Application Note

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Exact Structure Identification of Isomeric N-Glycans by High-Resolution Ion Mobility LC/Q-TOF

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Abstract

This application note describes the assignment of exact structures of N-glycan released from glycoproteins of complex biological sample using ion mobility-mass spectrometry (IM-MS) exploiting unique arrival time distributions (ATDs) for each glycan. Glycans are derivatized with 2-aminobenzoic acid, separated by porous graphitized carbon chromatography, and then analyzed with IM-MS in multiplexing mode. After high-resolution demultiplexing, distinctive IM ATDs are obtained, which can be used for exact structure assignment.

Introduction

Glycosylation is structurally the most complex post-translational modification of proteins playing key roles in many biological and disease processes. To understand the biology of glycans at a molecular level, it is essential to determine exact glycan structures in complex biological samples. Current analytical methods entail glycan release, derivatization and purification, followed by chromatographic separation and MS. Accurate mass measurements are used for compositional assignment and additional MS/MS fragmentation experiments can provide more detailed structural information. The assignment of exact structures from MS/MS spectra remains, however, very difficult. Glycans are usually branched and due to their isobaric nature, it is difficult to interpret multistage MS data. Recently, it was shown that IM ATDs of N-glycans resemble conformational populations in the gas phase and that these unique conformer distributions can be used for identification of isomeric glycans using a glycan ATD database.[1] In this application, IM-MS with multiplexing and high-resolution demultiplexing, combined with the use of well defined N-glycan standards, is applied for fast and unambiguous assignment of exact isomeric glycan structures derived from red blood cells using conformer distribution fingerprints (CDFs).

The N-glycans used in this application consist of a core pentasaccharide modified by branching N-acetylglucosamine moieties. These branching points can be extended by several N-acetyl-lactosamine (galactose(β1,4)N-acetylglucosamine, LacNAc) repeating units (Figure 1).

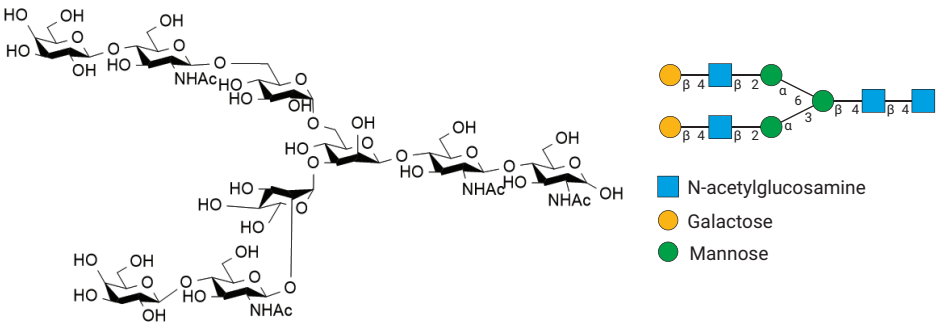

Figure 1. N-glycan structure (left). The representation of the molecular structure is commonly simplified by the use of symbols for the building blocks and annotation of linkages (right).

Experimental

N-glycan sample preparation

Analytical standards were synthesized as described before.[2,3] The amino acid and the first core N-acetylglucosamine were cleaved using commercially available Endo F2 (New England Biolabs) and the synthetic standards were labeled with 2-aminobenzoic acid (2-AA) using standard procedures. Samples were purified using porous graphitized carbon (PGC) solid phase extraction (SPE) cartridges before subjecting to LC/IM-MS.[4] Guinea pig red blood cells were isolated from fresh blood samples by centrifugation and washed with phosphate buffered saline. Lysis of the cells was performed in de-ionized water as previously described[5], and release of N-glycans was achieved by using commercial Endo F2. Isolation and purification of glycans was done by subsequent C_{18} and PGC SPE using commercially available cartridges.[5] Samples were labeled by reductive amination using 2-AA and sodium cyanoborohydride in DMSO with acetic acid for 2 hours at 65 °C and purified first by minitrap G-10 followed by PGC SPE cartridges. Before injection, samples were treated with an aqueous acetic acid solution (2 M, 72 hours, 65 °C) to remove sialic acid residues, and excess solvent was removed *in vacuo*.

LC/IM-MS analysis

LC/IM-MS analyses were performed on an Agilent 1290 Infinity II LC coupled to the Agilent 6560 ion mobility LC/Q-TOF, equipped with an Agilent Jet Stream source. LC separation was obtained on a Thermo Fisher Scientific Hypercarb PGC column (100 × 2.1 mm, 3 µm). The settings for LC and IM-MS are shown in Tables 1 and 2.

Data analysis

Multiplexed IM data was demultiplexed using PNNL PreProcessor Beta 3.0 software[6] and high-resolution IM data were obtained using HRdm 2.0 software.[7]

Table 1. LC conditions on the Agilent 1290 Infinity II LC.

Parameter	Value
Column	Hypercarb PGC, 100 × 2.1 mm, 3 µm
Column Temperature	75 °C
Solvent A	50 mM ammonium formate (pH 4.4)
Solvent B	Acetonitrile
Gradient	0 min 65% A and 35% B 1 min 65% A and 35% B 7 min 59% A and 41% B
Flow Rate	0.2 mL/min
Injection Volume	1 µL

Table 2. IM-MS settings on the Agilent 6560 ion mobility LC/Q-TOF.

Parameter	Value
Source	Agilent Jet Stream
IM-MS Polarity Mode	Negative
Capillary Voltage	3,500 V
Drying Gas Flow	8 L/min
Gas Temperature	300 °C
Nebulizer Pressure	50 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nozzle Voltage	1,000 V
Fragmentor	400 V
m/z Range	100 to 1,700
IM Trap Fill Time	3,900 µs
IM Trap Release Time	250 µs
IM Multiplexing Pulsing Sequence Length	4 bit
IM Maximum Drift Time	60 ms
IM Transient Rate	18 IM transients/frame
IM Drift Tube Entrance Voltage	−1,400 V
IM Drift Tube Exit Voltage	−250 V
IM Drift Tube Gas Type	Nitrogen
IM Drift Tube Pressure	3.95 Torr
IM Trap Funnel Pressure	3.80 Torr

Results and discussion

The extracted ion chromatograms of 2-AA labeled N-glycans, derived from guinea pig erythrocytes, showed the presence of isomeric N-glycans (Figures 2A and 2C).

Elucidation of exact structures for each peak, however, was difficult by MS/MS only. Using IM with CDFs, the identification was performed fast and unambiguously. A library with high-resolution CDFs of N-glycan standards 1 to 8 with different poly-LacNAcs chains (Figures 2 and 3) was obtained by direct infusion of aqueous solutions into the IM-MS instrument and high-resolution demultiplexing.

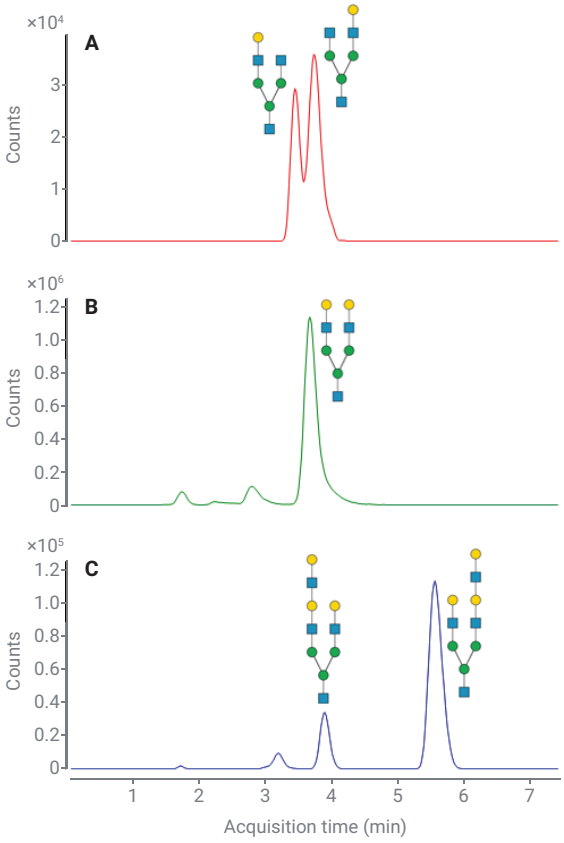

Figure 2. Extracted ion chromatograms of 2-aminobenzoic acid-labeled N-glycans, derived from guinea pig erythrocytes, as $[M-2H]^{2-}$ ions at *m/z* 697.25 (A), *m/z* 778.28 (B), and *m/z* 960.84 (C). Exact structures were assigned using conformer distribution fingerprints of the compounds.

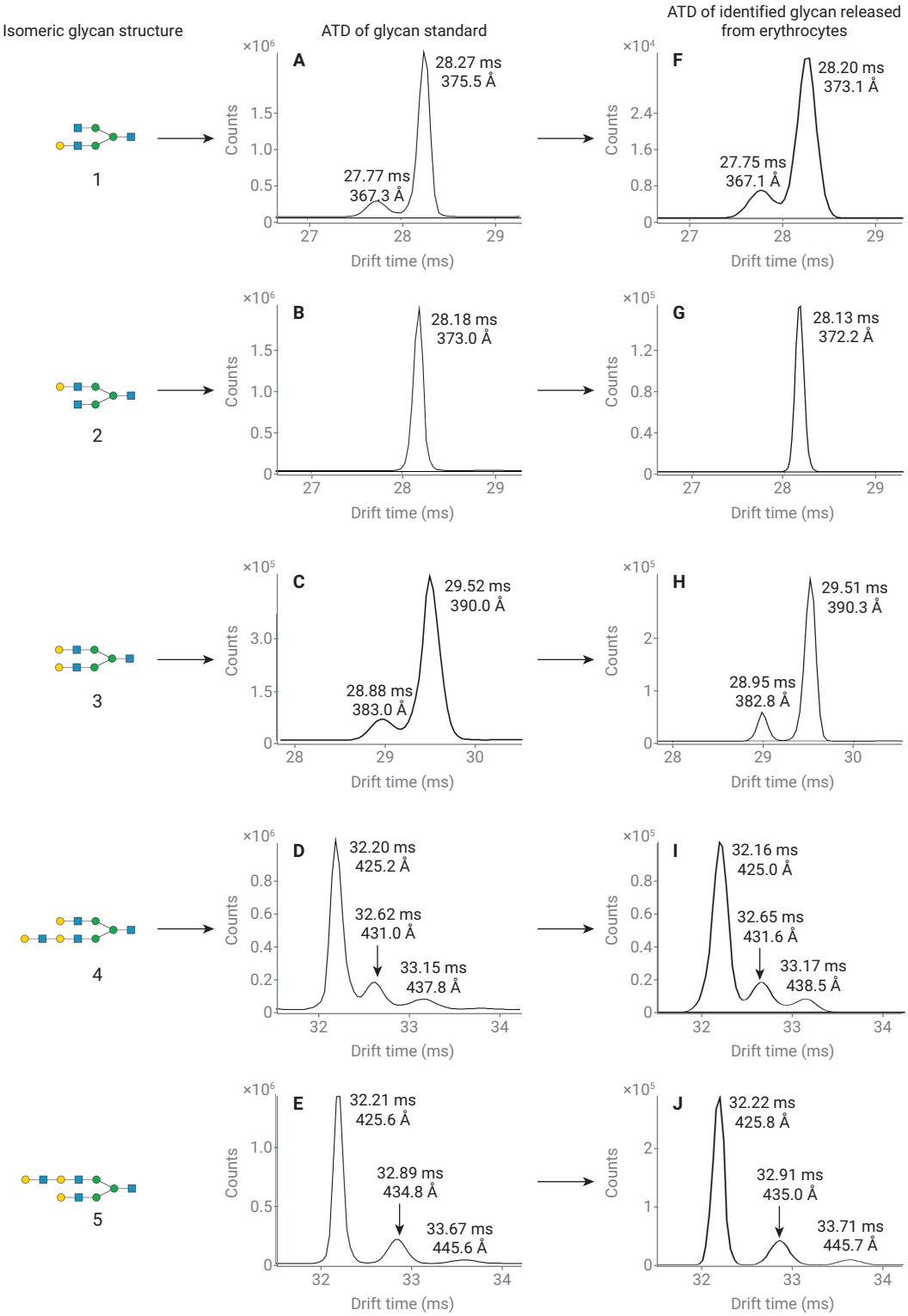

Figure 3. High-resolution arrival time distributions of N-glycan standards 1 to 5 (A to E), including isomeric structures, showed unique conformer distribution fingerprints (CDFs). Exact structures of N-glycans derived from red blood cells (F to J) were obtained by matching CDFs and collision cross section (CCS) values.

Distinctive CDFs were obtained for all standards (Figures 3A to 3E and Figures 4A to 4C), with resolutions up to 267 (Ω/ΔΩ), which enabled exact structure identification of the N-glycans with poly-LacNAcs chains (Figures 2F to 2J).

Standards 6 to 8 (Figure 4) showed unique CDFs for all three isomers, but these compounds were not detectable on the red blood cells used.

Conclusion

IM conformer distribution fingerprints allow fast and unambiguous assignment of exact N-glycan structures.

References

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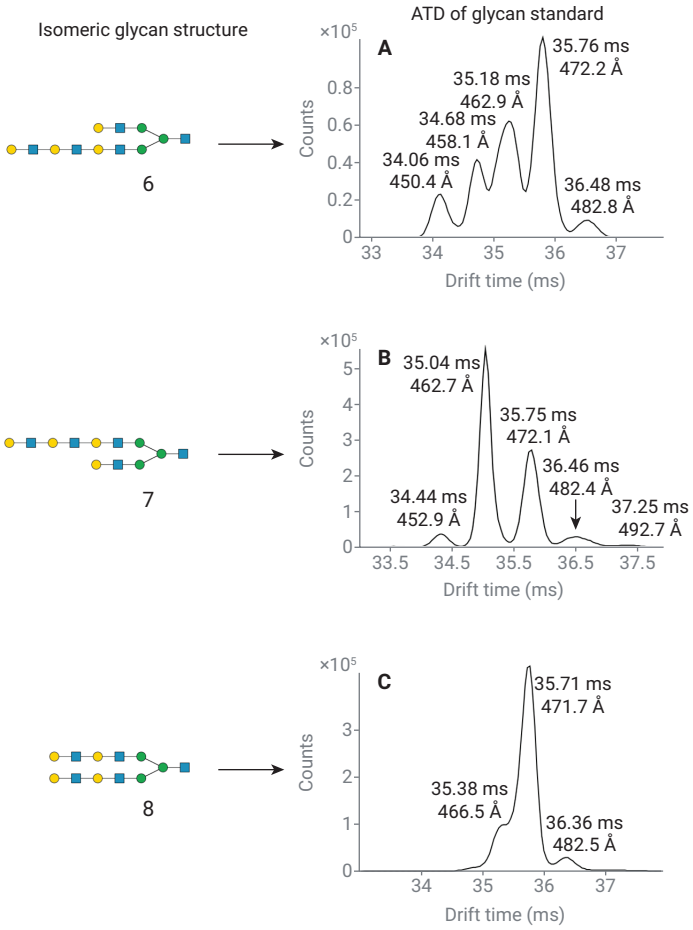

Figure 4. High-resolution arrival time distributions of isomeric structures 6 to 8 showing distinctive collision cross-section values and detailed conformer distribution fingerprints for fast and unambiguous identification in biological samples.

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