Qualification of a Process Analytical Technology for Quantifying Sialic Acid On Therapeutic Proteins Using Two Instrument Platforms

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Abstract

Sialic acid serves a critical role in mediating the effectiveness of recombinant therapeutic proteins. It has been well established that cell culture conditions, host cell type and media components can alter sialic acid content and the distribution of sialic acid species. These considerations underscore the importance of monitoring both sialic acid content and its various molecular species over the course of any therapeutic protein process development effort.

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 and a monochromator-based fluorescence plate reader. Optimization of the optical parameters for both assay platforms was performed, as well as evaluations of different microtiter plates used in the assay. Both instruments gave similar results with a wide variety of sialylated glycoproteins, including immunoglobulins; results are consistent with previously reported values for sialic acid content using well-established, but significantly more complex and time consuming methods. Detection as low as 0.2 nanomoles of sialic acid can be made with an inter-assay relative error of about 5%. At the 1.0 nmol level, the signal-to-background ratio was ~10:1. The assay is designed for maximal flexibility; assays can be carried out on a small number of analytical samples or up to 90 data points at one time for process development purposes.

Introduction

The method provides a sensitive, high-throughput approach to sialic acid quantitation, based on an enzyme-coupled reaction, converting released sialic acid to hydrogen peroxide, which reacts stoichiometrically with a dye to produce an intense fluorescent signal. Sample digestion, conversion, detection and quantitation are performed in a single well of a 96-well microtiter plate for fast and simple processing (ready for data analysis in ~70 minutes).

The procedure employs enzymatic digestion to release sialic acid from the glycoprotein. Sialidase digestion is advantageous, compared with acid hydrolysis, because it rapidly releases sialic acid under moderate conditions. Sialidase A™-51 (a 51 kDa isoform of Sialidase A, full length, 88 kDa) was selected as a means to release the sialic acid from the protein substrates because its smaller size allows access to more sterically hindered sialic acid residues; its broad substrate specificity allows cleavage of all molecular species of sialic acids, including O-acetylated and N-glycolyl forms (Figure 1, Step 1). However, all sialic acids may not be equally accessible to the enzyme, so it is necessary to qualify each protein substrate by optimizing the conditions for cleavage (amount of enzyme and/or the time of incubation) that give maximal values. After complete release of sialic acid, the sample is treated with conversion enzymes, sialic acid aldolase and pyruvate oxidase in a single step. The hydrogen peroxide generated is quantified with horseradish peroxidase in the presence of the reacting dye (Figure 1, Step 2).

The enzyme-coupled method offers a number of advantages over standard procedures:

- rapid quantitation of total sialic acid released from intact proteins as well as free sialic acid
- broad range of detection of sialic acid levels, from 0.2 -1.0 nanomole
- adaptable to high-throughput automation

Materials and Methods

Release Enzyme: Glyko® Sialidase A-51 (recombinant gene from *Arthrobacter ureafaciens*, expressed in *E. coli*; ProZyme product code GK80045). Conversion Enzymes and Cofactors: pyruvate oxidase *(Aerococcus* sp.); N-acetylneuraminic acid aldolase (*E. coli*); flavin adenine dinucleotide (FAD); and thiamine triphosphate (TPP). Detection Reagents: horseradish peroxidase; dye prepared in 1:1 (v/v) DMSO solution. Sialic Acid Standard: Sialic acid (*E. coli*, 98% pure) was thoroughly dried over P_2O_5 for 1 week under vacuum. A stock solution, in water, was prepared at a concentration at 100 m*M*. Instruments: Fluorescence intensity determinations were made on a Wallac Victor² 1420 Multilabel Counter (PerkinElmer) using an optimized filter set, 530DF30 and 590DF35 (Omega Optical) and a monochromator-based Synergy™ 4 Multi-Detection Microplate Reader (BioTek Instruments). 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 liberated from the target proteins by enzyme digestion using 20 mU of Sialidase A-51 and 2 - 60 µg of protein substrate. Digestions were performed in 96-well microtiter plates in 50 m*M* phosphate buffer (pH 6.0) in a total volume of 50 µl for 30 minutes at 37C°. The Sialic Acid Standard was added to wells at various levels and water added to a total volume of 50 µl. Reconstituted conversion enzymes and cofactors were added to each well and incubated at 37°C for 30 minutes in a total volume of 100 µl. Fluorescence intensity of the sample was developed by the addition of the dye solution and horseradish peroxidase after 10 minutes of incubation at room temperature.

Figure 1 Enzyme-Coupled Assay for Sialic Acid Quantitation on Glycoproteins Using Sialidase Release

Step 1: Release of Sialic Acid by Digestion of the Protein with Sialidase 51

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:

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

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

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

Dye + H_2O_2 ⟶ Reporter Dye

Results

Figure 2 Optimization of Optical Parameters for a Filter-Based Instrument

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

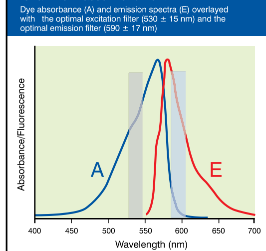

Figure 3 Determination of Optimal Excitation and Emission Settings Using a Monochromator-based Instrument

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.

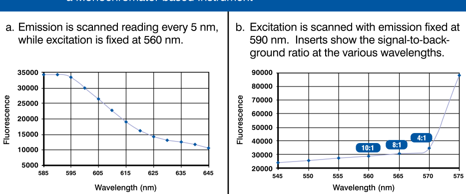

Figure 4 Sialic Acid Standard Curves Using Fluorescence Detection: Filter-based *vs.* Monochromator-based Instruments

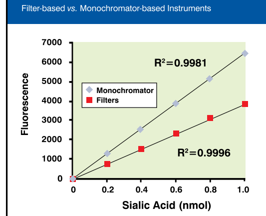

Table 1 Sialic Acid Content of Typical Glycoproteins: Enzyme-coupled Assay Using Filter-based and Monochromator-based Fluorometers for Detection *vs.* Reported Values

Glycoprotein	MW (kDa)	Amount of Protein Analyzed	Sialic Acid (mol/mol protein)[2]		
			Reported	Monochromator	Filters
Fetuin (bovine)	48	0.023 nmol/1.2 µg	13 - 17[a-c]	16.9±1.3	17.3±1.5
Transferrin (human)	78	0.115 nmol/8.9 µg	3.8[d]	4.4±0.2	4.0±0.2
Transferrin (bovine)	78	0.111 nmol/8.6 µg	2[e,f] - 3.5[c]	2.3±0.1	2.1±0.1
IgG (monkey)[1]	150	0.392 nmol/59 µg	1.1[g]	1.4±0.1	1.3±0.1

[1]Sialic acid is almost exclusively NGNA.

[2]Sialic acid values from triplicate determinations ± standard deviations.

[a](Townsend *et al.*, 1997); [b](Spiro, 1960); [c](Rohrer *et al.*, 1997); [d](Regoeczi *et al.*, 1979); [2](Hudson *et al.*, 1973); [f](Richardson *et al.*, 1973) and [g](Raju *et al.*, 2000).

Figure 5 Assay Performance Using High Protein Binding (■) *vs.* Non-Binding Surface (■) Microtiter Plates

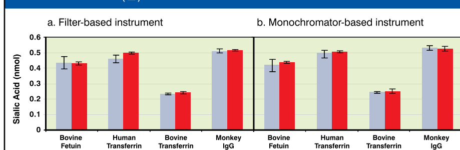

Table 2 Assay Performance Using High Protein Binding *vs.* Non-Binding Surface Microtiter Plates

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

Discussion of Results

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 2). An excitation filter at 530 nm with a band width of 30 nm (excitation maximum of the dye is 565 nm) combined with an emission filter at 590 nm with a band width of 34 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 ~10:1.

In related experiments, optimization of the optical parameters were determined 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 5 nm. At these settings, the signal-to-background ratio was ~10:1.

Linearity of response using the Sialic Acid Standard. 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 4). 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 using the enzyme-coupled method. Good correlation with published values were obtained for all proteins, including an immunoglobulin (Table 1) for both instruments.

Evaluation of different microtiter plates on assay performance. The effect of both high protein binding and Non-Binding Surface microtiter plates on the assay were evaluated, using both instruments (Figure 5a and b). With both types of instruments, the signals for the various proteins were in close agreement and no significant differences were observed using 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 a sensitive, rapid analysis (~70 minutes) on as many as 90 data points at one time, and is adaptable to a completely automated approach.

NOTE: The method is available in kit form as the GlykoScreen™ Sialic Acid Quantitation Kit (ProZyme product codes GS300 & GF57).

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