An ultrafast, batch-to-batch comparison of monoclonal antibody glycosylation

Application benefits

- High-throughput screening method for unambiguous identification of glycoforms
- 20 times reduction in separation time compared to standard profiling methods
- Excellent batch-to-batch reproducibility for method development of novel mAbs and biosimilar products

Goal

To develop a high-throughput screening method using HILIC UHPLC separation of 2AA-labelled glycans in a model mAb as a proof of concept for a more general approach to mAb glycoprofiling capable of identifying differences in high abundance glycoforms. The separation must be rapid but requires sufficient resolution to allow batch-to-batch differences in the glycan profile to be identified and subsequently characterized by high-resolution, accurate-mass mass spectrometry.

Introduction

One of the fastest growing fields in the pharmaceutical industry is the market of therapeutic glycoproteins (glycosylated proteins), which are produced by living cell systems. These include monoclonal antibodies (mAbs) and other...
recombinant protein products (e.g., fusion proteins, growth factors, cytokines, therapeutic enzymes, and hormones), which are approved or under development as therapeutics. Glycosylation is a critical quality attribute (CQA) for development and manufacturing of therapeutic mAbs in the biopharmaceutical industry and, therefore, needs to be assessed to ensure desired product quality, safety, and efficacy. Different glycosylation variants have been shown to affect stability, pharmacokinetics, serum half-life, immunogenicity, and effector functions.

Glycoforms on biopharmaceutical glycoproteins are affected by the culture conditions as well as the cell type by which they are produced. Biopharmaceutical glycosylation monitoring for correct structure during production and for quality control is required by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Current glycoanalytical methods are laborious and time-consuming; therefore, more rapid and high-throughput (HTP) methods are required. HTP techniques for in-depth monitoring of glycoform distributions must be integral components for the implementation of quality by design (QbD) approaches. To meet this need, novel approaches toward HTP monitoring are required.

Rituximab (MabThera®) is a genetically engineered chimeric mouse/human monoclonal antibody representing a glycosylated immunoglobulin with human IgG1 constant regions and murine light-chain and heavy-chain variable region sequences. The antibody is produced by mammalian (Chinese hamster ovary) cell suspension culture and purified by affinity chromatography and ion exchange, including specific viral inactivation and removal procedures.

This application note presents a proof of concept for an ultrafast N-glycan analysis approach to glycoprofiling of the main glycoforms of mAbs by HILIC chromatography and the Vanquish Horizon UHPLC system with fluorescence detection. Time for analysis is reduced considerably in comparison to the standard method applied for full detailed characterization (the standard methods can take up to 55 minutes). The analysis is completed in 2.5 minutes and can be applied for the comparison of mAbs glycosylation expressed under various cell culture conditions, as well as for the evaluation of antibody culture clones and various production batches.

**Experimental**

**Chemicals and reagents**
- Deionized (DI) water, 18.2 MΩ•cm resistivity
- Fisher Scientific™ Acetonitrile, HPLC grade (P/N 10407440)
- Fisher Scientific™ Optima™ Formic acid, LC-MS (P/N 10596814)
- Fisher Scientific Ammonium hydroxide (P/N 10508610)
- Fisher Scientific Ammonium bicarbonate (P/N 10207183)
- Thermo Scientific™ Virtuoso™ Vial kit (P/N 60180-VT402)
- PNGase F (purchased from a reputable supplier)
- Ultra 0.5 mL centrifugal filters MWCO 10 kDa (purchased from a reputable supplier)
- Fisher Scientific Sodium cyanoborohydride (P/N 10082110)
- Fisher Scientific Glacial acetic acid (P/N A/0360/PB17)
- Fisher Scientific Dimethylsulfoxide (DMSO) (P/N 10213810)
- Anthranilic acid (2-AA) (purchased from a reputable supplier)
- Fisher Scientific Tris(hydroxymethyl)methylamine hydrochloride (P/N 10060390)
- Fisher Scientific Urea (P/N 10132740)

**Equipment**
- Thermo Scientific™ UltiMate™ 3000 RS system, including pump:
  - LPG-3400RS Rapid Separation Quaternary Pump (P/N 5040.0036)
  - WPS-3000TRS Rapid Separation Thermostatted Well Plate Autosampler (P/N 5840.0020)
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
  - FLD-3400RS Rapid Separation Fluorescence Detector with Dual-PMT (P/N 5078.0025)
  - SR-3000 Solvent Rack (P/N 5035.9200)
  - 2 µL Micro Flow Cell (P/N 6078.4330)
Thermo Scientific™ Vanquish™ Horizon UHPLC system, including:

- System Base Vanquish Horizon (P/N VH-S01-A)
- Binary Pump H (P/N VH-P10-A),
- Column Compartment H (P/N VH-C10-A),
- Split Sampler HT (P/N VH-A10-A) with 25 µL (V=50 µL) Sample Loop
- Fluorescence Detector F (P/N VF-D50-A)

Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer

Thermo Scientific™ Accucore™ 150-Amide-HILIC 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)
Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)
Thermo Scientific™ SpeedVac™ Concentrator (P/N SPD121p)

Buffers preparation

- Ammonium formate (50 mM, pH 4.4): 1.8 g of formic acid was dissolved in 1 L of DI water. pH was adjusted to pH 4.4 with ammonium hydroxide solution.
- 8 M urea in 0.1 M Tris-HCl, pH 8.5 (UA buffer): 1.57 g of Tris-HCl was dissolved in 100 mL DI water and pH adjusted to 8.5 with 1 M NaOH. 24 g of urea was dissolved in 50 mL 0.1 M Tris-HCl, pH 8.5.
- Ammonium bicarbonate (50 mM, pH 7.8): 0.39 g of ammonium bicarbonate was dissolved in 100 mL of DI water.

N-glycan release, labelling, and clean up

1. 100 µg of protein was denatured using 8 M urea in 0.1 M tris buffer pH 8.0 (UA solution) and subsequently reduced and alkylated using 10 mM DTT and 50 mM IAA prepared in UA solution, respectively.
2. Following buffer exchange into 50 mM ammonium bicarbonate using 10 kDa MWCO filters, N-glycan release was performed by incubation of the reduced and alkylated sample with 1000 units of PNGase F overnight at 37 ºC.
3. Released glycans were collected from the deglycosylated proteins by centrifugation through 10 kDa MWCO filters and subsequently reduced to dryness via vacuum centrifugation.
4. Glycans were converted to reducing aldoses by reconstitution in 50 µL of 1% formic acid and reduced to dryness.
5. Dried glycans were derivatized with 5 µL 2-aminobenzoic acid (2-AA) via reductive amination with sodium cyanoborohydride in 30% (v/v) acetic acid in DMSO at 60 ºC for 5 hours.

4. Excess labelling reagent was removed by HILIC purification using an UltiMate 3000 RS system. Samples were loaded in 80% acetonitrile / 20% 50 mM ammonium formate pH 4.4 (v/v) onto an Accucore 150-Amide-HILIC 2.1 × 50 mm column at 0.5 mL/min for 2.5 minutes. Labelled glycans were eluted in 20% aqueous acetonitrile for 2.5 minutes, monitored by fluorescence detection, λex/em = 350/425 nm, and evaporated to dryness.

UHPLC-fluorescence N-glycan profiling

Labelled N-glycans were separated by hydrophilic interaction UHPLC-FLD on a Vanquish Horizon UHPLC. 2AA-labelled glycans were separated using a linear gradient. Glycans were injected in 5 µL 80% v/v acetonitrile and stored at 10 ºC prior to injection.

Separation conditions

Column: Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 50 mm (P/N 16726-052130)
Mobile phase A: Ammonium formate 50 mM, pH 4.5
Mobile phase B: Acetonitrile
Flow rate: 2.2 mL/min 395 backpressure at starting conditions
Column temperature: 60 ºC
Injection volume: 5 µL

Table 1. Mobile phase gradient for UHPLC-fluorescence N-glycan profiling.

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<th>%A</th>
<th>%B</th>
<th>Flow (mL/min)</th>
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N-glycan analysis by LC-MS

Glycan samples were injected into a Q Exactive Plus MS equipped with a HESI ion source. Samples were diluted in 75% acetonitrile prior to analysis.
Separation conditions

Column: Accucore 150-Amide-HILIC 2.6 µm, 2.1 x 150 mm (P/N 16726-152130)
Mobile phase A: Ammonium formate 50 mM, pH 4.5
Mobile phase B: Acetonitrile
Flow rate: 0.4 mL/min
Column temperature: 40 °C
Injection volume: 11 µL

Table 2. Mobile phase gradient.

<table>
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<th>Flow (mL/min)</th>
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<td>0.4</td>
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MS conditions

Ionization: HESI Negative Ion
Scan range: 500 to 2000 m/z
Source temperature: 300 °C
Sheath gas flow: 20 Arb
Auxiliary gas flow: 10 Arb
Spray voltage: 3.8 kV
Capillary temperature: 320 °C

Data processing and software

Chromatographic software: Thermo Scientific™ Chromeleon™
MS data acquisition: Thermo Scientific™ Xcalibur™ software 2.2 SP1.48

Results and discussion

A commercial chimeric IgG1 mAb was analyzed on an Accucore 150-Amide-HILIC HPLC column (2.1 x 50 mm) within 2.5 min, offering an ultrafast method for glycoprofiling of the main glycoforms. Figure 1 shows the separation of the eight most abundant 2AA-labelled N-glycans, which include bi-antennary structures with variable degrees of core fucosylation and galactosylation and a high mannose structure. The structural assignment for each peak was confirmed by accurate MS data.

Retention time precision is essential in labelled glycan analysis for peak identification. Precision of retention time was determined for seven consecutive injections of rituximab 2-AA N-glycans. Figure 2 shows excellent retention time precision for the eight dominant glycan structures.

Figure 1. Chromatographic separation of commercial chimeric IgG1 mAb (rituximab) 2AA-labelled N-glycans on an Accucore 150-Amide-HILIC column (2.1 x 50 mm, 2.6 µm).
The developed method was applied for batch-to-batch comparison of rituximab glycosylation. One of the batches corresponded to the drug product perfusion solution in water for injection (10 mg/mL) opened in-house. The other two batches were the surplus products obtained at the hospital after patients’ treatment, which were donated to the research institute for research purposes. Figure 3 shows glycan profile comparison of the three batches. Chromatographic profiles were normalized to the main signal (peak 4). Differences are noticeable in the relative abundance for peaks 6, 7, and 8 for both surplus batches compared to the freshly opened drug product.
Conclusions

• A fully integrated workflow for glycan profiling was successfully demonstrated using the combination of the Vanquish Horizon UHPLC system equipped with fluorescence detection and the Accucore 150-Amide-HILIC column to provide rapid resolution of N-glycoforms from biotherapeutics for ultrafast glycan profiling.

• This process was effectively applied to the rapid separation of labeled glycans for batch-to-batch glycoprofiling comparison of a model mAb, offering a potential approach to process development of novel mAbs and biosimilar products.

• Orthogonal structural confirmation of glycans using the Q Exactive Plus MS allowed rapid and unambiguous profiling.

References