Sample to Result Workflow for the Investigation of Biosimilars vs. Innovator Cetuximab by Charge Variant Analysis using Microchip CE-MS

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Introduction

Biosimilar drug development relies on a variety of analytical methods, mass spectrometry (MS) perhaps foremost among them, for characterization and comparability studies. Charge Variant Analysis (CVA) is key to biosimilarity assessment by providing information on the Critical Quality Attributes (CQAs) that can be measured and is frequently performed by a separation technique coupled to intact mass analysis. Capillary Zone Electrophoresis (CZE) in native conditions offers robust and efficient separation of charge variants with minimum method development. In this study, of a therapeutic protein with complex glycosylation, the combination of charge-based separation, mass detection, and assignment of variants by software can all be displayed on a dashboard that can be updated as more analyses and batches are made. Heterogeneity by sample for a given method can then be compared. Here we show the use of CZE, high resolution mass spectrometry (HRMS), and advanced charge deconvolution and mass matching software to compare Cetuximab (the first FDA-approved mAb with Fab glycosylation) with two biosimilars.

Methods: Experimental

Samples: The Cetuximab innovator and biosimilar #1 were obtained from LGM Pharma. Biosimilar #2 was obtained from Absolute Antibody.

Instrumentation: The Cetuximab innovator and biosimilar #1 were obtained from LGM Pharma. Biosimilar #2 was obtained from Absolute Antibody.

CZE Inlet PATsmart™ ZipChip® (Repligen)
Settings: ZipChip protocol: Intact Charge Variant Analysis
Field Strength: 500 V/cm
Injection volume: 1 nL
Pressure Assist Start Time: 0.5 min
Analysis time: 20 min
Consumables: Native Antibodies Kit* (Repligen)
The background electrolyte (BGE) was modified with 4% DMSO. "High Resolution Native" (HRN) chip (Repligen).

Mass Spectrometer: Orbitrap Exploris™ 240 MS with BioPharma Option (Thermo Fisher Scientific).
Settings: Scan Range (*m/z*): 2,500 8,000
Resolution setting: 30,000 at *m/z* 200
Sheath gas: 2
In source CID (V): 125
Normalized AGC Target (%): 300
RF Lens (%): 60
Microscans: 5

Charge Deconvolution Algorithm: Charge deconvolution mathematically inverts the physical process that ionizes molecules and produces an *m/z* spectrum Most algorithms (ReSpect™ UniDec) take a "forward" approach to the problem, iteratively computing masses and charge distributions that together give an *m/z* spectrum close to the observed *m/z* spectrum Protein Metrics software takes a "backward" and "parsimonious" approach (Bern et al., 2018), assigning charges to the *m/z* spectrum to produce the *m* spectrum.

Software: Intact workflow in Protein Metrics Byos®
Settings: Mass range: 143,000 163,000. *m/z* range: 4000 9000. **Match tolerance:** 5 Da

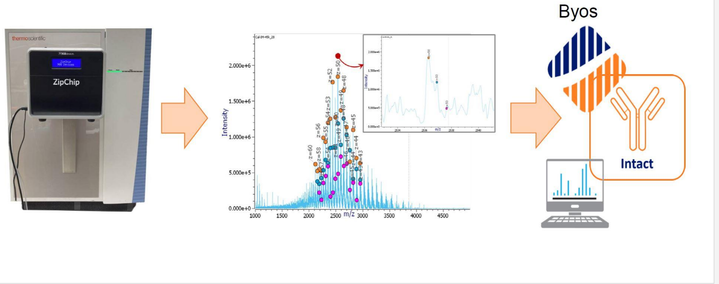


Figure 1. Charge variant analysis workflow with CE-MS and Data Analysis

*The Native Antibodies Kit has since been replaced by the Charge Variant Analysis Kit (p/n: 850-00052)

Results: Cetuximab Innovator

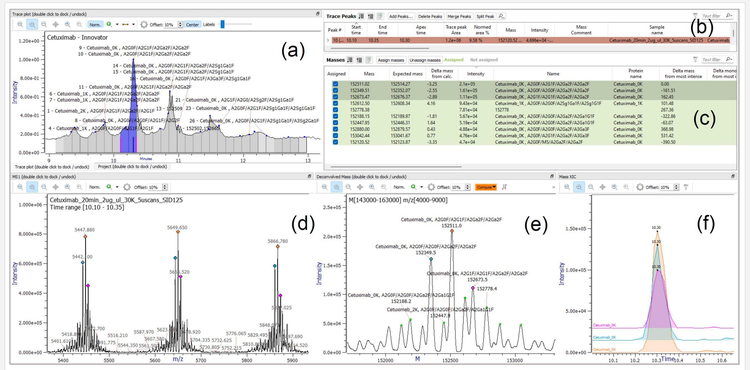


Figure 2. Overall view of the working space for the Intact workflow in Byos.

In the Trace plot window (a), the MS dataset is processed in 0.25 min wide slices overlapping by 0.125 min (50%); slice boundaries are shown as vertical lines in the Trace plot chromatogram. The selected time slice, shaded purple in (a), is also highlighted in the Trace Peaks window. The Masses window (c) shows deconvoluted masses from the selected slices, labeled with assignments that match within a user set mass tolerance. Selected rows in the Masses window are displayed and identified with colored markers in the raw *m/z* and deconvoluted *m* spectra, (d) and (e). Finally, XICs of the selected masses are displayed in (f).

Using the list of glycans published by Füssl et al., (2020) more than 80% of the components contributing to the total intensity of the CE-MS data of the innovator are annotated. In (3a) this is rendered in Byosphere® dashboards, and the proteoforms of the Innovator and Biosimilars directly compared. Basic variants associated with the C Terminal lysine (1-K or 2-K) migrate before the main peak (3b). The acid variants are associated with glycans containing sialic acids (3c).

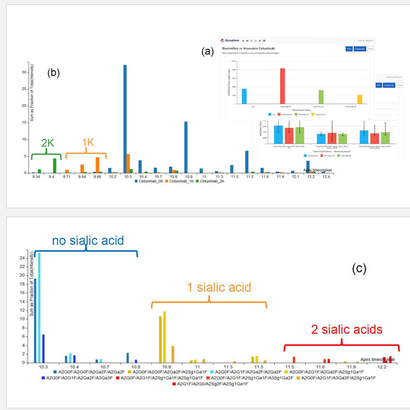
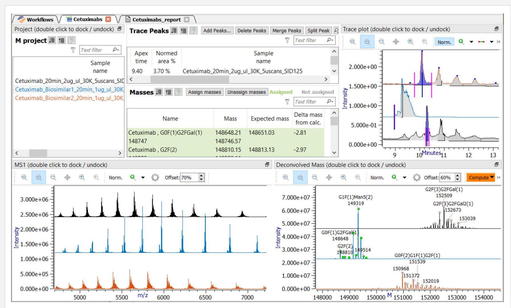


Figure 3. Results for the Innovator product.

Results: Innovator versus Biosimilars



The innovator drug is shown in black, biosimilar #1 in blue, and biosimilar #2 in red. The total ion electropherogram (TIE) for the innovator (upper right, bottom plot) shows charge variants with overall charge +2 (9.4 min) representing proteoforms with neutral glycans and two C-terminal K's, +1 (9.8 min) for proteoforms with neutral glycans and one C-terminal K, and so forth. The selected, most intense elution peak at 10.3 min represents proteoforms with neutral glycans and no C-terminal K's. The most intense mass peak for this elution peak is at 152,509 (lower right, top plot), matching G2F(3)G2FGal1(1) (the same overall composition as [A2] G0F / G1F / Ga2F / Ga2F in Figs. 2 and 3). Later elution peaks represent negatively charged proteoforms; the 10.9 min forms have one sialic acid, and the 11.5 min forms have two sialic acids.

Figure 4. Comparison of cetuximab innovator to the biosimilars.

Biosimilar #1 shows a completely different TIE than the innovator drug with no clear distinction of charge variants. The *m/z* and *m* spectra show fewer proteoforms, and given the intact masses, either the glycosylation sites are occupied by unusually small N-glycans or only two of the four sites are occupied, as shown by the mass matching in Figure 5 below.

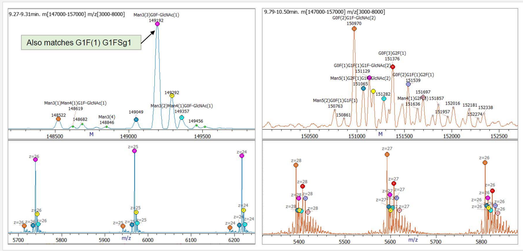


Figure 5. Biosimilar #1

Figure 6. Biosimilar #2

Biosimilar #2 shows less abundant C-terminal K forms than the innovator drug, along with some shift in the time of the neutral and basic forms. The most abundant proteoform in the neutral charge elution peak has mass ~159,070, matching (within 3 Da) glycan composition HexNAc(10)Hex(14)Fuc(4), which could be G0F(2)G1F GlcNAc(2) as shown to the left.

Discussion

ZipChip CZE coupled to HRMS for mAb analysis in native mode enables separation of charge variants producing simpler *m/z* spectra and hence greater resolution of proteoforms. Protein Metrics Intact Mass could identify more than 90 different glycoforms for the innovator drug from a single CE-MS injection; such an analysis is of course made possible by previous analyses of cetuximab (Ayoub, 2013; Füssl, 2020). Native charge is also valuable information beyond simply alleviating spectrum crowding. For example, (Bern et al., 2018) misidentified Fab glycoforms in an uncrowded spectrum of Cetuximab Fd, due to the within 1 Da mass coincidence of Hex(1)Fuc(1) and NeuGc(1).

Biosimilar cetuximabs turned out to be quite dissimilar. Biosimilar #1 has a drastically different charge variant profile than the innovator drug (Fig. 4), and it seems likely that not all four N-glycosylation sites (N88 on the Fd and N299 on the Fc part) are occupied in the most abundant proteoforms (Fig. 5). Biosimilar #2 has a charge variant profile similar to that of the innovator drug, but its proteoform masses are quite different in each of the charge variant peaks. Fig. 6 shows the neutral charge peak, but the acidic variants are equally diverged from the innovator.

As shown in Fig. 6, Intact Mass software could match the observed masses to combinations of common glycans such as Man5, G0F, G1F, and G0F and G1F missing GlcNAc, but more confident matches would be obtained from simpler mass spectra, for example, spectra of reduced or IdeS digested heavy chains. Biosimilar #2 had some mass peaks matching calculated masses involving alpha Gal and NeuGc (nonhuman motifs prevalent on the innovator drug), but these were at much lower abundance than on the innovator.

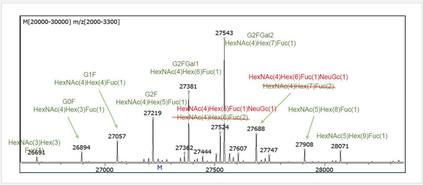


Fig. 7: Without charge separation and MS2 (Bern et al, 2018) misinterpreted Fd mass peaks.

Conclusion

- ZipChip coupled to HRMS provides a streamlined analytical approach to rapidly assess the heterogeneity of the investigated therapeutic proteins through the comprehensive interrogation of the different charge variants and their intact MS spectra.
- The performed analyses of the charge variant profiles of the Innovator product and its Biosimilars instantly reveal analytical differences and similarities supporting the assessment of biosimilarity.
- The separation achieved by the Chip based CE platform offers a deeper and more comprehensive investigation of the detected species.
- The Byos platform Intact workflow offers efficient identification, inspection, and relative quantification of charge variants, while reliably detecting even the low abundance species and highlighting even subtle differences between innovator and biosimilar products.

References

Bern et al., J. Proteome Research, 2018. 17, 3. 1216-1226.
Ayoub et al. mAbs, 2013. 5, 5. 699-710;
Füssl et al., Anal. Chem. 2020, 92, 7, 5431-5438.

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