An Enzyme-based Sialic Acid Quantitation Assay for Rapid Screening of Therapeutic Glycoproteins During Process Development: A Potential Process Analytical Technology

Thomas G. Warner, PhD

Presented at WCBP 2007: 11th Symposium on the Interface of Regulatory and Analytical Sciences for Biotechnololgy Health Products January 29 - 31, 2007, Washington, DC, USA

ABSTRACT

Sialic acid serves a critical role in mediating the effectiveness of recombinant therapeutic proteins, especially those intended for intra-vascular administration. The presence or absence of this 9-carbon carbohydrate can dramatically effect the pharmacokinetics of the protein, as well as its immunogenicity; most recently, sialic acid has been directly implicated in the function and effectiveness of therapeutic immunoglobulins (Scallon *et al.*, 2006; Kaneko *et al.*, 2006). It has clearly been demonstrated that cell culture conditions; the cell type used for expression host; and cell culture media components can alter sialic acid content and the distribution of sialic acid species. Given these potential variations in sialic acid levels during process optimization and for biosynthesis of antigenic forms of this carbohydrate, continual monitoring of both sialic acid content and its various molecular species is a mandatory requirement of any 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 (or colorimetric), enzyme-coupled method for sialic acid screening, which utilizes low levels (10 - 50 µg) of protein in a 96-well plate format. Detection as low as 200 pmols of sialic acid can be made with an inter-assay relative error of about 5%. Sialic acid on recombinant proteins with very low levels of sialic acid, as found on monoclonal antibodies, can be readily quantitated. Intra-assay variations are about 5 - 7%. Assays on ~90 samples can be made in about 70 minutes. Sialic acid content was determined for 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.

INTRODUCTION

Although a number of methods for quantitation of sialic acid have been developed, one of the most frequently employed is analysis by high-performance liquid chromatography (HPLC) of pre-column derivatized, fluorescently 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, the method is cumbersome and time consuming, particularly when analysis of multiple samples is required. Moreover, the method is not readily adapted to a high-throughput modality.

The method described herein represents 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 that is intensely fluorescent (or absorbant). This method allows sample digestion, conversion, detection and quantitation to be performed in a single well for fast and simple processing (ready for data analysis in ~70 minutes). A comparison of the steps and time requirement for the DMB-HPLC procedure *vs.* the enzyme-coupled procedure is shown in Table 1.

The procedure described herein employs enzymatic digestion to release sialic acid from the glycoprotein. Digestion with Sialidase A™ is advantageous, compared with acid hydrolysis, because it is rapid and releases sialic acid under moderate conditions. However, all sialic acids may not be equally accessible to the enzyme, so it is necessary to qualify a specific protein substrate by optimizing the conditions for cleavage (amount of enzyme and/or the time of incubation) that give maximal values.

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 200 - 1,000 pmol (fluorescence) to 1 - 5 nmol (absorbance) of sialic acid per sample
- flexible detection, adaptable to high-throughput automation
- enzymatic cleavage allows rapid analysis (~70 minutes) with minimal, if any, degradation of sialic acid

Figure 1 - The Enzyme-coupled Method

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 (or absorbant) and can be readily quantitated.

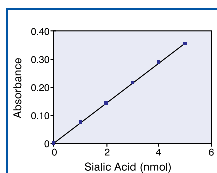

Figure 2 - Sialic acid standard curve at the 1 - 5 nmol scale using absorbance detection at 560 nm. ($y = 0.0726x$ and $R^2 = 0.9992$).

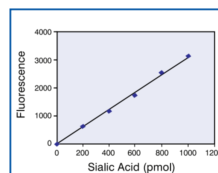

Figure 3 - Sialic acid standard curve at the 200 - 1000 pmol scale using fluorescence detection. ($y = 3.0918x$ and $R^2 = 0.9965$).

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_20_5 for 1 week under vacuum. A stock solution, in water, was prepared at a concentration at 100 m*M*. Instruments: Absorbance determinations were made on a Multiskan microtiter plate reader (Labsystems) at 560 nm. Fluorescence determinations were made on a Victor² 1420 Multilabel Counter (PerkinElmer) using an optimized filter set, 530DF30 and 590DF35 (Omega Optical, Brattleboro, VT, USA). Microtiter Plates: Clear, flat bottom plates (Corning) were used for absorbance measurements. Black flat bottom plates, non-treated polystyrene (Corning-Costar) were used for fluorescence measurements. DMB-HPLC Assay of Sialic Acids (Hara *et al.*, 1989): Assays were performed according to the method described in the Signal™ DMB Labeling Kit (ProZyme product code GKK-407) with a GlycoSep™ R HPLC Column (ProZyme product code GKI-4727).

The Method: Sialic acid was liberated from the target proteins by enzyme digestion or mild acid hydrolysis (2 *M* HOAc, 80°C, 3 hours). For enzyme digestions, 20 mU of Sialidase A-51 were employed using from 10 - 200 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 37°C. Solutions of the Sialic Acid Standard were added to the 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 (absorbance) was developed by the addition of the dye solution and horseradish peroxidase followed by 10 minutes of incubation at room temperature.

Table 1 - Time Requirement of DMB-HPLC *vs.* the Enzyme-coupled Method

Parameter	DMB-HPLC (Acid Release)	Enzyme-coupled Method	
		(Acid Release)	(Enzyme Release)
Digestion	3 hr	3 hr	30 min
Drying	3 hr	3 hr	n/a
Labeling	3 hr	15 min	15 min
Detection single sample multiple samples[1]	 30 min 9 hr	 5 min 5 min	 5 min 5 min
Data analysis	20 min	20 min	20 min
Total time	~10 - 18 hr	~7 hours	70 min

[1] Two samples in triplicate plus the standard curve (6 points) in duplicate.

Table 2 - Sialic Acid Requirement of Typical Glycoproteins: Comparison of the Enzyme-coupled Assay (Absorbance Detection) and Standard Reported Values

Glycoprotein	MW (Da)	Amount of Protein Analyzed	Sialic Acid (mol/mol protein)		
			Reported	Enzyme Release	Acid Release (n = 1)
Fetuin (bovine)	48,000	0.25 nmol/12 µg	13 - 17 [a-c]	13 ±0.8	13.9
Transferrin (human)	78,000	0.46 nmol/36 µg	3.8 [e]	4.2 ±0.2	4.5
Transferrin (bovine)	78,000	0.46 nmol/36 µg	2 [f,g] - 3.5 [c]	1.9 ±0.1	2.4
Thyroglobulin (bovine)	669,000	0.018 nmol/12 µg	28 - 30 [d]	28 ±1.5	29.8
IgG (monkey)[1]	150,000	1.2 nmol/180 µg	1.1 [h]	n/d	1.1

[1] Sialic acid is almost exclusively NGNA.

[a] (Townsend *et al.*, 1997); [b] (Spiro, 1960); [c] (Rohrer *et al.*, 1997); [d] (Spiro and Spiro, 1965); [e] (Regoeczi *et al.*, 1979); [f] (Hudson *et al.*,1973); [g] (Richardson *et al.*, 1973) and [h] (Raju *et al.*, 2000).

Comparison of DMB-HLPC *vs.* Enzyme-coupled Method (Absorbence Detection) for Two Glycoproteins

Table 3 - Fetuin (bovine)

Method	Sialic Acid (mol/mol protein)	
	mean	rel error
DMB-HPLC	12.2	±3.2%
Enzyme-coupled (acid release)	12.6	±3.4%
Enzyme-coupled (enzyme release)	13.7	±2.2%

Table 4 - Transferrin (human)

Method	Sialic Acid (mol/mol protein)	
	mean	rel error
DMB-HPLC	3.3	±15.1%
Enzyme-coupled (acid release)	3.9	±13.7%
Enzyme-coupled (enzyme release)	3.7	±1.8%

Table 5 - Inter-Assay Precision of the Enzyme-coupled Method

Protein	Average Value (mol/mol protein)	Relative Error	Std Dev
Fetuin (bovine)	13.6	±2.2%	0.3
Transferrin (human)	3.4	±4.9%	0.2

Table 6 - Intra-Assay Precision of the Enzyme-coupled Method

Sample	Average Value (mol/mol protein)	Relative Error	Std Dev
Sialic Acid Standard	n/a	±2.2%	n/a
Fetuin (bovine)	13.2	±1.9%	0.3
Transferrin (human)	3.4	±4.5%	0.3

RESULTS

The overall scheme for the formation of free sialic acid and its subsequent conversion to hydrogen peroxide is shown in Figure 1. 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 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. The enzymes together gave complete conversion of sialic acid to hydrogen peroxide from about 0.23 nmol of fetuin (~3 nmol of sialic acid) in about 30 minutes at 37°C.

The assay gave good linearity of response using the Sialic Acid Standard at various levels. With absorbance detection, the linear range occurred between 1 and 5 nmol, with correlation coefficients of about 0.996 (Figure 2). With fluorescence detection, the linear range occurred between 100 to 1000 pmols, with correlation coefficients of about 0.996 (Figure 3). Relative error between readings was about 5%.

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 2). The sialic acid content of fetuin and human transferrin were determined using the DMB/HPLC method (n = 3) and the 96-well plate version with absorbance detection of the enzyme-coupled method (n=5). Release of sialic acid was achieved with the latter method using both sialidase treatment and acid hydrolysis. Results are shown in Table 3 and Table 4.

The sialic acid assay in the microtiter plate format gives results with standard glycoproteins which are comparable to those obtained with HPLC analysis (Table 5 and Table 6). However the latter is extraordinarily more time consuming and involved compared with the microtiter plate approach.

CONCLUSIONS

Enzyme-based sialic acid quantitation performed in the microtiter plate assay format with either absorbance or fluorescence detection provides sensitivity as well as precision comparable to other methods, which are considerably more involved and costly. Moreover, conventional methods, such as DMB-HPLC assays, are not readily adapted to high-throughput modes. In this regard, the microtiter plate assay is advantageous as it allows for as many as 90+ data points to be acquired in about 70 minutes. In addition, the enzyme-based method is adaptable to a completely automated approach.

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

REFERENCES

Hara, S., Yamaguchi, M., Takemore, Y., Furuhata, K., Ogura, H. and M. Nakamura. Determination of mono-O-acetylated N-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. **Anal Biochem 179:** 162-166 (1989).

Hudson, B. G., Ohno, M., Brockway, W. J. and F. J. Castellino. Chemical and physical properties of serum transferrin from several species. **Biochemistry 12:** 1047-1053 (1973).

Kaneko, Y., Nimmerjahn, F. and J. V. Ravetch. Anti-inflammatory activity of Immunoglobulin G resulting from Fc sialylation. **Science 313**: 670-673 (2006).

Raju, T. S., Briggs, J. B., Borge, S. M. and A. J. S. Jones. 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 10:** 477-486 (2000).

Regoeczi, E., Taylor, P., Deanne, M. T., Marz, L. and M. W. C. Hatton. Three types of human asialo-transferrin and their interactions with rat liver. **Biochem J 184:** 399-407 (1979).

Richardson, N. E., Buttress, N., Feinstein, A., Stratil, A. and R. L. Spooner. Structural studies on individual components of bovine transferrin. **Biochem J 135:** 87-92 (1973).

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

Scallon, B. J., Tam, S. H., McCarthy, S. G., Cai, A. N. and T. S. Raju. Higher levels of sialylated Fc glycans in immunoglobulin G molecules can adversely impact functionality. **Mol Immun** (in press 2006).

Spiro, R. G. and M. J. Spiro. The carbohydrate composition of the thyroglobulins from several species. **J Biol Chem 240:** 997-1001 (1965).

Spiro, R. G. Studies on Fetuin a glycoprotein of fetal serum. **J Biol Chem 235:** 2860-2869 (1960).

Townsend, R. R., Manzi, A., Merkle, R. K. Rohde, M. F., Spellman, M., Smith, A. and S. A. Carr. Quantitative Monosaccharide Analysis: A Multi-Center Study. Association of Biomolecular Resource Facility, http://www.abrf.org/ABRFNews/1997/mar97monosach.html

ProZyme® and Glyko® are registered trademarks of ProZyme, Inc, San Leandro, CA, USA.

Sialidase A™, Signal™, GlycoSep™ and GlykoScreen™ are trademarks of ProZyme, Inc, San Leandro, CA, USA.