Monosaccharide profiling by intact mass analysis - method qualification of a new approach for the characterization of highly glycosylated biopharmaceuticals

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Introduction

The development and production of recombinant biosimilars is a challenging field due to their structural complexity. When implementing a manufacturing process for biosimilars, differences in the protein structure or post-translational modifications can occur. Therefore a characterization program is required following the biosimilar guidelines.

Of great interest are protein glycoconjugates which can have a great influence on efficacy and safety of the biopharmaceutical. Glycosylations are usually analyzed after isolation from the protein by HPAEC-PAD or HILIC-FLD. Alternatively, LC-ESI-MS analysis of the intact protein can be employed to yield the glycosylation pattern or monosaccharide profile. Here, we analyzed Ustekinumab, Erythropoietin, and Enanercept with an UHPLC-QTOF mass spectrometer. The method was qualified by comparison to data achieved by HILIC-FLD and HPAEC-PAD.

Overview

- Qualification of monosaccharide profiling by intact mass analysis with ESI-MS for Ustekinumab, Erythropoietin, and Enanercept
- Comparison to standard methods for glycosylation analysis (HPAEC-PAD and HILIC-FLD)
- Monosaccharide profiling by ESI-MS is highly reproducible, time efficient, and easy to perform

Methods

All analyses were performed in six replicates for each sample.

Monosaccharides by HPAEC-PAD

The samples were rebuffered by ultratritration, hydrolyzed with TFA, dried and reconstituted in pure water.

The separation of monosaccharides was performed with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS 3000 system (Dionex) with an isocratic elution. Separation column Dionex CarbPAC™ PR-20 (3 x 150 mm) and guard column Dionex AmexTrap (3 x 150 mm) were used.

The calibration (standard and peak assignment by retention time) was performed using a defined monosaccharide mixture containing N-acetylglucosamine, glucosamine, galactose, glucose, and mannose with 2.5-400 pmol per injection for each monosaccharide.

Sialic Acids by HPAEC-PAD

The samples were rebuffered by ultratritration, hydrolyzed with hydrochloric acid, dried and reconstituted in pure water.

The separation of sialic acids was obtained with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS 3000 system (Dionex) with a linear gradient with increasing concentration of sodium carbonate. Separator column Dionex CarbPAC™ PR-20 (3 x 150 mm) and guard column Dionex AmexTrap (3 x 30 mm) were used.

The calibration (standard and peak assignment by retention time) was performed using a defined sialic acid mixture (NeuAc and Neu5Ac) in the concentration range from 5-500 pmol per injection.

N-Glycans by HILIC-FLD

The samples were rebuffered by ultratritration, N-glycans were released from the protein enzymatically by PNGase F at enzyme specific conditions. The N-glycan pool was desalted and separated from the protein by rotating glycans on an SPE spin-column. The glycans were labeled using 2-Aminobenzamide (ZAB) for subsequent fluorescence detection.

The separation of N-glycans was performed with hydrophobic interaction chromatography (HILIC) with ESI detection (FLD, Excitation 330 nm and Emission 422 nm) on a Waters UPLC H-Class instrument with a linear gradient. Separator column ACQUITY UPLC BEH Glycan (2.1 x 150 mm) was used.

The peak assignment was performed by retention time using a defined N-glycan mixture (ZAB tagged N-Glycan Library, Prolyn).

Intact mass analysis by ESI-MS

Erythropoietin samples were precipitated with acetone. Ustekinumab samples were reduced with dithiothreitol at 60°C in the presence of 1 M urea. Enanercept samples were enzymatically deglycosylated with PNGase F at enzyme specific conditions and concentretively reduced with dithiothreitol at 60°C in the presence of 1 M urea.

The intact mass was analyzed by LC-ESI-MS using the nanoACQUITY UPLC® system (Waters) coupled to a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Xevo TQ-S, Waters). The separation of the peptides was performed with reversed phase (RP) chromatography. Separator column ACQUITY UPLC BEH C18 (150 μm i.d. x 100 mm, 1.7 μm particle size, 30 A pore size, Waters). MS acquisition was performed in positive ion mode with external mass calibration.

The quantitative level of each identified glycan structure/composition was assigned by comparing the MS signal intensity of the corresponding mass peak. The monosaccharide composition was calculated based on the composition of the corresponding glycan structure.

- Protegen Protein Services (PPS) has established a qualified workflow to measure the monosaccharide composition of Ustekinumab, Erythropoietin, and Enanercept through intact mass analysis by ESI-MS.
- Once set up, monosaccharide profiling by intact mass analysis is more time efficient and less complicated, making it the perfect method for glycosylation analysis of a large number of samples (e.g. clone selection).
- ESI-MS shows a high reproducibility for the determination of the monosaccharide composition of highly glycosylated biopharmaceuticals. This is crucial for comparability studies, where even small differences can be detected by a low number of replicate measurements.

Figure 1: HILIC-FLD chromatogram overlay of the six replicate measurements of Ustekinumab showing the N-glycan distribution.

Figure 2: Deconvoluted mass spectrum of Ustekinumab under reducing conditions with annotated glycosylated isoforms (a). The six replicate measurement of Ustekinumab show a high reproducibility (b).

Figure 3: N-glycan (a) and monosaccharide distribution (b) for Ustekinumab determined with HILIC-FLD and ESI-MS showing the deviation of the six replicate measurements is indicated by error bars.

Figure 4: Overlay of the six HPAEC-PAD runs for determination of the monosaccharide composition of Erythropoietin (a). The stoic acid composition was determined in a second experiment, the respective six HPAEC-PAD runs are shown in (b).

Figure 5: Deconvoluted mass spectrum of Erythropoietin (a). The annotated glycosylated isoforms are exemplary, overall 20 isoforms were used for the calculation of the monosaccharide distribution. The six replicate measurement of Erythropoietin show a high reproducibility (b).

Figure 6: Monosaccharide distribution for Erythropoietin determined with HPAEC-PAD and ESI-MS. The respective standard deviation of the six replicate measurements is indicated by error bars.

Figure 7: Overlay of the six HPAEC-PAD runs for determination of the monosaccharide composition of Enanercept (a). The stoic acid composition was determined in a second experiment, the respective six HPAEC-PAD runs are shown in (b).

Figure 8: Deconvoluted mass spectrum of Enanercept, N-deglycosylated and under reducing conditions (a). The annotated O-glycosylated isoforms are exemplary, overall 12 isoforms were used for the calculation of the monosaccharide distribution of the O-glycans. The six replicate measurement of Enanercept show a high reproducibility (b).

Figure 9: Monosaccharide distribution for Enanercept determined with HPAEC-PAD and ESI-MS. For ESI-MS it was only possible to determine the monosaccharide composition of all O-glycans. The monosaccharide distribution of N-glycans were estimated with a N-glycan distribution taken from literature4. The respective standard deviation of the six replicate measurements is indicated by error bars.

HILIC-FLD and ESI-MS analysis show a similar distribution of N-glycans for Ustekinumab. The largest deviation was determined for GlcNAc (36.9% with HILIC-FLD vs. 34.9% with ESI-MS).

Regarding the reproducibility of the N-glycan distribution, the determination of the N-glycans by ESI-MS shows a higher average CV than HILIC-FLD (5.3% vs. 1.8%).

The reproducibility of the determination of the monosaccharide composition shows a similar picture (average CV for ESI-MS 1.0% vs. 0.2% for HILIC-FLD).

The largest deviation was determined for Fucose (4.4% with HILIC-PAD vs. 3.9% with ESI-MS).

Regarding the reproducibility of the determination of the monosaccharide composition, ESI-MS shows a lower average CV than HILIC-PAD (0.2% vs. 1.2%).


Conclusion

- Qualification of monosaccharide profiling by intact mass analysis - method qualification of a new approach for the characterization of highly glycosylated biopharmaceuticals.