Novel LCMS-Compatible icIEF Fractionation For Characterization of Innovator and Biosimilar mAbs

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Abstract

Monoclonal antibodies (mAbs) are an important class of biotherapeutics, though their development is complex and expensive. Many companies opt to develop biosimilar mAbs, which share the same protein sequence as the innovator but may have differences in post-translational modifications (PTMs), which can affect safety and efficacy. Regulatory agencies require evidence that there are no clinically relevant differences between innovator and biosimilar.

One of the gold standard methods for charge profile characterization is icIEF. This method is traditionally optical-only, meaning incompatible with mass spectrometry analysis for investigation of charge variants species. However, the new MauriceFlex™ instrument helps to overcome this obstacle, as it can separate charge variants, and then mobilize them in LC-MS compatible ammonium acetate buffer for subsequent analyses.

Here we present a simple workflow for investigation of charge variants using the MauriceFlex icIEF instrument to separate, mobilize, and collect fractions for LC-MS analysis using the BioAccord™ System (UHPLC coupled to an easy-to-use compact TOF mass spectrometer), operated under a compliance-ready informatics platform with automated acquisition and data processing.

Materials and Methods

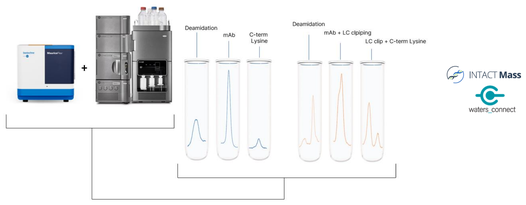


Figure 1. MauriceFlex instrument is used for icIEF separation and fraction collection of protein samples. Collected fractions are then analyzed via the BioAccord LC-MS System.

Samples

This study used Benlysta™ (Belimumab Innovator), approved for lupus and lupus nephritis, and a Biosimilar candidate (research-grade) to characterize charge variants using MauriceFlex fractionation, followed by LC-MS analysis of selected fractions via the BioAccord LC-MS System (Figure 1).

icIEF Analysis Method

The innovator and biosimilar samples were prepared at a final concentration of 0.1 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (4:1), 20% SimpleSol, 5 mM arginine, and Maurice pI markers 7.05 and 9.50. The samples were loaded onto the MauriceFlex instrument along with the Maurice cIEF cartridge and focused for 1 min at 1500 V, then 12 min at 3000 V.

icIEF Fractionation Method

Samples were prepared at a final concentration of 2 mg/mL in an ampholyte solution containing Pharmalytes (4%) 8-10.5 and 3-10 (4:1), 30 mM arginine, 30% SimpleSol, Maurice pI markers 7.05 and 9.50, and Simple Western™ pI markers 7.00 and 9.70. The samples were loaded onto the MauriceFlex instrument along with the MauriceFlex icIEF Fractionation Cartridge and focused for 10 min at 250 V, 10 min at 500 V, 10 min at 1000 V, and 25 min at 1500 V. The detected peaks mobilized for 25 min at 1000 V, followed by fraction collection for 45 sec at 1000 V. Fractions were collected into plate wells containing 40 µL 5 mM Ammonium Acetate. Arginine was added (Final Conc: 100 mM) to each well after fractionation. All data were analyzed using Compass for iCE software.

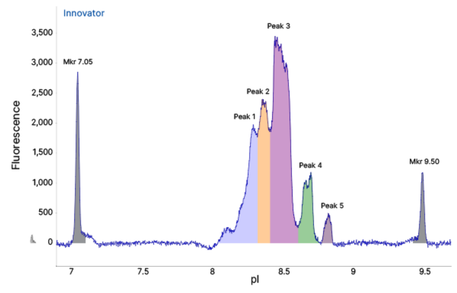
LC-MS Analysis

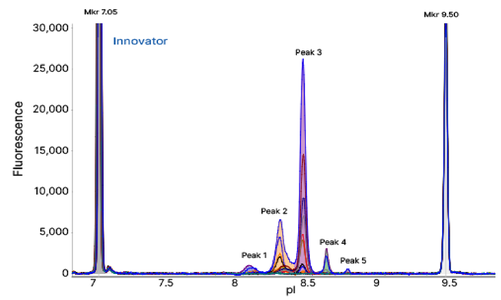
The 96-well plate collected from the MauriceFlex system was transferred to the BioAccord System for LC-MS analysis without any buffer exchange or additional sample preparation. The BioAccord System was equipped with the Waters MassPREP™ Micro Desalting Column (2.1 x 5 mm, P/N 186004032). Mobile Phase A was water with 0.1% formic acid and Mobile Phase B was acetonitrile with 0.1% formic acid. Separation was achieved using a gradient of 5-90% Mobile Phase B over 1.5 minutes (Total method time of 4 minutes). Column temperature was set to 80°C. The RDa™ Detector settings were as follows: default capillary voltage of 1.5 kV, default desolvation temperature of 550°C, and a cone voltage of 70 V. Mass spectra of each fraction were acquired, automatically deconvoluted, and masses matched within 40 ppm via INTACT Mass App within the waters_connect™ Informatics Platform.

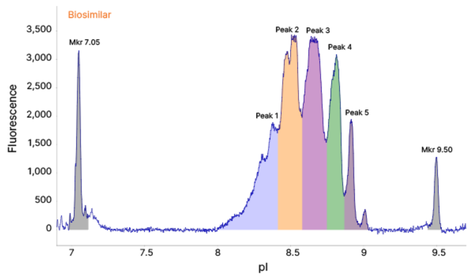
Results and Discussion

icIEF Charge Variant Separation

The Benlysta (belimumab) innovator and biosimilar samples were analyzed for charge heterogeneity using MauriceFlex icIEF Fractionation Method, where five major peaks were detected for each (Figure 2). It is interesting to note that there is both an apparent pI value shift, as well as an overall different ratio, of the mAb species. These observations underscore the need for further investigation. The ability to collect these fractions into MS-compatible ammonium acetate buffer enables the user to analyze them with LC-MS without any additional sample preparation, which decreases the overall time from question to results. Each collected fraction was reinjected to MauriceFlex using the icIEF Analysis Method to compare to the unfractionated Innovator or Biosimilar sample, to confirm charge variant species (estimated pI) and to estimate a concentration for each fraction.







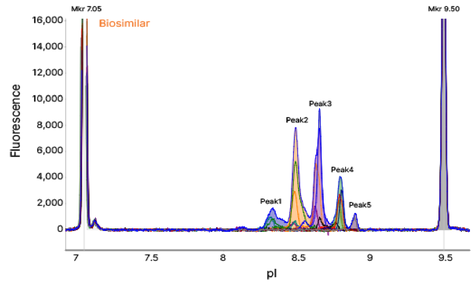


Figure 2. MauriceFlex Fractionation. (A) Charge separation of Benlysta (belimumab) Innovator and (B) research-grade belimumab biosimilar sample with MauriceFlex icIEF Fractionation cartridge. Five major peaks were detected in both samples; however, an overall pI shift is observed for the biosimilar sample. Each mAb was fractionated using the same method, producing 14 fractions for (A) the Innovator and 16 for (B) the biosimilar candidate.

Figure 3. Collected fractions reinjected for icIEF analysis, overlaid with unfractionated sample (blue trace). A small sample of each collected fraction was reinjected for icIEF analysis using the icIEF Analysis Method. When compared to the unfractionated sample, the user can confirm charge species and estimate a concentration for each fraction.

LC-MS Mass Identification

The 96-well collection plate from MauriceFlex was transferred to the BioAccord System for mass identification. The sample manager was equipped with a 50 µL sample extension loop for extra flexibility to accommodate any lower-concentration icIEF fractions. The fast 4-minute LC-MS acquisition method using the MassPREP Micro Desalting Column enables high throughput sample analysis without concern of sample carryover.

Results for the analysis are shown in Figures 4 and 5. Figure 4 displays a representative Total Ion Chromatogram (TIC) (4A) and combined raw MS spectrum for the integrated TIC peak (4B). All peak integrations, MaxEnt1 mass deconvolutions, and mass matches were performed automatically in the INTACT Mass App within waters_connect Informatics Platform. Figure 5 displays mass deconvolutions for one acidic, one basic, and one main peak fraction for the Innovator and Biosimilar samples. The differences in the icIEF profiles are confirmed with mass identification of species such as C-terminal lysine and significant mAb clipping species observed in the Biosimilar sample.

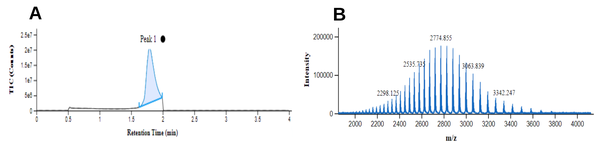


Figure 4. Example Total Ion Chromatogram (TIC) and Combined Raw MS spectrum. (A) Example TIC from the fast 4-minute LC-MS acquisition for one of the selected MauriceFlex fractions and (B) Respective combined raw MS spectrum for the peak in TIC above.

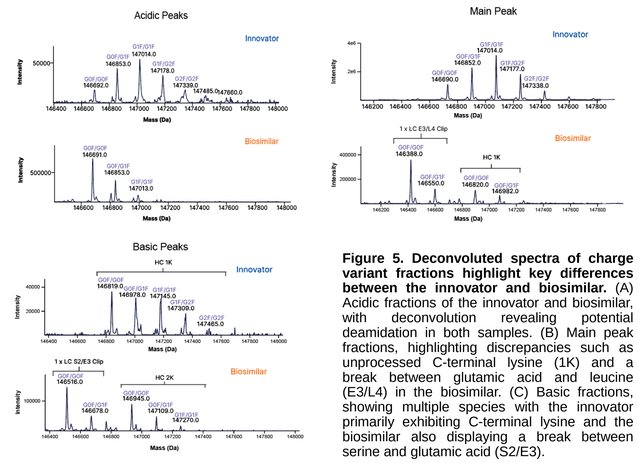


Figure 5. Deconvoluted spectra of charge variant fractions highlight key differences between the innovator and biosimilar. (A) Acidic fractions of the innovator and biosimilar, with deconvolution revealing potential deamidation in both samples. (B) Main peak fractions, highlighting discrepancies such as unprocessed C-terminal lysine (1K) and a break between glutamic acid and leucine (E3/L4) in the biosimilar. (C) Basic fractions, showing multiple species with the innovator primarily exhibiting C-terminal lysine and the biosimilar also displaying a break between serine and glutamic acid (S2/E3).

icIEF Peak	Innovator	Biosimilar Candidate
Peak 1	Possible mAb deamidation, free LC, LC dimer*	Possible mAb deamidation, free LC, LC dimer*
Peak 2	mAb, possible mAb deamidation	mAb
Peak 3	mAb, free LC, LC dimer*	mAb + 1 x LC E3/L4 clip, mAb, mAb + 1K species
Peak 4	mAb + 1K species	mAb + 1x LC S2/E3 clip, mAb + 2K species
Peak 5	Insufficient MS signal	mAb + 1 x LC S2/E3 clip + 1K species

Table 1. A summary of various species detected after LC-MS analysis of select acidic, main, and basic fractions collected after icIEF. *Free LC and LC dimers are not indicated in the spectra (Figure 3).

Summary

- Efficient and successful workflow to separate, collect, and characterize mAb charge variants.
- The MauriceFlex System enables icIEF-based separation and fractionation into MS-compatible ammonium acetate buffer.
- The BioAccord LC-MS System was operated under compliance-ready waters_connect Informatics Platform for easy LC-MS analysis of collected fractions.
- This workflow facilitates easy investigation of differing charge variant species observed between an innovator Benlysta (belimumab) and a biosimilar candidate sample.
- A variety of charge variant species were identified (see Table 1), from unprocessed C-terminal lysine variants to significant mAb clipping species in the biosimilar candidate sample.