Label-Free Analysis by UHPLC with Charged Aerosol Detection of Glycans Separated by Charge, Size and Isomeric Structure

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Overview

Purpose: To develop fast and sensitive UHPLC methods suitable to directly measure the glycan content and profile of glycoproteins.

Methods: N-linked glycans were released from proteins by PNGase-F. The released glycans were separated by ultra high performance liquid chromatography (UHPLC) on a new UHPLC platform that integrates the charged aerosol detector into the system for increased performance and ease of use. The mixed mode analytical column employs both weak anion exchange and reversed-phase separation mechanisms to resolve glycans based on charge, isomerism and size. The native glycans were detected directly without labeling by using charged aerosol detection.

Results: Glycans released from various proteins were analyzed including those from bovine fetuin and alpha acid glycoprotein. Quantitative performance including precision, detection limits and dynamic range is presented. Figures of merit include sensitivity at the low-nanogram on-column level, dynamic range over two orders of magnitude, and peak area precision averaging less than three percent RSD.

Introduction

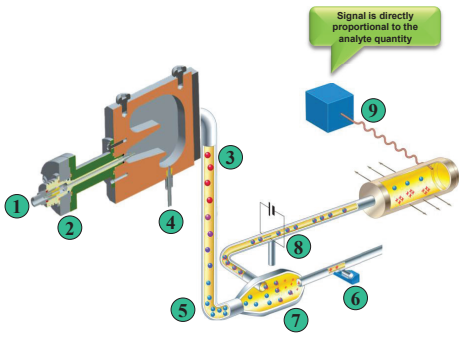
Glycoproteins of biological, diagnostic or therapeutic interest owe key aspects of their normal function to the oligosaccharides attached to the protein backbone. Changes in the number, type, composition or linkage pattern of these glycans may serve as a biomarker of disease or influence the efficacy of a biotherapeutic product.[1] For this reason, the ability to correctly identify and measure these glycans is of scientific interest, and to do so reliably, quickly and inexpensively is of practical benefit.

Glycans are polar molecules that are weakly retained on reversed phase liquid chromatography columns, but more strongly retained by porous graphitic carbon, HILIC, anion-exchange, or mixed-mode columns. Because N-linked glycans are not well-detected by UV/Vis absorption detectors, the separated glycans may be detected directly by pulsed amperometry or mass spectroscopy, or by fluorescence after labeling with 2-AA or 2-AB. Nearly every combination of separation and detection mode has been demonstrated, but the most common combinations for separation and detection of glycans are HILIC-FLD[2] and HPAE-PAD[3] for quantitation, and HILIC-MS and Mixed-mode-MS[4] for identification and characterization.

This work explores direct detection of native glycans as an alternative to the common techniques for glycan analysis that rely on derivatization reactions to render glycans detectable. The lack of a detectable chromophore in native glycans is overcome by using HPLC with charged aerosol detection, a detector that can quantitatively measure any non-volatile compound.

Figure 1 depicts the operation of the charged aerosol detector. At the top left (1) the mobile phase from the LC column entering the detector is nebulized by combining with a concentric stream of nitrogen gas or air (2). The fine droplets carried by bulk gas flow to the heated evaporation sector (3) are desolvated to form dry particles (5) from any nonvolatile or semivolatile species. Any remaining large droplets drain away to waste (4). The dry analyte particles combine with another gas stream that has been charged by a high voltage Corona charger (6). The charged gas transfers positive charge to the analyte particle's surface (7). The charged analyte particles pass through an ion trap (8) that removes any high mobility species and pass to a collector where they are measured by a sensitive electrometer. The signal produced (9) is directly proportional to the quantity of analyte.

FIGURE 1. Charged aerosol detector and principle of operation.



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Methods

Liquid Chromatography

Thermo Scientific™ Vanquish™ UHPLC system with:
- Vanquish Charged Aerosol Detector H:
  - Evaporation Temperature: 50 °C
  - Power function: 1.00
  - Data collection rate: 10 Hz
  - Signal Filter: 5 sec

Reagents: HPLC- or LCMS-grade or better

Data Analysis

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

Separation:

Column:	Thermo Scientific™ GlycanPac™ AXR-1 1.9 µm, 2.1 × 100 mm
Column Temp:	30 °C
Flow Rate:	0.4 mL/min
Injection Vol.:	2 µL
Mobile Phase A:	Deionized water
Mobile Phase B:	100 mM ammonium formate pH 4.4
Gradient: Time, %B:	-8, 4; 0, 4; 35, 39 (slope = 1 mM/min)

Sample prep:
OligoStandard;
Add 500 µL of HPLC grade water to one vial of Thermo Scientific™ OligoStandard™ Sialylated Fetuin N-linked alditols (Thermo Fisher Scientific P/N 043604). Vortex to dissolve and transfer to a polypropylene HPLC autosampler vial.

Glycan Standards:
Sialylated glycan standards were purchased from Prozyme. Monosialylated A1 (GKC-124300), di-sialylated A2 (GKC-224300), and tri-sialylated A3 (GKC-335300). Reconstitute one vial (10 µg) of each glycan standard with 50 µL deionized water. Vortex to mix and transfer to polypropylene autosampler vial.

Alpha acid glycoprotein (Sigma G3643) and fetuin from fetal bovine serum (ICN) were prepared by dissolving 4 mg +/- 1 mg in 1 mL HPLC grade water.

Protein PNGase F digestions were performed by using QA-Bio PNGase F Deglycosylation kit (QA-Bio P/N E-PNG01) per the manufacturer's instructions. Briefly, add 35 µL of protein solution to a plastic centrifuge tube. Add 10 µL 5x Reaction Buffer 7.5 and 2.5 µL of Denaturation Solution. Heat at 100°C for 5 minutes. Cool. Add 2.5 µL of Triton X-100 and mix. Add 2.0 µL of PNGase F to the reaction. Incubate 18 hours at 37°C. Centrifuge at 6720 x g for 10 min and inject the supernatant.

Method Development

Three method parameters were optimized during development of this UHPLC-CAD method*. First, from a starting concentration of 4 mM ammonium formate, the gradient slope was optimized by comparing glycan resolution and total run time for gradient slopes ranging from 0.5 – 3 mM/min. The optimum gradient slope was 1 mM/min, as seen in Figure 2, so this was chosen for the final method.

Secondly, mobile phase composition was optimized by examining the S/N of analytes after inclusion of from 0 – 20% acetonitrile or methanol in the mobile phase. Although the signal increased with increasing organic solvent, the S/N ratio varied only slightly and peaked at 1 or 5 % (Figure 3). Given the only modest increase in S/N provided by added solvent, we chose to omit the solvent for the sake of simplicity.

Finally, the effect of evaporation tube temperature was considered by examining S/N for the glycan analytes at evaporation temperature settings of 35, 50 and 80 °C. Although S/N clearly decreased at 80 °C, the differences between 35 °C and 50 °C were less pronounced (Figure 4). 50 °C was chose as the evaporation temperature for the final method.

*(For convenience, these initial experiments were performed on a Thermo Scientific Dionex™ UltiMate™ 3000 RSLC system using a Thermo Scientific™ Dionex™ Corona™ Veo™ charged aerosol detector with performance similar to the Vanquish Charged Aerosol Detector H).

FIGURE 2. Optimizing mobile phase gradient slope.

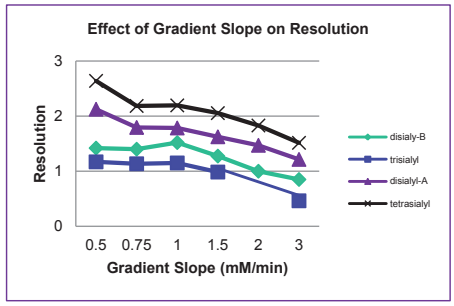


FIGURE 3. Optimizing mobile phase composition.

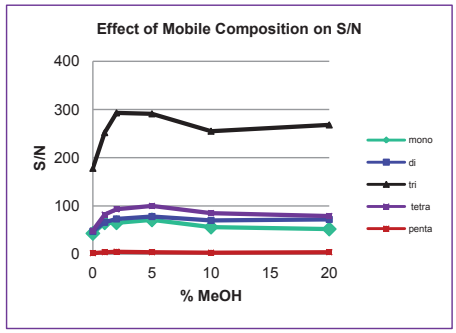
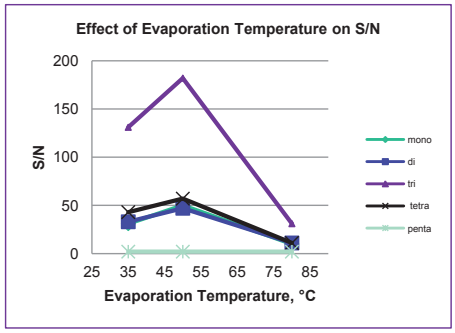


FIGURE 4. Optimizing evaporation temperature.



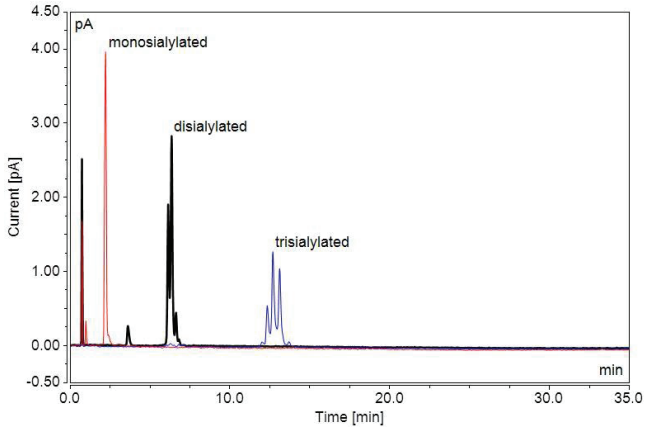
High Resolution Separation by Charge, Size and Structure

Quality control labs profile a protein's glycan pool to assess lot-to-lot variability, degradation or level of impurities. In the separation shown in Figure 5, glycans in a standard mixture are separated according to charge, size and structure. The native glycans are separated by UHPLC using a binary gradient consisting of water and a volatile ammonium formate buffer and measured directly by using the Vanquish Charged Aerosol Detector H. The elution order is neutral glycans first, followed by glycans with a single negative charge (monosialylated), glycans with two negative charges (disialylated), and so on. The glycans comprising each charge group are separated by ion-exchange interactions. Within each well-separated charge group, glycans differing in size or isomeric structure are further resolved by reversed phase interactions.

There is no need to use fluorescent labeling when using charged aerosol detection, as may be necessary with other means of detecting these compounds. Because of the uniform response of the charged aerosol detector, the relative peak area accurately reflects the amount (pmol) within each charge group.

Note that under these conditions the neutral glycans are not well separated from the void peaks. To better resolve neutral glycans, use a shallower gradient, or derivatize the glycans to increase hydrophobicity (e.g., 2-AB)[2] or to introduce a negative charge (e.g., 2-AA).

FIGURE 5. Direct charged aerosol detection of bovine fetuin N-linked alditols in a standard mixture separated with high-resolution on the GlycanPac AXH-1 column.



Performance

Calibration curves for three major charge groups of the bovine fetuin N-linked alditol standard (analyzed in triplicate) are presented in Figure 6. Standards were prepared at concentrations ranging from 1 to 200 pmol/µL; using 2 µL injections the mass on column ranged from 2 to 400 pmol. The data were fit to a quadratic equation, yielding coefficients of determination, R^2, greater than 0.995 for all three analytes.

Table 1 presents a summary of the method's performance, including the coefficients of determination and the limits of detection for the three major charge groups of the bovine fetuin N-linked alditol standard. Also included is the precision obtained for 7 replicates of the fetuin oligosaccharide alditols standard (55 pmol/µL total sialylated fetuin N-linked alditols, nominal).

FIGURE 6. Calibration data for direct detection of N-linked glycans by HPLC-Charged Aerosol Detection.

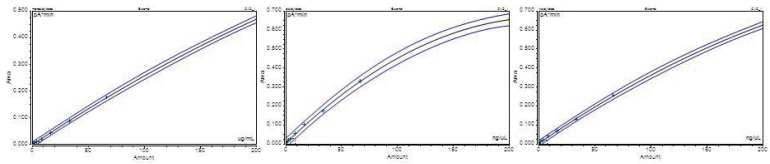


TABLE 1. Method performance for Direct Detection of Glycans by HPLC-Charged Aerosol Detection.

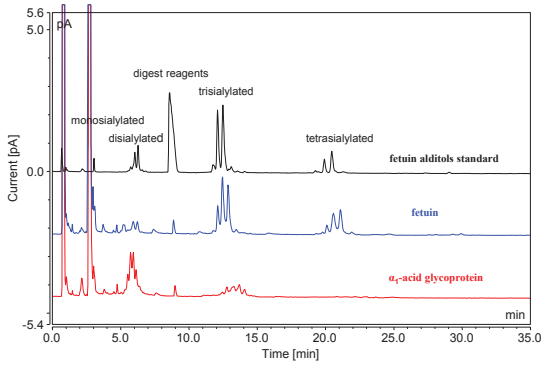
Component	Amount (ng/µL)	Amount[1] (pmol)	Ret. Time[2] (%RSD)	Peak Area[2] (%RSD)	LOD[3] (ng/µL)	R^{2*}
Monosialylated	12	6	0.11	2.7	7.3	0.999
Disialylated	22	10	0.04	1.2	8.8	0.997
Trisialylated	115	40	0.03	2.8	7.4	0.995
Tetrasialylated	19**	5	0.03	1.7	--	--
1. Estimated from nominal MW of the glycan calibration standards						
2. for n = 7 replicates						
3. Hubaux-Vos method						
* 8 levels, in triplicate (duplicate for highest concentration), quadratic fit with no offset						
** Estimated by peak area response factor for disialylated glycan standard						

Protein Digests

Charged aerosol detection is clearly highly sensitive, able to detect glycans at the low picomole concentration level. Because charged aerosol detection is universal, there might be concern that the reagents and reaction products remaining after endoglycosidase treatment would interfere with detection of the released glycans. To test for such interference, we treated two proteins by using a commercial glycan release kit and then analyzed for glycans by using UHPLC-CAD. Figure 7 shows that although additional peaks related to the reaction procedure are evident, they are well resolved from the glycan analytes and do not interfere with reliable quantification of the glycans.

This demonstrates that where ultimate sensitivity is not required[3], UHPLC-CAD obviates the need to spend time or money on 2-AB labeling and delivers a clean chromatogram with no concern for reaction side products.

FIGURE 7. Direct charged aerosol detection of α_1-acid glycoprotein and bovine fetuin N-linked glycans released by PNGase F separated with high-resolution on the GlycanPac AXH-1 column



Conclusion

- The HPLC method developed to measure native glycans is precise, with retention time precision better than 0.1% RSD and peak area precision averaging 2.1 % RSD for the major sialylated N-glycans of bovine fetuin.
- Charged aerosol detection enables sensitive, direct measurement of glycans with no need to perform labeling reactions. Detection limits for native glycans are in the low pmol (ng on-column) range.
- By responding directly to any non-volatile compound, charged aerosol detection is able to measure both native and fluorescently labeled glycans, yielding simple, accurate and precise estimates of relative concentration even in the absence of pure primary standards.

References

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