Direct Measurement of Sialic Acids Released From Glycoproteins, by High Performance Liquid Chromatography and Charged Aerosol Detection

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Poster Note 64686

Overview

Purpose: To develop a fast and sensitive HPLC method for direct measurement of the sialic acid content of glycoproteins.

Methods: An isocratic HPLC charged aerosol detector (CAD) method was developed for direct determination of sialic acids from glycoproteins following their release by neuraminidase digestion.

Results: N-acetyl-neuraminic acid and N-glycolyl-neuraminic acid were measured in less than 12 min with detection limits of approximately 3pmol on column. The method was evaluated using human and bovine transferrin.

Introduction

Sialic acid is a generic term covering a number of N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. Two important sialic acids are N-acetyl-neuraminic acid (Neu5Ac or NANA) and N-glycolyl-neuraminic acid (Neu5Gc) and these can be found in a number of biochemically relevant molecules including glycoproteins, proteoglycans and glycolipids. Sialic acid plays a number of vital physiological functions and is involved with neural transmission, synaptogenesis, and immunity (1). Sialylation can also improve the qualities of therapeutic glycoproteins, such as circulatory half-life, biological activity, and solubility (2). Therefore, it is important to determine the sialic acid content of such protein when assaying for pharmaceutical therapeutic function and efficacy.

Typically, sialic acids are not measured as part of the intact glycoprotein but, rather, are usually determined following their release using acid hydrolysis or enzymatic digestion. Released sialic acids can then be measured by a number of analytical methods. Spectrometric methods, although easy to perform, tend to overestimate sialic acids content due to the presence of numerous interferences. Chromatographic approaches have the advantage of separating individual sialic acids from interfering compounds and thus can determine sialic acids content more accurately. A common high performance liquid chromatography (HPLC) method uses fluorescent labelling, but derivatization procedures can be time consuming. Presented here is an approach for the direct determination of sialic acids using HPLC with charged aerosol detection. The lack of a detectable chromophore in sialic acids is overcome by the universal nature of the CAD that can quantitatively measure any non-volatile compound. Prior to their detection, sialic acids were first resolved on a Thermo Scientific™ Acclaim™ Trinity™ P2 column that uses cation exchange, anion exchange and HILIC separation mechanisms on the same stationary phase.

FIGURE 1. Charged Aerosol Detector and Principle of Operation.

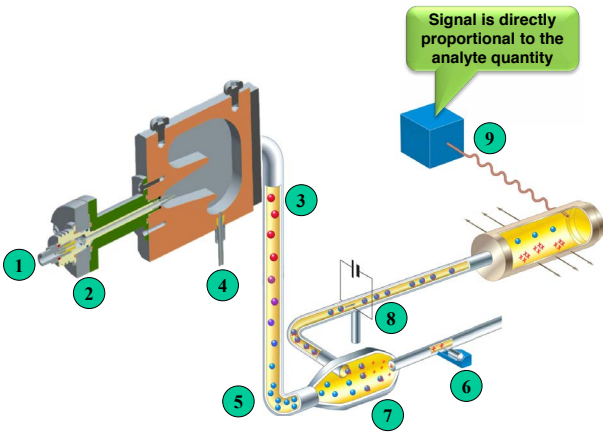

Figure 1 depicts the operation of the charged aerosol detector. At the top left (1) the liquid mobile phase from the LC column enters the detector, where it is nebulized by combining with a concentric stream of nitrogen gas or air (2). The fine droplets are carried by bulk gas flow to the heated evaporation sector (3) where desolvation occurs forming dried particles (5) from any nonvolatile or semivolatile species. Any remaining large droplets drain away to waste (4). The dry analyte particles exit the evaporation tube and interact with another gas stream that has been ionized by a high voltage Corona charger (6). Charge is transferred to the particles (7). After passing through an ion trap (8) that removes any high mobility ionized species, the remaining charged particles pass to a collector and the charge is measured by a sensitive electrometer. The signal produced (9) is directly proportional to the quantity of analyte.

Methods

Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with:
- DPG-3600RS pump
- WPS-3000 TRS autosampler
- TCC-3000RS column compartment
- Thermo Scientific™ Dionex™ Corona™ Veo™ Charged Aerosol Detector:
  - Evaporation Temperature: 60 °C
  - Power function: 1.00
  - Data collection rate: 10 Hz
  - Filter: 10 sec

Column:	Acclaim Trinity P2 column, 3 µm Guard: 2.1 × 10 mm Analytical: 2.1 x 100 mm
Column Temperature:	30 °C
Flow Rate:	0.3 mL/min
Injection Volume:	5µL
Mobile Phase:	65/25/10 Acetonitrile/water/100mM ammonium formate pH4
Reagents:	HPLC- or LCMS-grade or better

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2

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Sample preparation

Sialic acid standard:
A 1mg/mL Neu5Ac and Neu5Gc stock solution was prepared in 18MΩ-cm water, then diluted in sample solvent (60:40 acetonitrile:40mM ammonium acetate, pH5) prior to analaysis.

Protein:
Human transferrin (Fisher CB40204) was prepared by dissolving 16.2 mg standard in 1 mL 18MΩ-cm water. Bovine transferrin (Fisher ICN 15233580) was prepared by dissolving 10 mg standard in 0.6 mL 18MΩ-cm water. Each solution contained approximately 200 nmol/mL of protein.

Neuraminadase digest:
5U Neuraminidase (Fisher ICN15173805, 5U) was dissolved in 1 mL 18MΩ-cm water. A 100 µL volume of neuraminidase solution was then mixed with a 100 µL volume of 200 mM ammonium acetate (pH5) buffer. A 100µL volume of human or bovine transferrin solution was added, mixed, and incubated at 37ºC for 24 hours. At the end of incubation, a 300 µL volume of acetonitrile was added in order to stop the reaction. The solution was then centrifuged at 12,000 rpm for 10 min prior to analysis of the supernatant.

Results

Separation of Sialic Acids

Several different columns with HILIC mechanism were evaluated for separation of Neu5Ac and Neu5Gc. The Acclaim Trinity P2 column was found to provide the best peak shape and lowest column bleed. The Acclaim Trinity P2 column is a mix-mode column with HILIC, weak cation exchange and strong anion exchange separation mechanisms. With a pKa at 2.6, negatively charged sialic acids were retained and separated on the Trinity P2 column under acidic pH, based on charge and hydrophilic affinity. Figure 2 shows an example chromatogram of separation of the two sialic acids as well as related impurity peaks.

The effect of pH and buffer type on separation of the sialic acid peaks from interference by impurities was evaluated. As shown in Figure 3, with ammonium formate, the resolution between the impurities and the main sialic acid peaks was slightly better at pH4 than pH5. With ammonium acetate at pH4.5, the impurity peak seems to be broadened. The background current (a parameter that affects limits of detection) was found to be 2pA, 4pA and 5.8pA for mobile phase with pH4, pH4.5 and pH5, respectively. The final method used an ammonium formate buffer at pH4 for best resolution, lowest background current and thus the best sensitivity.

FIGURE 2. Separation of Sailic Acids Neu5Ac and Neu5Gc on an Acclaim Trinity P2 Column.

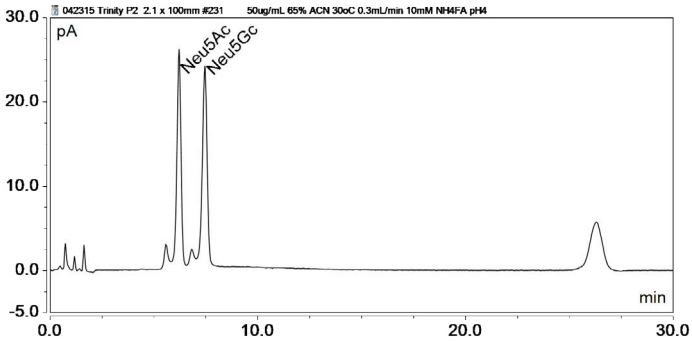
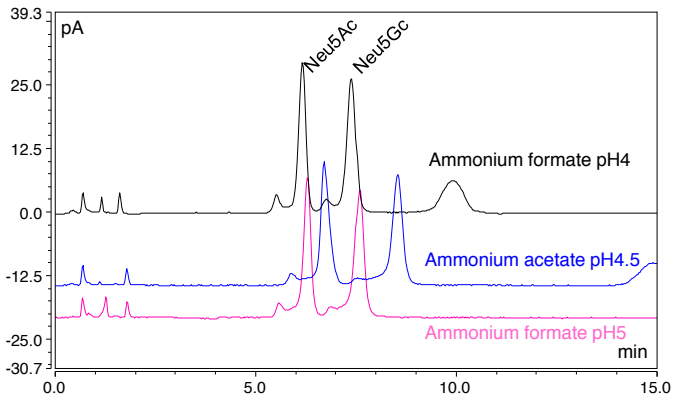


FIGURE 3. Effect of pH and Buffer Type on The Separation of Neu5Ac, Neu5Gc and Impurities.



Method Performance

Calibration curves for the two sialic acids standards (analyzed in triplicate) are presented in Figure 4. Standards were prepared at concentrations ranging from 1 to 20 µg/mL of each sialic acid. With 5 µL injection volumes the mass on column ranged from approximately 15 to 160 pmol. Data were linear in this range, with coefficients of determination, R^2, greater than 0.99 for both analytes.

Detection limit for both analytes was determined to be ~1ng (~3pmol on column). A chromatogram showing detection of 1ng of neu5Ac and Neu5GCc on column at S/N above 3 is presented in Figure 5.

A summary of the method's performance, including precision of retention time and peak area for the 30 pmol calibration standard, the coefficient of determination, and the limits of detection for Neu5Ac and Neu5Gc standard is presented in Table 1.

FIGURE 4. Calibration Data for Direct Detection of Neu5Ac and Neu5Gc by HPLC with Charged Aerosol Detection.

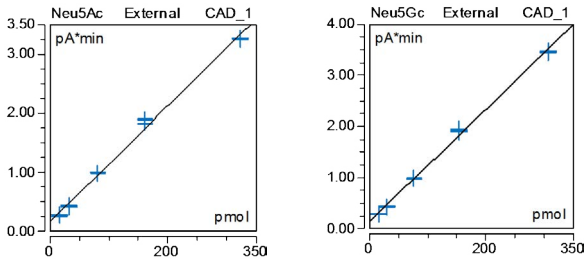
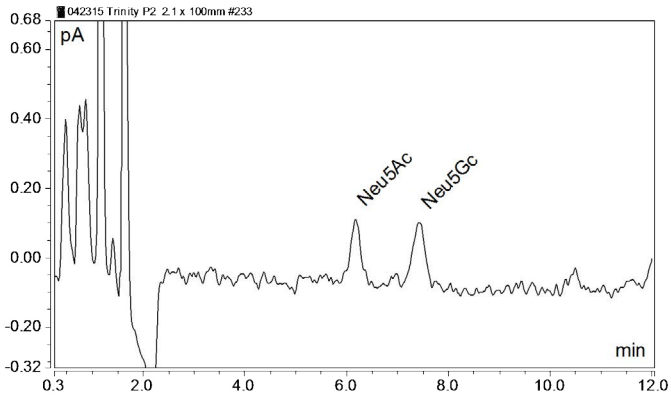


TABLE 1. Method performance for Direct Detection of Neu5Ac and Neu5Gc by HPLC-Charged Aerosol Detection.

Component	Amount (pmol)	Ret. Time* (%RSD)	Peak Area* (%RSD)	LOD (pmol)	R^2
Neu5Ac	32	0.10	1.69	3	0.9953
Neu5GC	30	0.13	2.8	3	0.9975

* n = 6 replicates

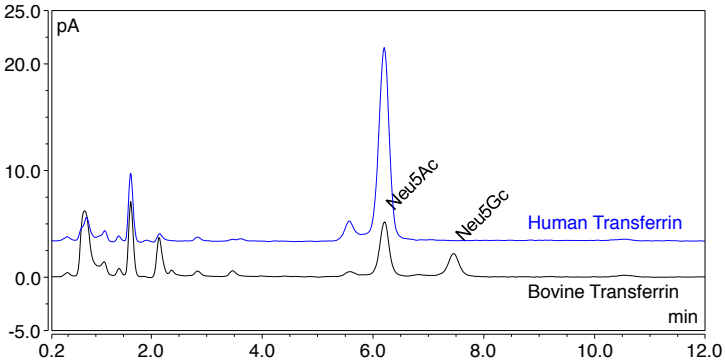
FIGURE 5. Detection of 1ng (approximate 3pmol) Neu5AC and Neu5GC on an Acclaim Trinity P2 Column with Charged Aerosol Detection.



Sialic Acids in Glycoproteins

Sialic acids from human and bovine transferrin were released with nuraminidase enzyme digestion and analyzed with the presented method. Both Neu5Ac and Neu5Gc were observed in the bovine transferrin, while human transferrin contains no Neu5Gc (as expected). See Figure 6.

FIGURE 6. Direct Detection of Enzymatically Released Sialic Acids (Neu5Ac and Neu5Gc) from Human and Bovine Transferrin, by HPLC with Charged Aerosol Detection.



Conclusion

- Charged aerosol detection enables sensitive, direct measurement of sialic acids Neu5Ac and Neu5Gc with no need to perform labeling reactions. Detection limits are 1ng, (approximately 3pmol on column) for both Neu5Ac and Neu5GC.
- The method is precise, with retention time precision better than 0.13% RSD and peak area precision of less than 3% RSD for Neu5Ac and Neu5Gc at 30pmol on column. Response was linear in the range of 15 to 160pmol on column.
- The application of this method to biopharmaceutical samples was illustrated with enzyme hydrolyzed human and bovine transferrin samples.

References

1. Padler-Karavani, V.; Yu, H.; Cao, H.; Chokhawala, H.; Karp, F.; Varki, N.; Chen, X.; Varki, A. Diversity in Specificity, Abundance, and Composition of Anti-Neu5Gc Antibodies in Normal Humans: Potential Implications for Disease. *Glycobiology 2008, 18, 818–830*.
2. Byrne, B.; Donohoe, G.G.; and O'Kennedy, R., Sialic Acids: Carbohydrate Moieties that influence the biological and physical properties of biopharmaceutical Proteins and Living Cells. *Drug Discovery Today*, 2007, 12, 319-326.

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