INNOVATOR AND BIOSIMILAR INFLIXIMAB: COMPARABILITY ASSESSMENT OF THE HOST CELL PROTEINS AND PROTEIN HIGHER ORDER STRUCTURE

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INTRODUCTION

- HOS and HCP comparability analysis between an innovator and a biosimilar mAb (infliximab and inflectra). Both studies were carried out using a micro flow ACQUITY UPLC system interfaced to a high resolution mass spectrometry (Synapt HDMS).
- Overall, the biosimilar attributes mirrored those of the innovator product to a very high degree
- Protein tertiary structure analysis was conducted using Hydrogen Deuterium Exchange Mass Spectrometry (HDX MS), results showed the conformation for both mAbs are highly comparable except a minute difference in CH2 domain.
- Regular peptide mapping and released glycan analysis were performed to confirmed the results of HDX MS. No significant difference was found between two mAbs in terms of PTMs. Some differences were observed for the lower abundance glycans.
- The HCP study concludes that two HCPs (epidermal growth-factor like protein 8 and WD repeat containing protein 37) were found in both the innovator and the biosimilar mAbs.

METHODS AND MATERIALS

Sample preparation:
Two high-purity therapeutic monoclonal antibodies (mAbs), expressed in murine cell cultures, multiple batches of the innovator (**Infliximab**, 21 mg/mL) and its biosimilar (**Inflectra**, 10 mg/mL) were analyzed for all experiments.

Hydrogen Deuterium Exchange MS (HDXMS)

The labeling reaction was initialized by adding 15-fold of phosphate buffer (pH 6.8) to protein stock (~14 µM). After variable reaction times (30 sec, 1 min, 10 min, 60 min and 240 min), the labeling reaction was quenched by adding pre-chilled quenching buffer with TCEP and GdnHCl. All the sample preparation were operated by HDX-2 Automation utilizing PAL RTC Robotics and timing was scheduled by Chronos (Axel Semrau)

LC condition
Quenched solution flew through Enzymate™ column (Waters, 2.1 x 30 mm, 130 Å, 5 µm) to complete online digestion and then went to cold chamber inside of HDX manager for separation. Analytical column was ACQUITY UPLC BEH C18 column, 1.7 µm 1.0 x 100 mm. The trap column was an ACQUITY VanGuard column, BEH 18 1.7 µm 2.1 x 5 mm.

MS and data processing
- Data were collected by Waters Synapt G2S HDMS instrument.
- Undeuterated control was processed using PLGS 3.0.2 for peptide identification. DynamX 3.0 was used to measure the deuterium uptake of each peptides and generate all visualization graphs.

Host cell protein (HCP)

2D-LC configuration
An M-class ACQUITY™ UPLC® system with 2D technology was used for peptide separations. A reversed-phase/reversed-phase (RP/RP) 2DLC method, using the pH of the mobile phases to change the selectivity of peptide separations in two separate dimensions, was developed:
- **First Dimension (1D) pH=10**: 1.0 mm x 50 mm XBridge C_{18} column (5 µm particles), 10 µL/min flow. Mobile phase: 20 mM ammonium formate in water (Solvent A) and ACN (Solvent B).
- **Online dilution (**1:10) of the eluent from 1D before analyte trapping onto the trap column.
- **Trap column**: 0.3 x50 mm packed with 5-µm Symmetry C_{18} particles.
- **Second Dimension (2D) pH=2.4**: 0.3 mm x 150 mm analytical column CSH C_{18} 1.7 µm, kept at 60 °C and operated at 10 µL/min. Fractions were eluted in ten steps (Each step was mixed in a 1:10 ratio with 0.1% TFA in water (pH=2.1) before trapping.

MS and data processing

Data independent, alternate scanning 2D-LC/HDMS^E experiments were performed on a SYNAPT G2-S mass spectrometer:
- Acquisition time was 0.5 sec, m/z range: 100-1,990 amu.
- Fixed CE at 5 V for low-energy MS scans; drift-time specific CEs were applied for the high-energy scans
- For the IMS separations a fixed wave velocity (650 m/s) and a fixed wave height (40 V) were employed
- Data Processing: ProteinLynx Global Server (PLGS) 3.0.2.

HDX configuration

Figure 1. *ACQUITY UPLC® M-Class System with HDX Technology and HDX-2 Automation. DynamX 3.0 enables automated processing of global (Intact), local (peptide), and residue (AA, ETD) level HDX MS data.*

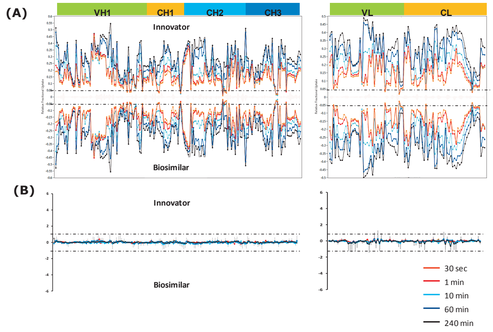

Figure 2. *(A) A butterfly plot of the relative fractional exchange data for Innovator (top) versus biosimilar (bottom), as a function of peptide order. The standard deviation between two measurements across all peptides was less than ±0.05 Da,*

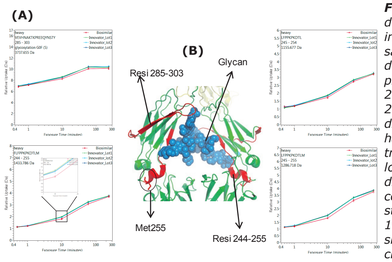

Figure 3. *Comparison of deuterium incorporation of innovator and biosimilar samples. (A) Representative deuterium incorporation profiles of regions (residues 244-255, 245-254, 245-255, 285-303) show minute difference. The experiments have been repeated in triplicate runs. (B) The location of the region displayed minor difference is colored in red in the model structure of IgG1 (PDB: 1HZH). Glycosylation is shown in blue. Met255 is circled and shown in stick.*

RESULTS

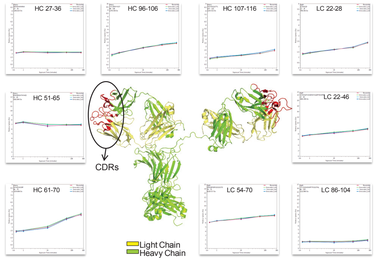

Figure 4. *Representative peptides covered the complementarily determining regions (CDRs) of infliximab displayed identical conformation and dynamics. The heavy chain and light chain structures are colored in the 3D model of IgG1 (PDB: 1HZH) in green and yellow, respectively. The CDRs are colored in red. The deuterium incorporation curves of the sample peptides, which covered all the CDRs, are showed.*

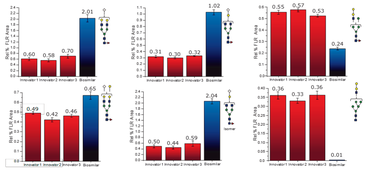

Figure 5. *Differences in the normalized relative intensities (based on FLR data) for selected glycans featuring immunogenic glycans.*

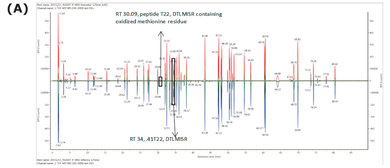

Figure 6. *(A)BPI chromatograms of peptides generated by trypsin digestion of innovator lot 1 (top) and the biosimilar mAb (bottom). (B) The oxidation level of M255 cross all samples.*

(B)

Peptide	Sequence	Oxidation %			
		Biosimilar	Innovator 1	Innovator 2	Innovator 3
252-258	DTLMISR	2.41	1.75	1.51	1.07

CONCLUSION

- In this study, we focus on two main areas of mAb characterization for comparative purposes: structure and impurities present in the final products.
- HCP study showed that the biosimilar mAb has higher level of HCP compare to the innovator (2-4 fold higher). Two HCPs (epidermal growth-factor like protein 8 and WD repeat containing protein 37) were found in both the innovator and the biosimilar mAbs.
- HDX MS study showed the conformation for both mAbs are highly comparable except a minute difference in CH2 domain.
- Our study shows great promise in adapting these analytical capabilities into biosimilar drug development process.

HCP configuration

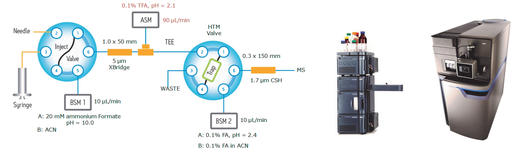

Figure 7. *Fluidic configuration for the two-dimensional high pH/ low pH RP/RP chromatographic setup employing on-line dilution.*

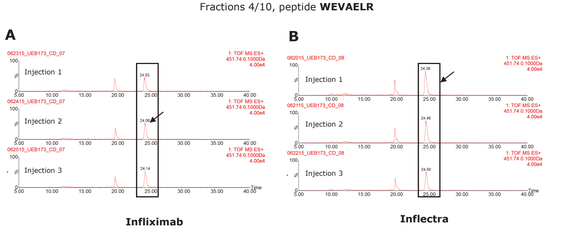

Figure 8. *Reproducibility of the 2D-LC chromatographic system: (A,B) Extracted mass chromatograms generated for the monoisotopic peak of a low-abundance HCP peptide detected in both mAbs: peptide WEVAELR (m/z = 451.74, +2) from epidermal growth-factor like protein 8, eluting in Fraction 4/10.*

Infliximab

No	Accession Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	Amount on column ng	Concentration ng/mL	Concentration ppm	RSD (%)
1	P04489	Glycogen phosphorylase rabbit (PHO) - 1000 fmoles	43.2	97.1	1000	97	1942	92	0.8
2	P00330	Alcohol dehydrogenase yeast (ADH) - 5000 fmoles	44.6	36.7	1981	73	1454	69	3.2
3	P02769	Bovine serum albumin (BSA) - 250 fmoles	21.9	66.3	296	20	392	19	10.3
4	Q63ZQ1	Epidermal growth-factor-like protein 8	8.2	33.3	792	26	527	25	14.3
5	Q8C8E1	WD repeat-containing protein 37	4.8	55.1	198	16	317	15	23.1
6	P00924	Enolase 1 yeast (ENL) - 50 fmoles	15.7	46.6	102	5	95	5	10.8
						Total mAb	ng/mL	845	
						Total mAb	ppm		60
						mAb	purity		99.996%

Inflectra

No	Accession Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	Amount on column ng	Concentration ng/mL	Concentration ppm	RSD (%)
1	P00489	Glycogen phosphorylase rabbit (PHO) - 1000 fmoles	35.7	97.1	1000	97	1884	188	0.0
2	P00330	Alcohol dehydrogenase yeast (ADH) - 5000 fmoles	40.7	36.7	2077	76	3043	304	4.5
3	Q63ZQ1	Epidermal growth-factor-like protein 8	8.2	33.3	687	23	915	92	18.9
4	P02769	Bovine serum albumin (BSA) - 250 fmoles	19.1	66.3	294	19	780	78	13.1
5	Q8C8E1	WD repeat-containing protein 37	4.8	55.1	141	8	513	51	35.0
6	P00924	Enolase 1 yeast (ENL) - 50 fmoles	18.3	46.6	58	3	108	11	26.5
						Total mAb	ng/mL	1228	
						Total mAb	ppm		123
						mAb	purity		99.988%

Table 1. *Comparison of HCPs identified and quantified in Infliximab and Inflectra. The same 2 HCPs were detected in both samples: epidermal growth-factor like protein 8 and WD repeat containing protein 37.*

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