Poster Note 71817

Label-Free Profiling of O-linked Glycans by HPLC with Charged Aerosol Detection

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Introduction

Purpose

The goal of this work was to develop a quantitative profiling assay for O-linked glycans released from glycoproteins by reductive β-elimination. While glycan release under reducing conditions helps to reduce peeling reactions, the resulting O-linked glycan alditols can't be derivatized easily with a fluorescent label. Charged aerosol detection does not require a fluorophore or chromophore for sensitive, accurate quantification, and so UHPLC-CAD provides a simple, direct approach to separate and quantify native glycans. The method uses a volatile mobile phase fully compatible with mass spectrometry in case further characterization is desired.

Methods

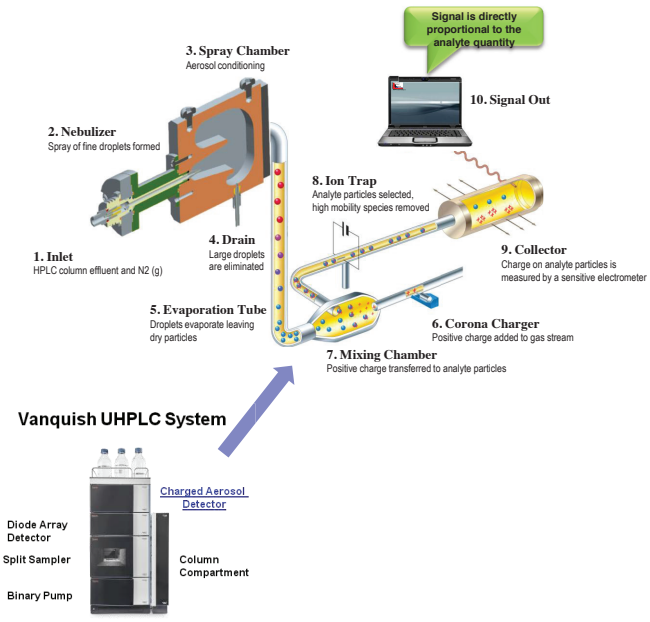
O-linked glycans are released from proteins by reductive β-elimination. The released glycans are separated by ultra high performance liquid chromatography (UHPLC) on a new UHPLC platform that integrates the charged aerosol detector into the system for increased performance and ease of use (Figure 1). The mixed mode analytical column employs both weak anion exchange and HILIC separation mechanisms to resolve glycans based on charge, size and polarity. The reduced glycans are detected directly without labeling by using charged aerosol detection.

Results

O-linked glycan pools released from various proteins were analyzed including those from bovine fetuin, bovine submaxillary mucin, and IgG. 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 three percent RSD.

Figure 1 depicts the charged aerosol detector and its placement within the UHPLC system. At the top left (1) the mobile phase from the LC column enters the detector and is nebulized by combining with a concentric stream of nitrogen gas or air (2). The fine droplets selected in the spray chamber (3) are swept to the heated evaporation tube (3) and desolvated to form dry particles (5) from any nonvolatile or semivolatile species. 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 (9) where charge is measured by a sensitive electrometer. The signal produced (10) 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: Reagent-grade or LCMS-grade

Data Analysis

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

Separation:

Column:	Thermo Scientific™ GlycanPac™ AXH-1 1.9 µm, 2.1 × 100 mm
Column Temp:	30 °C, Still Air mode
Flow Rate:	0.5 mL/min
Injection Vol.:	0.2 - 2 µL
Mobile Phase A:	95:5 acetonitrile:deionized water
Mobile Phase B:	50 mM ammonium formate pH 4.4
Gradient: Time, %B:	-8, 2; 0, 2; 60, 42 (slope = 0.67 mM/min)

Sample prep:
Thermo Scientific™ Dionex™ OligoStandard™ Sialylated Fetuin N-linked alditols were from Thermo Fisher Scientific (P/N 04360). Added 500 µL of HPLC grade water to one vial, vortexed to dissolve and transferred to a polypropylene HPLC autosampler vial.

Galβ(1-3)GalNAc-α-Thr (TCI G0340) from VWR was dissolved in deionized water at a concentration of 1 mg/mL. The O-linked Core 1 Glycan was released during the reductive β-elimination procedure.

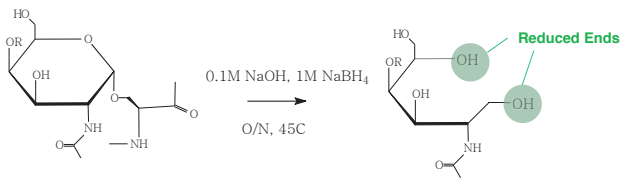
Mucin, bovine submaxillary type 1-S (Sigma M3895), Anti-IgG H&L Rabbit Polyclonal Antibody (VWR RL105-4102),fetuin from fetal bovine serum (Sigma F3004) and a commercial monoclonal antibody from a private donor were prepared by dissolving 10 mg +/- 1 mg in 1 mL HPLC-grade water.

O-glycan pools were released by reductive β-elimination as follows. Briefly, dispensed a volume of solution containing 500 µg of protein into a 1.5 mL screw-cap tube and dried in a SpeedVac. Added 125 µL 1.0 M NaBH4 in 0.1 M NaOH. Heated at 45°C for 18-24 hours. Centrifuged 1 min to unite condensate with bulk liquid. Neutralized by adding 12 µL 4 M acetic acid, followed by an additional 12 µL 4 M acetic acid. Desalted with AG50W-X8, and centrifuged through 10 kDa spin filter. Dried in SpeedVac, and reconstituted in 100 µL deionized water.

Release of O-glycans

O-linked glycans cannot be released by a single enzyme like the PNGase F typically used to release N-linked glycans. Instead, O-linked glycans are released by chemical methods such as reductive β-elimination [1]. Chemical reduction produces glycans with one end reduced to an alditol that cannot be fluorescently tagged (Figure 2). But this presents no problem for charged aerosol detection, because the charged aerosol detector easily detects the native glycan. Because of the uniform response of the charged aerosol detector, the relative peak area accurately reflects the amount of each nonvolatile analyte.

FIGURE 2. Alditols produced by reductive β-elimination cannot be labeled.



Results

Method Development

Compared to N-linked glycans separated under the same conditions, the O-linked glycans investigated in this work typically elute earlier and with less resolution. After preliminary method scouting to evaluate alternative columns such as the GlycanPac AXR-1 and Thermo Scientific™ Accucore™ 150 Amide HILIC columns, a suitable method for small, polar, neutral and charged O-linked glycans was developed on the GlycanPac AXH-1 column. This somewhat generic method maximized retention of small, neutral and singly charged glycans, while allowing plenty of time to elute possible large and/or more highly charged glycans. The final method was used to analyze both the O-linked (upper trace) and N-linked (lower trace) glycan pools released from bovine fetuin, which are compared in Figure 3. The O-linked glycan peak identifications are tentatively assigned until confirmed by MS.

FIGURE 3. Fetuin N- and O-linked *native* glycans analyzed by UHPLC-CAD on the GlycanPac AXH-1.

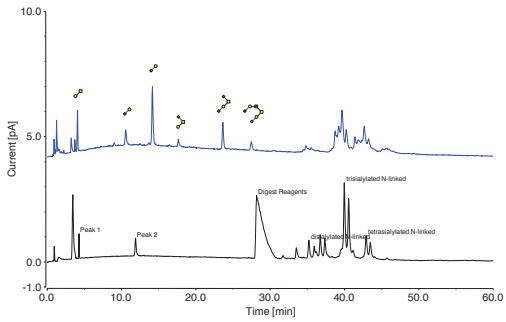


Figure Legend:

Linkage Type: - - - α-type ─ β-type
Symbol for Sugar ■ N-acetylglucosamine ■ N-acetylgalactosamine ● Galactose ◆ Sialic Acid

Performance

Lacking sufficient O-linked glycan core standard, we evaluated method performance by analyzing the well characterized and readily available sialylated fetuin N-linked alditol standard shown in the lower trace of Figure 3. Table 1 presents a summary of the retention time and peak area precision for two peaks eluting within the retention time window corresponding to the O-glycans plus the trisialylated and tetrasialylated N-glycans. The limits of detection for O-linked glycan standards were not determined for this work, but similar methods for N-linked glycans have yielded limits of detection in the low ng/µL range and quadratic calibration curves over the range from 1–200 ng/µL (2–400 ng on-column) [2].

TABLE 1. Method performance for Label-free Profiling of O-linked Glycans by UHPLC with Charged Aerosol Detection.

Component	Ret. Time[1] (%RSD)	Peak Area[1] (%RSD)
Peak 1	0.06	3.3
Peak 2	0.04	3.7
Trisialylated	0.01	3.5
Tetrasialylated	0.02	1.9
Average	0.03	3.1

1. for n = 7 replicates

Protein Digests

Charged aerosol detection is clearly highly sensitive, able to detect glycans at the low ng/μL (pmol/μL) concentration level. Because charged aerosol detection is universal, there might be concern that the reagents and reaction products remaining after reductive β-elimination would interfere with detection of the released glycans. To test for such interference, we treated several proteins by reductive β-elimination and then analyzed the released glycans by using UHPLC-CAD. Figures 4 and 5 show that although additional peaks related to the reaction procedure may be 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 easily detects native O-linked glycans and delivers a clean chromatogram without complicated sample cleanup to remove reaction side products.

FIGURE 4. Direct charged aerosol detection of the O-linked glycan pool released form mucin by reductive β-elimination, and separated with high-resolution on the GlycanPac AXH-1 column.

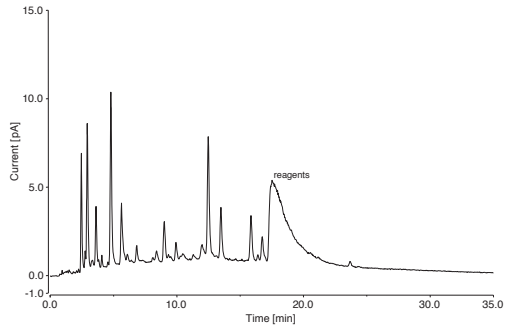
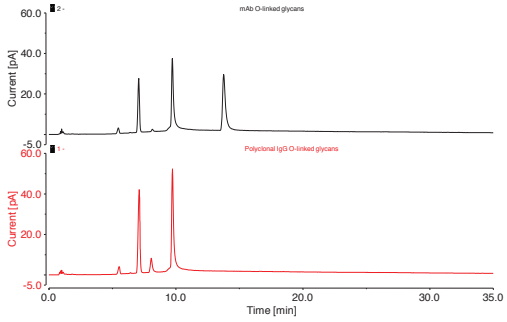


FIGURE 5. Direct charged aerosol detection of the O-linked glycan pools released from a mAb (top trace) and a polyclonal IgG (bottom trace) by reductive β-elimination, and separated with high-resolution on the GlycanPac AXH-1 column.



High Resolution Separation by Charge, Size and Polarity

Quality control labs profile a protein's glycan pool to assess lot-to-lot variability, degradation or level of impurities. In the separations shown in Figures 3, 4 and 5, O-glycans are separated according to charge, size and polarity. The native glycans are separated on the GlycanPac AXH-1 by using a binary gradient of acetonitrile and a volatile ammonium formate buffer. 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 polarity are further resolved by HILIC interactions. This mixed mode separation may be preferred in some cases for its simple MS compatible mobile phase versus HPAE-PAD, and for different selectivity versus HILIC methods [4].

Future Steps

This preliminary work shows that HPLC with charged aerosol detection is well suited to analyze O-linked glycan pools released from glycoproteins by reductive β-elimination.

Future work will include:

- A more rigorous evaluation of method performance parameters such as limits of detection and linear dynamic range, using authentic O-glycan core standards.
- The development of an optimized workflow for O-glycan release, cleanup, separation and detection that is simple and robust enough for routine quality control purposes.
- Further investigation into coupling charged aerosol detection for quantitative analysis with mass spectrometry for glycan identification [5,6].

Conclusion

- O-glycans released by reductive β-elimination to preserve structural integrity can be quantitatively measured by a simple direct method that minimizes sample manipulation and cleanup.
- Released glycans are separated with high resolution by charge, size and polarity on a mixed mode column employing both ion exchange and HILIC separation modes, using a simple, mass spectrometry compatible mobile phase.
- The UHPLC method developed to measure native O-linked glycans is precise and sensitive. Retention time precision is better than 0.1% RSD and peak area precision averages 3.1 % RSD. 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 quantify unlabeled O-linked glycans. The uniform response of charged aerosol detection also provides simple, accurate and precise estimates of relative concentration even in the absence of pure primary standards.

References

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