2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography

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Introduction

mAbs represent a class of highly advanced, but expensive, pharmaceutical products. These circumstances led to the development of less costly biosimilars of innovator biopharmaceuticals[1]. Comparability studies focusing on quality attributes are of utmost importance to guarantee product equivalence. One important quality attribute is the charge variant profile, which can be determined using ion exchange chromatography (IEX). To qualify the charge variant pattern of the mAbs, further analysis is necessary, for example, by identification with mass spectrometry (MS). However, identification using online MS analysis after IEX is not a straightforward procedure, mostly due to the incompatibility of the most common ion exchange buffers with the electrospray ionization process.

The Agilent 1290 Infinity II 2D-LC solution enables automated desalting, denaturation and, if required, additional separation by the addition of reversed-phase chromatography (RP) in the second dimension. Figure 1 shows the plumbing diagram of the 2-position/4-port-duo valve with two 6-position/14-port valves with 12 preinstalled 40 µL loops.

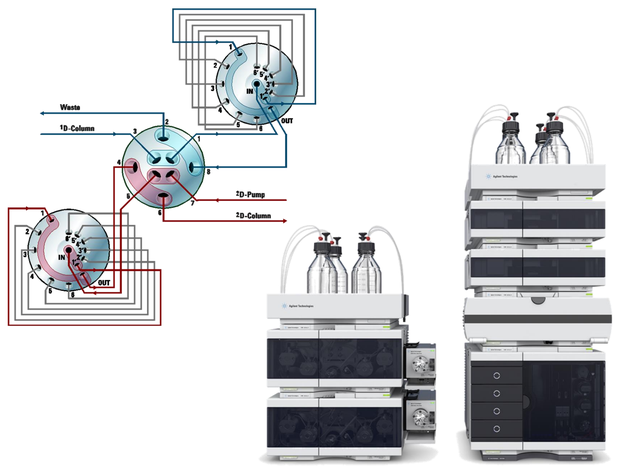

Figure 1 – Plumbing diagram of the 2-position/4-port-duo valve with two 6-position/14-port valves with 12 preinstalled 40 µL loops together with the 1290 Infinity II 2D-LC solution

Here, we demonstrate charge variant analysis of rituximab innovator and biosimilar using multiple heart-cutting 2D-LC analysis with MS detection.

Experimental

Systems

The Agilent 1290 II 2D-LC solution was comprised of following modules:

- Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A)
- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler
- 2x Agilent 1290 Infinity II Multicolumn Thermostats (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port duo-valve (2D-LC) valve head (G4236A)
- 2x Agilent 1290 Infinity Valve Drives (G1170A) with 2x multiple heart-cutting valves (G4242-64000), equipped with 40-µL loops
- 2x Agilent 1290 Infinity II Diode Array Detectors (G7117B) with 10-mm Max-Light cartridge cells (G4212-60008)

Agilent 6530 Accurate Mass QTOF LC/MS system

Samples

The mAbs samples used were rituximab innovator as well as rituximab biosimilar. Both samples were purchased in local pharmacies in India.

Columns

First Dimension: Agilent Bio mAb, non-porous, 2.1 x 250 mm, 5 µm, PEEK (p/n 5190-2411)
Second Dimension: AdvanceBio RP-mAb C4, 2.1 x 75 mm, 3.5 µm (p/n 797775-904)

Analytical conditions – Table 1, 2 & 3

Injection Volume: 6 µl
Thermostat Multisampler: 10 °C

Table 1 - Chromatographic conditions 1D

Solvents	A: Water, B: 1700 mM NaCl C: 30 mM NaH2PO4, D: 60 mM Na2HPO4
Flow rate	0.25 mL/min
Gradient (quaternary salt gradient, calculated with **BufferAdvisor**) 1D stop time	0 minutes - 7.59 % A 0.0 % B 84.82 % C 7.59 % D 30 minutes – 1.31 % A 11.8 % B 73.85 % C 13.08 % D 35 minutes – 1.31 % A 11.8 % B 73.85 % C 13.08 % D 75 minutes
Temperature MCT	22°C
Wavelength DAD	280 nm/ 4 nm, Ref.: 360 nm/ 100 nm
Peak width	>0.025 min (0.5 s response time) (10 Hz)

Table 2 - Chromatographic conditions 2D Multiple Heart-cutting

Solvents	A: Water + 5 % FA, B: Acetonitrile with 5 % FA
Flow rate	1 mL/min
Gradient	0.0 minutes: 10 %B 2.5 minutes: 60 %B 2.75 minutes: 90 %B
2D parameter mode 2D gradient stop time 2D cycle time	Heart-cutting 3.0 minutes 4.5 minutes
Temperature MCT	70°C
Wavelength DAD	280 nm/ 4 nm, Ref.: 360 nm/ 100 nm
Peak width	Peak width 2D: > 0.0063 minutes (0.13 seconds response time) (40 Hz)

Table 3 – MS Parameter

Gas temp	300 °C
Sheath gas temp	400 °C
Gas flow	13 l/min
Sheath gas flow	12 l/min
Nebulizer	45 psi
Vcap	5000 V
Nozzle	2000 V
Fragmentor	170
Skimmer	65 V
Oct 1 RF Vpp	750
Mode	MS

Results and Discussion

Rituximab biosimilar and innovator charge variants were analyzed with weak cation exchange chromatography (WCX) using a flat salt gradient. Only one main peak was visible for the innovator, while three prominent peaks were found for the biosimilar (Figure 2). Due to the 1D peak pattern, selected peak areas were transferred to the RP column in the second dimension using multiple heart-cutting 2D-LC. To qualify the identity of the three biosimilar peaks in comparison to the single main peak of the innovator, further MS analysis was necessary.

For this application, the second dimension is not primarily used for a further orthogonal separation dimension, but is rather regarded as a desalting step for intact mAbs to remove buffer residues from the first dimension using RP chromatography.

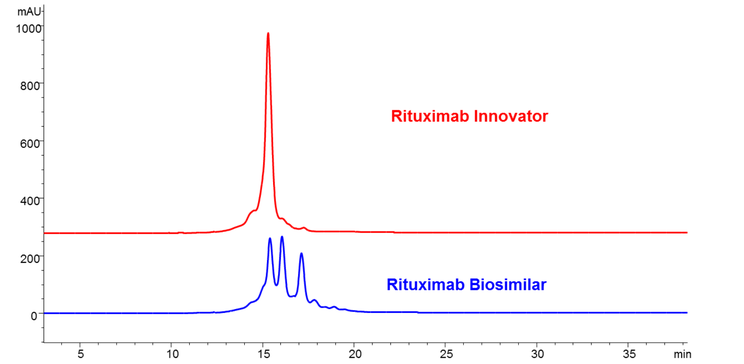

Figure 2 – Charge variant analysis of rituximab innovator (red) and biosimilar (blue)

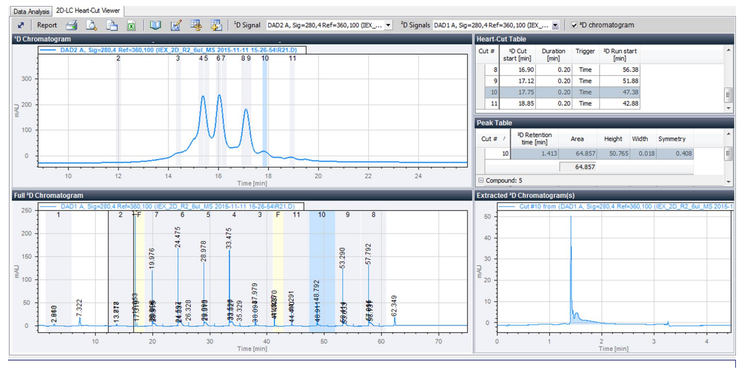

Figure 3 - Results of the multiple heart-cutting 2D-LC analysis presented in the 2D-LC viewer.

The transfer of the mAb into a denaturing RP system (acidified water and acetonitrile) enables straightforward intact protein MS analysis.

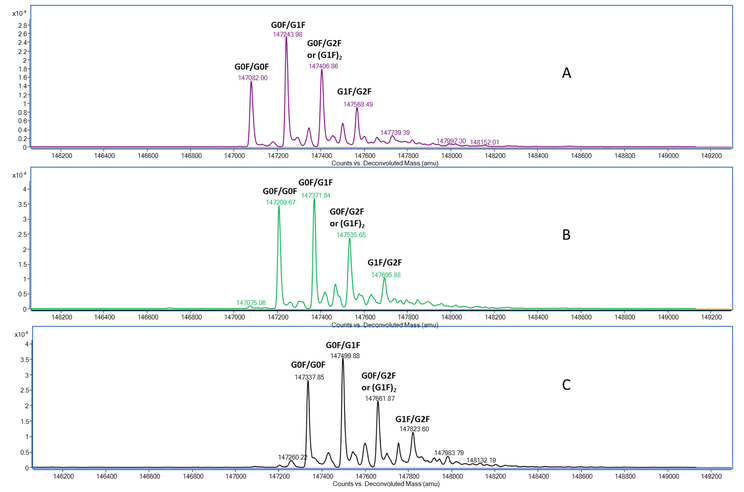

Figure 4 –Deconvoluted spectra of main peak 1 (A), 2 (B), and 3 (C) of the rituximab biosimilar.

With Q-TOF MS analysis, the mass of the first main peak of the biosimilar for G0F/G0F was found to be identical to G0F/G0F of the innovator. The G0F/G0F glycoforms of the second and third charge variants were shifted by about 128 Da (Figure 4), representing C-terminal lysine variants. To prevent buffer residues from entering and contaminating the MS source, an MS diverter valve time table was set up. Using the MS time table for switching between waste and the MS source is highly recommended when working with MS incompatible buffers.

Conclusions

To enable direct qualification of the charge pattern using online MS detection, a two-dimensional workflow was developed with RP in the second dimension. The addition of an RP second dimension enabled not only desalting (highly important after the phosphate/NaCl buffer system), but denaturation of the sample to provide straight forward MS analysis.

References:

1. *Hongwei, X. Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies, MAbs, 2010, 2(4), 379–394.*