ThP 486

A Comprehensive N-Glycan Profiling Analysis of Bevacizumab Biosimilar by UHPLC with Fluorescence Detection and Q-TOF Mass Spectrometry

Yonghai Lu [1], Jie Xing [1], Zhaoqi Zhan [1]

[1] Application Development & Support Centre (ADSC), Shimadzu (Asia Pacific), Singapore

1. Overview

- N-glycans were released from bevacizumab biosimilar by PNFase F, labeled with 2-aminobenzamide (2-AB), and measured by Nexera Bio UHPLC coupled with fluorescence detector and Q-TOF mass spectrometer.
- Nine 2-AB labeled N-glycans, including Man3, G0F-2GN, G0-GN, G0F-GN, G0, Man5, G0F, G1Fa, and G1Fb, were characterized and quantified.
- G0F was found to be the most abundant N-glycan that makes up 87.23% of the total N-glycans from bevacizumab biosimilar.
- The analytical system was validated for stability and repeatability (RSD < 2%).

2. Introduction

Gobal biopharmaceutical market is entering a new era of biosimilars - generic copies of commercialized monoclonal antibodies (mAbs), with the aim of providing less-expensive medication options. Thus far, more than 50 biosimilar products have been approved by USFDA and EMA. Despite this, biosimilar industry faces some significant hurdles. One of the major challenges is to produce biosimilars with the same/closest N-glycosylations as the reference mAb, as they play a crucial role in stability, bioactivity and immunogenicity of the product. That's why an appropriate characterization of biosimilar product is essential. In this work, we established a robust, sensitive and reproducible analytical system on the basis of a Nexera Bio UHPLC coupled with Fluorescence detection and Q-TOF Mass Spectrometry for N-glycan profiling analysis of a bevacizumab biosimilar sample.

3. Methods

Protein Solubilization: 1 mg/mL of bevacizumab biosimilar solution was prepared in Tris buffer. A 100 µL aliquot was loaded into a 10 kDa molecular weight cut-off (MWCO) to remove salts from the sample buffer. The recovered sample (~20 µL) was diluted to 100 µL with 25 mM ammonium bicarbonate solution.

Reduction and Alkylation: 2 µL dithiothreitol (DTT, 1M) solution was added to reduce disulfide bonds. The sample was incubated at room temperature for 60 min. Then, 4 µL iodoacetamide (IAA, 1M) solution was added for alkylation, and incubated in the dark for 60 min at room temperature.

Deglycosylation: 2 µL PNFase F (1000U) was added to release N-glycans from bevacizumab biosimilar, and incubated at 37 ºC overnight.

Extraction of N-glycans: N-glycans were extracted using LudgerClean™ EB10 cartridge by eluting with 4 × 200 µL of 50% acetonitrile with 0.1% trifluoroacetic acid. For details see the LudgerClean™ EB10 cleanup protocol [1]. The obtained sample was dried down by a centrifugal evaporator and reconstituted in 50 µL of acetonitrile.

2-AB Labeling : 10 µL 2-AB/acetic acid/ DMSO/ sodium cyanoborohydrate mixture with defined composition was used for labeling [2].

Purification of 2-AB Labeled N-glycans: LudgerClean™ S cartridge was applied to remove the excess labeling reagent. For details see the LudgerClean™ S cleanup protocol [3]. The obtained sample was dried down by a freeze dryer and reconstituted in 50 µL of 50% acetonitrile for LC/Fluorescence/MS analysis (**Table 1**).

4. Results

4.1 UHPLC/RF injection-to-injection reproducibility

The main purpose of UHPLC/RF analysis is to relatively quantify N-glycans. Injection-to-injection variability of UHPLC/RF system was evaluated as shown in **Figure 1**.

Table 1. LC/Fluorescence/MS conditions

LC conditions		
LC system	:	Shimadzu Nexera Bio UHPLC
Column	:	HALO®Glycan, 2.7 µm, 150 × 2.1 mm
Column temperature	:	60 ºC
Flow rate	:	0.4 mL/min
Mobile phase A	:	50 mM ammonium formate
Mobile phase B	:	Acetonitrile
Gradient program	:	0 min, 78% B, 50min, 55% B, 51 min, 20% B, 56 min, 20% B, 57 min, 78% B.
Injection volume	:	5 µL
Fluorescence conditions		
Fluorescence detector	:	Shimadzu RF-20A
Excitation	:	330 mm
Emission	:	420 mm
MS conditions		
MS system	:	Shimadzu LCMS-9030 (QTOF)
Interface	:	Heated ESI (+)
Interface voltage	:	4 kV
Interface temperature	:	300 ºC
Nebulizing gas	:	N2, 3 L/min
Heating gas flow	:	Zero air, 10L/min
DL temperature	:	250 ºC
Drying gas flow	:	N2, 10 L/min
Heat block temperature	:	400 ºC
MS mode	:	MS scan
Mass range	:	500 - 2500 m/z
MS mode	:	MS/MS scan
Collision Energies	:	50 ± 17 V
Mass range	:	100 - 2500 m/z

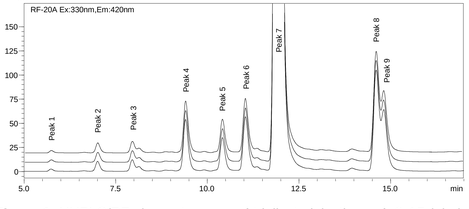

Figure 1. UHPLC/RF chromatograms of triplicate injections of 2-AB labeled N-glycans released from the same bevacizumab biosimilar product. It shows perfect alignment of chromatograms.
* Variations in peak area and retention time of three injections of the sample were <2% RSD for all peaks.

4.2 Characterization of N-glycans using LCMS-9030

In total, we characterized nine 2-AB labeled N-glycans from bevacizumab biosimilar, including Man3, G0F-2GN, G0-GN, G0F-GN, G0, Man5, G0F, G1Fa, and G1Fb (**Figure 2**). Proposed structures for the 2-AB labeled N-glycans are shown in **Figure 3**. **Table 2** shows accurate mass data of LCMS-9030. MS/MS spectra of N-glycans are shown in **Figure 4**. Accurate mass combined with MS/MS patterns provide high confidence in identification of N-glycans

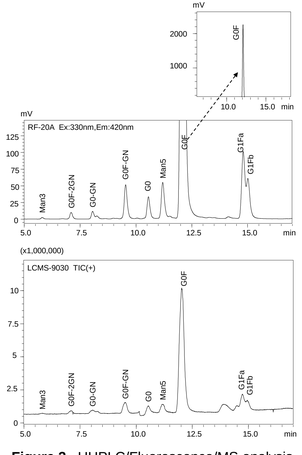

Figure 2. UHPLC/Fluorescence/MS analysis of 2-AB labeled N-glycans.
Top: fluorescence; bottom MS chromatogram.

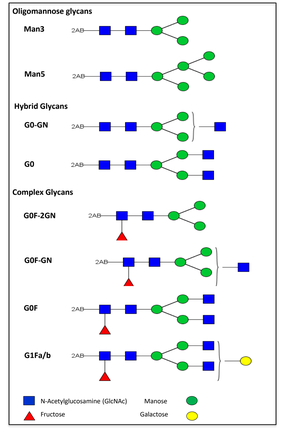

Figure 3. Proposed structures for 2-AB labeled N-glycans. GN = GlcNAc

4.3 Relative quantitation of N-glycans

Figure 5 shows the relative abundance of N-glycans of bevacizumab biosimilar. G0F was found to be the highest abundant N-glycan that made up 87.23% of the total N-glycans from bevacizumab biosimilar, and Man3 was the lowest abundant N-glycan that was only accounted for 0.7% of total N-glycans.

Table 2 Mass accuracy of LCMS-9030

2-AB N-glycans	Accurate mass	Exact mass	Mass error (ppm)
Man3	1031.4033	1031.4038	-0.48
G0F-2GN	1177.4636	1177.4617	1.61
G0-GN	1234.4830	1234.4832	-0.16
G0F-GN	1380.5404	1380.5411	-0.51
G0	1437.5638	1437.5625	0.90
Man5	1355.5083	1355.5095	-0.89
G0F	1583.6195	1583.6205	-0.63
G1Fa	1745.6724	1745.6733	-0.52
G1Fb	1745.6724	1745.6733	-0.52

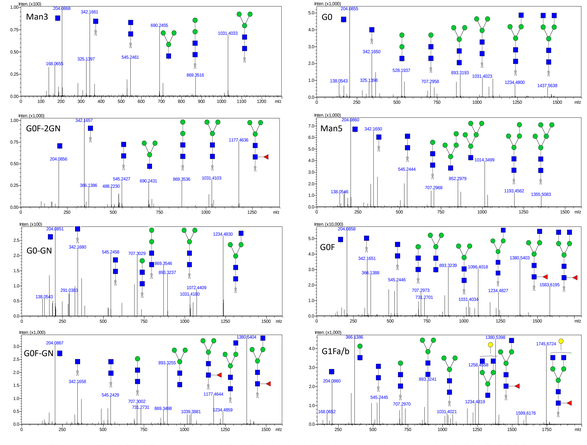

Figure 4. MS/MS spectra of 2-AB labeled N-glycans obtained by LCMS-9030.

5. Conclusion

In this work, we have demonstrated that the system comprising of Nexera Bio UHPLC coupled with RF-20A fluorescence detector and Q-TOF mass spectrometer is robust and reliable for N-glycan profiling and quantitation of bevacizumab biosimilar products, which enables confident and rapid identification of N-glycan compositions with an average mass error of < 1ppm. The tests for stability and repeatability of this analytical system are also satisfactory (RSD < 2%). This LC/MS Q-TOF system may become a tool of choice for mAb characterization.

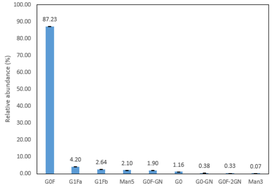

Figure 5. Relative contents of N-glycans in bevacizumab biosimilar.

References

1. https://www.ludger.com/docs/products/lc/eb/ludger-lc-eb10-ax-guide.pdf
2. Keser T, Pavić T, Lauc G, Gornik O. Comparison of 2-Aminobenzamide, Procainamide and RapiFluor-MS as Derivatizing Agents for High-Throughput HILIC-UPLC-FLR-MS N-glycan Analysis. *Front Chem* 2018 6:324. .
3. https://www.ludger.com/docs/products/lc/s/ludger-lc-s-ax-guide.pdf