidase in the presence of the reacting dye (Assay Mixture) (**Step 2**) for color development for fluorescence intensity or absorbance detection.

Optimization of optical parameters. For the filter-based fluorescence plate reader, the optimal filter set for excitation and emission was determined empirically by testing a variety of filter combinations (Figure 1). An excitation filter at 530 nm with a bandwidth of 30 nm (excitation maximum of the dye is 565 nm) combined with an emission filter at 590 nm with a bandwidth of 37 nm (emission maximum for the dye is 585 nm) gave maximal fluorescence intensity at 1.0 nanomoles of sialic acid with a signal-to-background ratio of about 10:1.

In related experiments, optimization of the optical parameters was achieved for a monochromator-based fluorescence plate reader using an iterative protocol. Initially the excitation was fixed at 560 nm and emission was determined at 5-nm intervals, using a 1 nmole sample of sialic acid (Figure 3a). In a second experiment, emission of 590 nm was held constant and the excitation was varied between 545 and 575 nm (Figure 3b). A significant decline in the signal-to-background ratio was observed as the excitation wavelength approached the emission maximum of 585 nm. Based on these experiments, subsequent assays were carried out at an excitation of 560 nm and emission at 590 nm, with a slit width of about 5 nm. At these settings, the signal-to-background ratio was about 10:1.

Linearity of response using sialic acid standard. A linear response for the sialic acid standard was observed with both fluorescence intensity and absorbance measurements. In most cases, the R-squared values were about 0.999+ (Figures 4 & 5). The time course for the assay was monitored with fluorescence intensity detection by following the change in slope of the linear regression curve of the standard at room temperature or 37°C. The maximum signal was obtained after 40 minutes of incubation at room temperature and 20 minutes at 37°C (Figure 6). The signal at 1000 pmol, relative to the background fluorescence intensity, remained relatively constant at about 30:1 for up to 70 minutes.

Using an earlier version of the assay, both filter- and monochromator-based measurements gave good linearity of response using the Sialic Acid Standard between 0.2 and 1.0 nanomole, with correlation coefficients of about 0.9996 and 0.9981, respectively (Figure 7). Relative error between the replicates at each concentration was 5% or less. Intra-assay error was about 5% (data not shown).

Determination of sialic acid levels on glycoproteins. Several well-characterized proteins were analyzed for sialic acid content. The results correlated with published values for all proteins, including an immunoglobulin, (Table 1). Detection as low as 200 pmol of sialic acid can be made with an inter-assay relative error of 2 – 3%. At the 1,000 pmol level, the signal-to-background ratio was >30:1. Detection using fluorescence intensity can be made at 50 – 1000 pmol of sialic acid and absorbance detection can be made at 1000 – 5000 pmol.

Evaluation of different microtiter plates on assay performance. The effect of both high protein binding and non-binding surface microtiter plates on the assay was evaluated, using both instruments (Figures 8a & 8b). The signals for the various proteins were in close agreement and no significant differences were observed between the two plate types. With some proteins, a small increase in the relative error between replicates was observed using the high protein binding plates (Table 2). However, these values were within the expected relative error (~5 %) for the assay.

Conclusions

Enzyme-coupled sialic acid quantitation on a variety of glycoproteins gave values in agreement with literature values, with detection levels in the 0.2 to 1.0 nanomole range. Both fluorescence readers gave similar results. Microtiter plate type did not significantly affect assay results with the proteins tested. The method is advantageous, providing sensitive, rapid analysis (~70 minutes) in a 96-well format, and is adaptable to a completely automated approach and therefore suitable for screening requirements.

This method has been shown to have superb assay precision as well as a high signal to background ratio (about 30:1). The format allows for the screening of multiple samples of recombinant therapeutic proteins during a process development effort such as cell-line selection, scale-up and cell-culture optimization.

NOTE: The method is available in kit form as the GlykoScreen™ Sialic Acid Quantitation Kit.

References

Hara S, Yamaguchi M, Takemori Y, Furuhata K, Ogura H, Nakamura M. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. Anal Biochem. 1989;179:162-6

Hudson BG, Ohno M, Brockway WJ, Castellino FJ. Chemical and physical properties of serum transferrin from several species. Biochemistry. 1973;12:1047-53

Kaneko YL, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of Immunoglobulin G resulting from Fc sialylation. Science. 2006;313:670-3

Raju TS, Briggs JB, Borge, SM, Jones AJS. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology. 2000;10:477-86

Regoeczi E, Taylor P, Deanne MT, Marz L, Hatton MWC. Three types of human asialo-transferrin and their interactions with rat liver. Biochem J. 1979;184:399-407

Rohrer JS, Thayer J, Weitzhandler M, Avdalovic N. Analysis of the N-acetylneuraminic acid and N-glycolylneuraminic acid contents of glycoproteins by high-pH anion-exchange chromatography with pulsed amperometric detection. Glycobiology. 1997;8:35-43

Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. Mol Immun. 2007;44(7):1524-34

Spiro RG. Studies on Fetuin, a glycoprotein of fetal serum. J Biol Chem. 1960;235:2860-9

Townsend RR, Manzi A, Merkle RK, Rohde MF, Spellman M, Smith A, and Carr SA, Quantitative Monosaccharide Analysis: A Multi-Center Study. Association of Biomolecular Resource Facility. http://www.abrf.org/ABRFNews/1997/March1997/mar97monosach.html