Protocol for ultra performance liquid chromatography-mass spectrometry N-glycan analysis of the native and desialylated human apo-transferrin

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Abstract

This protocol describes the method used for *N*-glycan analysis of the native and desialylated human apo-transferrin. The protein *N*-glycans were released with the addition of 1.2 U of PNGase F and overnight incubation at 37°C. The released *N*-glycans were labeled with 2-AB and purified using hydrophilic interaction liquid chromatography solid-phase extraction (HILIC-SPE). Fluorescently labeled *N*-glycans were separated by UPLC using Waters BEH Glycan chromatography column. All glycan structures were annotated with MS/MS analysis using an ESI-QTOF-MS system.

Keywords

transferrin, *N*-glycan analysis, UPLC, mass spectrometry

1. Materials and instrumentation

1) human apo-Transferrin protein (200 µg; Biorbyt, UK, cat. no. orb80927)
2) sodium dodecyl sulfate (SDS) (1.33% (w/v); Invitrogen, USA)
3) Igepal-CA630 (4% (v/v); Sigma Aldrich, USA)
4) PNGase F (1.2U; Promega, USA)
5) acetonitrile (ACN) (J.T. Baker, USA)
6) ethanol (70%; Sigma-Aldrich, USA)
7) ammonium formate (100mM, pH 4.4; prepared in-house using formic acid (Merck, Germany) and ammonia solution 25% (Merck, Germany))
8) *Labeling mixture*:
   a) 2-aminobenzamide (2-AB) (19.2 mg/mL; Sigma Aldrich, USA)
   b) 2-picoline borane (44.8 mg/mL; Sigma Aldrich, USA)
   c) dimethyl sulfoxide (Sigma Aldrich, USA) and glacial acetic acid (Merck, Germany) mixture (70:30 v/v)
9) plate shaker (GFL, Germany)
10) 0.2 µm GHP filter plate (Pall Corporation, USA)
11) vacuum manifold (Millipore Corporation, USA)
12) Acquity UPLC H-Class instrument (Waters, USA)
13) Empower 3 software, build 3471 (Waters, USA)
14) BEH Glycan chromatography column, 150 × 2.1 mm, 1.7 µm BEH particles (Waters, USA)
15) Synapt G2-Si ESI-QTOF-MS system (Waters, USA)
16) Eppendorf Centrifuge 5804 (Eppendorf, Germany)
17) Vacuum Concentrator (Thermo, USA)

2. Ultra performance liquid chromatography- mass spectrometry *N*-glycan analysis

2.1. Initial sample preparation

Initial samples were prepared by dissolving needed amount of protein concentrate corresponding to 200 µg of native or desialylated apo-transferrin in 50 µL of redistilled water, followed by drying in a vacuum centrifuge

2.2. *N*-glycan release

Samples were denatured with addition of 30 µl of 1.33% SDS (w/v) and by incubation at 65°C for 10 min. After denaturation, 10 µl of 4% (v/v) Igepal-CA630 was added to the samples, and the mixture was shaken 15 min on a plate shaker. *N*-glycans were released with the addition of 1.2 U of PNGase F and overnight incubation at 37°C.

2.3. Fluorescent labeling and HILIC-SPE clean up of released *N*-glycans

The released *N*-glycans were labeled with 2-AB using the labeling mixture. To each sample 25 µL of labeling mixture was added, followed by 2 h incubation at 65°C. Free label and reducing agent were removed from the samples using hydrophilic interaction liquid chromatography solid-phase extraction (HILIC-SPE). After incubation samples were brought to 96% of ACN by adding 700 µL of ACN and applied to each well of a 0.2 µm GHP filter plate. Solvent was removed by application of vacuum using a vacuum manifold. All wells were prewashed with 70% ethanol and water, followed by equilibration with 96% ACN. Loaded samples were subsequently washed 5× with 96% ACN. *N*-glycans were eluted with water and stored at – 20 °C until usage.

2.4. N- glycan hydrophilic interactions liquid chromatography coupled to mass spectrometry

Fluorescently labeled *N*-glycans were separated by hydrophilic interaction chromatography on Acquity UPLC H-Class instrument (Waters, USA) consisting of a quaternary solvent manager, sample manager and a fluorescence detector set with excitation and emission wavelengths of 250 and 428 nm, respectively. The instrument was under the control of Empower 3 software, build 3471 (Waters, Milford, USA). Labeled *N*-glycans were separated on a Waters BEH Glycan chromatography column, with 100 mM ammonium formate, pH 4.4, as solvent A and ACN as solvent B. Separation method used linear gradient of 70–53% acetonitrile at flow rate of 0.56 mL/min in a 25-min analytical run. The system was calibrated using an external standard of hydrolyzed and 2-AB labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units (GU). All glycan structures were annotated with MS/MS analysis by HILIC-UPLC coupled with a Synapt G2-Si ESI-QTOF-MS system (Waters). The instrument was under the control of MassLynx v.4.1 software (Waters). MS conditions were set as follows: positive ion mode, capillary voltage 3 kV, sampling cone voltage 30 V, source temperature 120°C, desolvation temperature 350°C, desolvation gas flow 800 l/h. Mass spectra were recorded from 500 to 3,000 m/z at a frequency of 1 Hz. MS/MS experiments were performed in a data-dependent acquisition (DAD) mode. Spectra were first acquired from 500 to 3,000 m/z and then two precursors with the highest intensities were selected for CID fragmentation (m/z 100 to 3,000 was recorded). A collision energy ramp was used for the fragmentation (LM CE Ramp Start 7 V, LM CE Ramp End 13 V, HM CE Ramp Start 97 V, HM CE Ramp End 108 V).

2.5. Glycan peak annotation

The glycan peaks resulting from the UPLC-fluorescence chromatography were processed with Empower 3 software (Waters) using an automated method with a traditional integration algorithm after which each chromatogram was manually corrected to maintain the same intervals of integration for all the

samples. Total area normalization was performed, where the area of each glycan peak was divided by the total area of the corresponding chromatogram. Glycan peaks were analyzed on the basis of their glucose units and compared to reference values in the "GlycoStore" database (available at: https://glycostore.org/) for structure assignment.[1,2] All glycan structures were further confirmed with MS/MS analysis by resulting from HILIC-UPLC-ESI-QTOF-MS analysis. Glycan compositions and structural features were assigned using software tools GlycoWorkbench[3] and Glycomode[4] according to obtained MS and MS/MS spectra.

2.6. Typical results

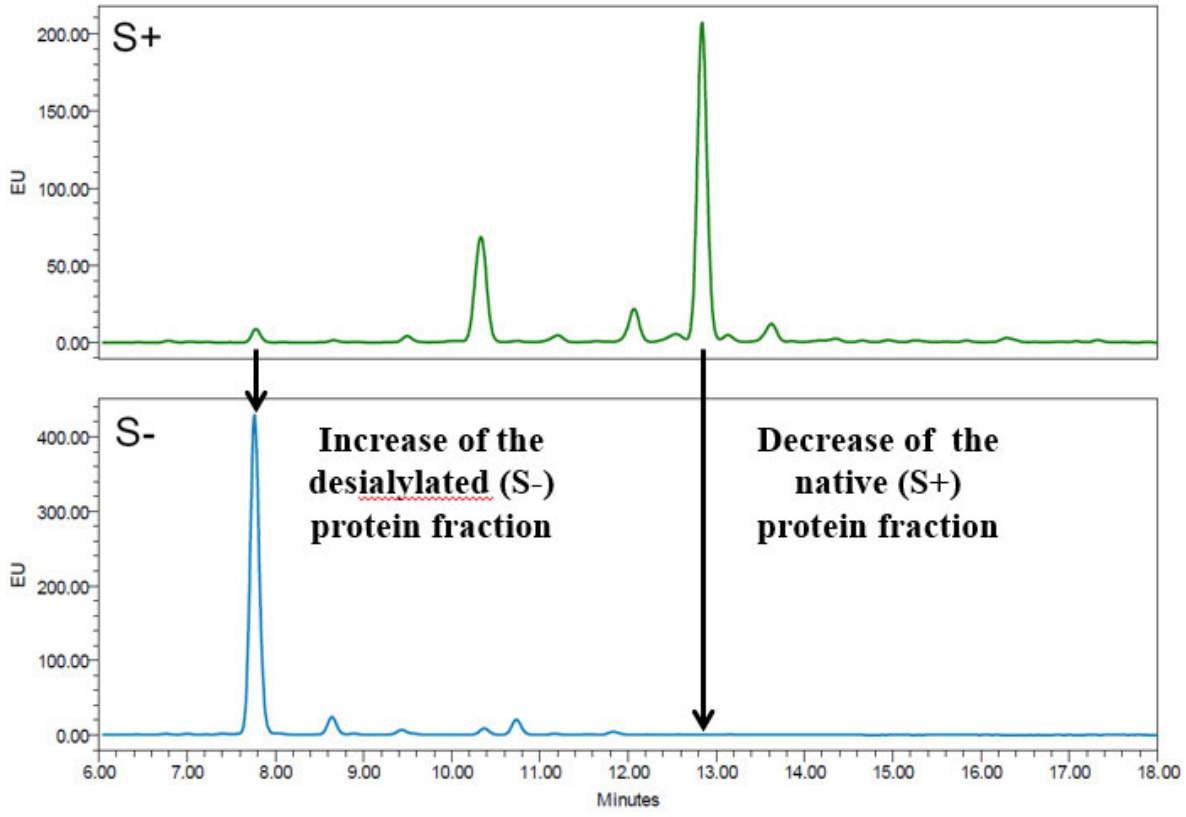

Figure 2.1 Typical ultra-performance liquid chromatography of fluorescently labelled and purified N-glycans released from native (S+) and desialylated (S-) human apo-transferrin.

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