Comparative Post Translational Modification in mAbdi2 and Innovator samples by LC-MS/MS workflow

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Introduction

The mAbdi2 is being developed as a biosimilar candidate. This study was designed to provide a comparative analytical assessment of the mAbdi2 and Innovator products to evaluate the post-translational modifications by LC-MS/MS workflow.

Methodology

The Bruker's maXis II-QTOF mass spectrometer was used in combination with an Elute UPLC in the positive ion polarity mode. Spectra were recorded in the mass range between 500-5000 m/z for Intact samples, and 300-3000 m/z for reduced samples. The de-glycosylation of the samples was performed by PNGase-F for overnight incubation at 37 ºC. The reduction of the samples was performed by 1M DTT, at 65 ºC for 15 Minutes. The spectra were deconvoluted using the Maximum Entropy algorithm. The mobile phase A 0.1% (v/v) formic acid in water and mobile phase B 0.1% (v/v) formic acid in acetonitrile.

For peptide mapping, samples were denatured with guanidine hydrochloride and then treated with dithiothreitol (DTT) to break disulfide linkages between cysteine residues for complete reduction. To prevent the disulfide bonds from re-forming, iodoacetamide (IAA) was used to modify the reactive cysteine-SH groups, forming carbamidomethylated cysteines. After alkylation, buffer exchange was performed for the removal of excess salts and trypsin was used for digesting the protein into peptides and identified by the mass spectrometer by Biopharma compass software.

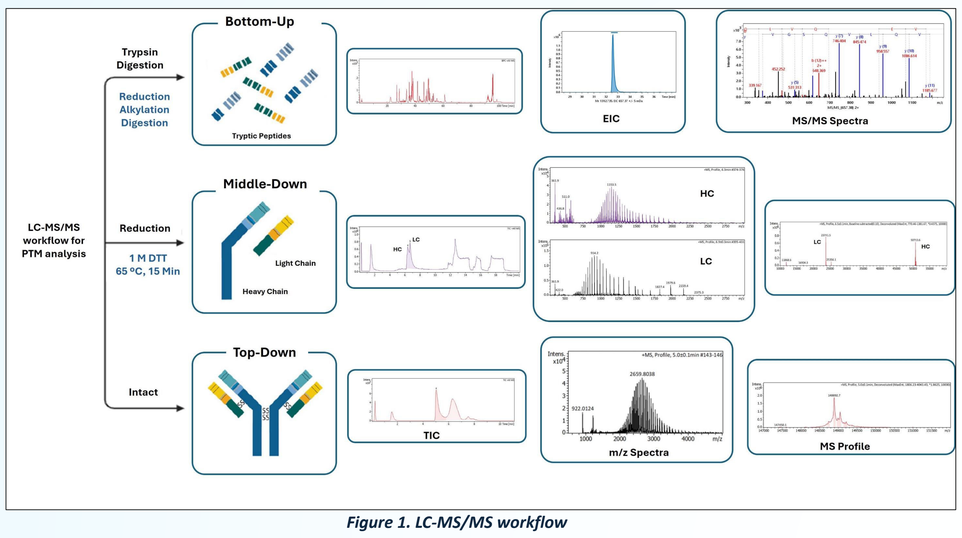
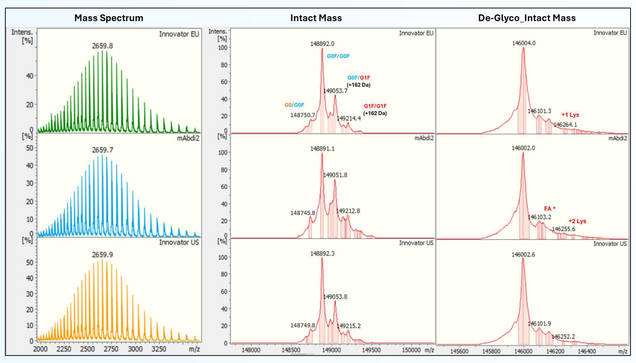


Figure 1. LC-MS/MS workflow

Results

Intact Mass Confirmation



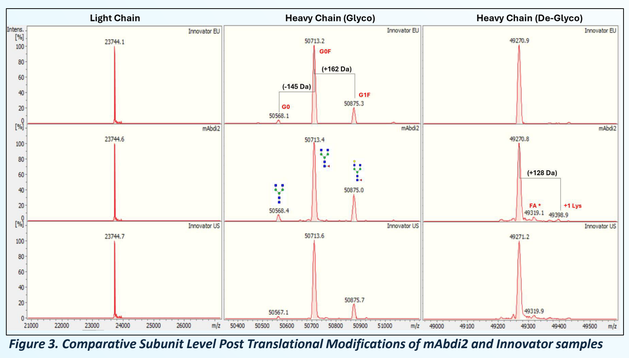
Sample Details (Glyco)	Glycan Forms (Mass, Da)		
	G0F+G0F-2 Lys+ 2 Pyro Q (Main Peak)	G0F+G1F -2 Lys+2 Pyro Q	G1F+G1F -2 Lys+2 Pyro Q
Theoretical mass	148887.0	149049.0	149211.0
Innovator EU	148892.0	149053.7	149214.4
mAbdi2	148891.1	149051.8	149212.8
Innovator US	148892.3	149053.8	149215.2

Sample Details (De-Glyco)	Component (Mass, Da)		
	2LC + 2HC +2 Pyro Q - 2 Lys (Main Peak)	2LC + 2HC – 1 Lys	2LC + 2HC +2 Pyro Q
Theoretical mass	145997.0	146159.0	146253.0
Innovator EU	146004.0	146164.3	146264.1
mAbdi2	146001.9	ND	146255.4
Innovator US	146002.6	146163.5	146252.2

- **A similar Intact mass profile was observed under Glyco & De-Glyco conditions for mAbdi2 & Innovator samples.**

Figure 2. Comparative Intact Level Post Translational Modifications of mAbdi2 and Innovator samples

Reduced Mass Confirmation



Sample Details	Component (Mass, Da)				
	Heavy Chain (Glyco)			Heavy Chain (De-Glyco)	
	G0-1 Lys +1 Pyro Q	G0F-1 Lys+1 Pyro Q (Main Peak)	G1F-1 Lys +1 Pyro Q	1 Pyro Q -1 Lys (Main Peak)	1 Pyro Q
Theoretical mass	50569.0	50715.0	50877.0	49271.0	49399.0
Innovator EU	50568.1	50713.2	50875.3	49270.9	ND
mAbdi2	50568.4	50713.4	50875.0	49270.8	49398.9
Innovator US	50567.1	50713.6	50875.7	49271.2	ND

- **A similar Light chain profile under all the tested conditions (23744.0 ± 1 Da).**
- **The Δ mass of 1445 Da was observed in the Heavy chain under Glyco & De-Glyco conditions.**
- **A comparative subunit mass profile was observed for mAbdi2 and Innovator samples.**
- **mAbdi2 Heavy chain shows the traces of C-Term Lys residue as compared with Innovator samples.**

Figure 3. Comparative Subunit Level Post Translational Modifications of mAbdi2 and Innovator samples

Tryptic Digest Sequence Confirmation

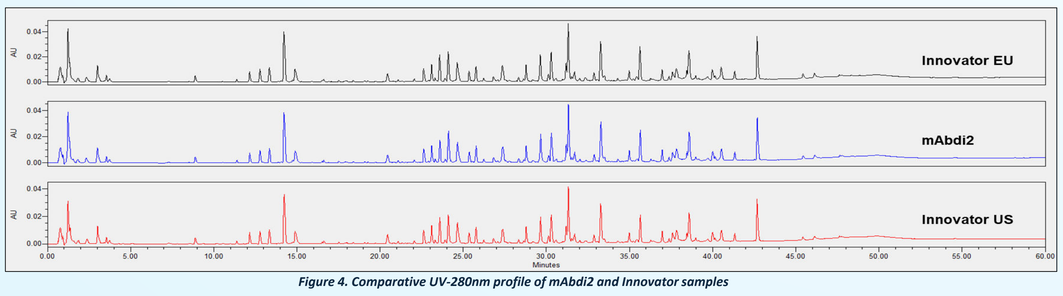
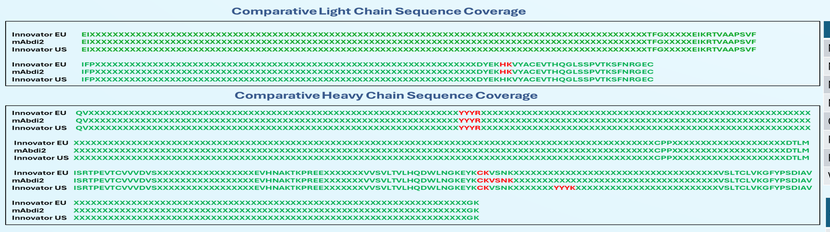


Figure 4. Comparative UV-280nm profile of mAbdi2 and Innovator samples



Parameter	Criteria
MS tolerance	20 ppm
MS/MS tolerance	0.01 Da
Missed Cleavage	2
Mass range	50-2500 (m/z)
Charge State	1-6
Polarity	+ Ve
Fixed Modification	Carbamidomethyl (C)
Variable Modification	Oxidation & Deamidation

Sample Details	Sequence Coverage (%)		
	Light Chain	Heavy Chain	Total
Innovator EU	99.1	97.8	98.8
mAbdi2	99.1	97.8	98.2
Innovator US	100.0	96.9	97.9

- **The peptide mapping was performed under De-Glyco reduced condition using Trypsin protease for digestion.**
- **The amino acid sequence was masked due to confidentiality.**
- **A similar Peptide Mapping Fingerprinting observed for all the tested samples with > 98% sequence coverage.**
- **Red highlighted region peptides were not identified under the defined search criteria.**
- **The short peptides could be lost when they are too hydrophilic and, as a result, won't reach the mass spectrometer.**

Disulfide Bond Mapping

- **To evaluate the Di-sulfide linkages, all the samples were digested under reduced and non-reduced conditions.**
- **Non-Reduced samples were used to identify the di-sulfide bonds within the samples.**
- **Whereas reduced samples were further alkylated and analyzed for identification of disulfide linked peptides.**

Non-Reduced PMF Data

Cystine Linkage	Peptide Mass (Da)			
	Expected Mass	Observed mass		
		Innovator EU	Innovator US	mAbdi2
LC Cys23-LC Cys92	4047.82	4047.83 (Δ ppm = 0.56)	4047.83 (Δ ppm = 0.02)	4047.82 (Δ ppm = 1.17)
LC Cys138-LC Cys198	3555.75	3555.75 (Δ ppm = 1.57)	3555.75 (Δ ppm = 0.08)	3555.75 (Δ ppm = 0.05)
HC Cys22- HC Cys96	2083.93	2083.93 (Δ ppm = 0.02)	2083.93 (Δ ppm = 0.95)	2083.93 (Δ ppm = 1.62)
HC Cys134-LC Cys218	1534.69	1534.69 (Δ ppm = 0.21)	1534.69 (Δ ppm = 0.3)	1534.69 (Δ ppm = 1.21)
HC Cys147-HC Cys203	2970.40	2970.41 (Δ ppm = 0.06)	2970.40 (Δ ppm = 0.73)	2970.40 (Δ ppm = 1.52)
HC Cys226-HC Cys226 HC Cys229-HC Cys229	5672.82	5672.83 (Δ ppm = 0.33)	5672.81 (Δ ppm = 2.21)	5672.81 (Δ ppm = 2.17)
HC Cys261-HC Cys321	3977.85	3977.85 (Δ ppm = 0.86)	3977.85 (Δ ppm = 1.01)	3977.84 (Δ ppm = 1.16)
HC Cys367-HC Cys425	3845.81	3845.81 (Δ ppm = 0.64)	3845.81 (Δ ppm = 0.01)	3845.81 (Δ ppm = 0.55)

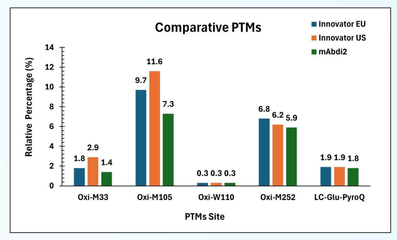
Reduced PMF Data (Disulfide bond-linked peptides)

Sr.No	Peptide Type	Region	Peptide Mass (Da)			
			Expected Mass	Observed Mass		
				Innovator EU	Innovator US	mAbdi2
1	Intra Chain	LC (19 - 24)	706.343	706.343	706.343	706.344
2		LC (66 - 96)	3457.541	3457.544	3457.541	3457.546
3		LC (131 - 146)	1796.888	1796.890	1796.888	1796.889
4		LC (195 - 211)	1874.920	1874.921	1874.921	1874.922
5		HC (20 - 38)	2395.077	2395.079	2395.080	2395.081
6		HC (85 - 98)	1707.756	1707.758	1707.757	1707.759
7		HC (137 - 150)	1422.702	1422.703	1422.703	1422.704
8		HC (200 - 213)	1663.762	1663.763	1663.762	1663.763
9		HC (256 - 288)	3787.768	3787.774	3787.770	3787.777
10		HC (361 - 370)	1160.622	1160.624	1160.623	1160.624
11		HC (417 - 439)	2801.244	2801.245	2801.243	2801.247
12		HC (321-322)	305.913	ND	ND	ND
13	Inter Chain	HC (125 - 136)	1286.644	1286.645	1286.645	1286.646
14		LC (216 - 218)	364.105	364.098	364.098	364.099
15	Hinge Region	HC (222 - 248)	2952.471	2952.470	2952.471	2952.475

Post Translational Modifications (PTMS, site and extent)

- **The PMF of the samples under Glyco & De-glyco reduced conditions were performed for the PTM analysis.**
- **The Oxidation, Deamidation, and Glycosylation site and extent of modifications were evaluated.**
- **The comparative N and C terminal modifications in the Light & Heavy chain for all the tested samples were presented.**

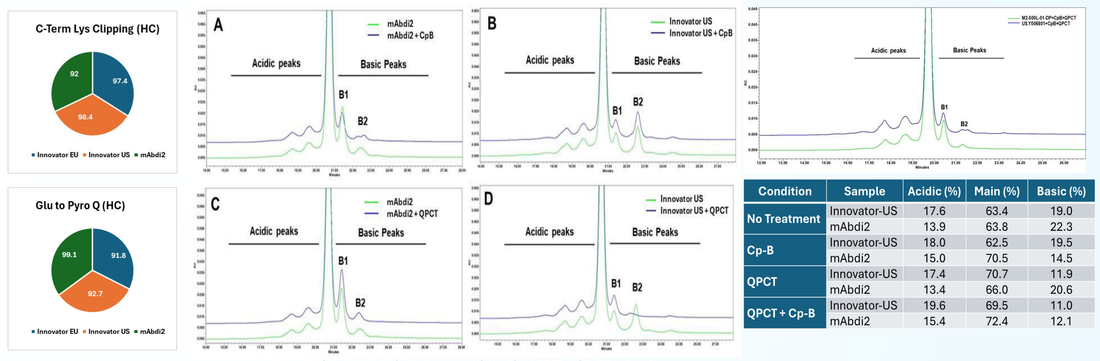
Peptide	Modification	Region	Theoretical Mass (Da)	Observed Mass (Da)	Δ Mass (Da)	Relative Peptide Modification (%)			MS/MS Spectra
						Innovator EU	Innovator US	mAbdi2	
AXXXXXXXXXMXXXR	Oxidation	HC (M33_CDR-1)	1920.9	1936.8	16	1.8	2.9	1.4	Yes
FXMXXXXXXXXXXXXXXXXXXK	Oxidation	HC (M105_CDR-3)	2384.1	2400.1	16	9.7	11.6	7.3	Yes
FXXXXXXWXXXXXXXXXXXXXK	Oxidation	HC (W110_CDR-3)	2384.1	2400.1	16	0.3	0.3	0.3	Yes
DTLMISR	Oxidation	HC (M252)	834.4	850.4	16	6.8	6.2	5.9	Yes
EXXXNSTXXXXXXXXXXXXXXXXK	Glycosylation	HC (N297)	3381.7	4826.2	1445	100	100	100	Yes
EXXXNSTXXXXXXXXXXXXXXXXK (De-Glyco PMF)	Deamidation	HC (N297)	3381.7	3382.7	1	100	100	100	Yes
EXXXXXXXXXXXXXXXXR	Glu to Pyro Glu	LC (N-Term)	1897.0	1879.0	-18	1.9	1.9	1.8	Yes
QVXXXXXXXXXK	Gln to Pyro Glu	HC (N-Term)	1312.7	1295.7	-17	91.8	92.7	99.1	Yes
SXXXXXGK	Lys Truncation	HC (C-Term)	803.5	675.4	-128	97.4	98.4	92.0	Yes



- **Similar PTMs observed in mAbdi2 & Innovator samples.**

N and C Terminal Modifications (Heavy Chain)

- **The mAbdi2 samples show comparatively less percentage of C-Term Lysine truncation.**
- **Whereas the relatively higher conversion of Glutamine (Q) to Pyro Glutamine (Pyro Q) in mAbdi2 was observed.**
- **For further confirmation, all the samples were treated with Carboxypeptidase B (CpB) & Glutaminyl-peptide cyclotransferase (QPCT) enzymes**
- **After treatment, samples were evaluated for charge variant profile and summarized below**



Condition	Sample	Acidic (%)	Main (%)	Basic (%)
No Treatment	Innovator-US	17.6	63.4	19.0
	mAbdi2	13.9	63.8	22.3
Cp-B	Innovator-US	18.0	62.5	19.5
	mAbdi2	15.0	70.5	14.5
QPCT	Innovator-US	17.4	70.7	11.9
	mAbdi2	13.4	66.0	20.6
QPCT + Cp-B	Innovator-US	19.6	69.5	11.0
	mAbdi2	15.4	72.4	12.1

Figure 5. Charge variants chromatograms with & without QPCT and Cp-B treatment

N-Term Q/E to Pyro Q Conversion v/s Relative Potency

- **To understand the conversion mechanism of Q/E to Pyro Q under physiological conditions, a PBS incubation study was conducted.**
- **The samples were buffer exchanged to PBS, pH 7.4, and incubated at 37°C for 2 weeks.**
- **The CEX-HPLC profile was evaluated at 0, 1, and 2-week time points.**
- **The same PBS incubation study samples were used to perform the relative potency by Reporter Gene Assay and results were summarized.**

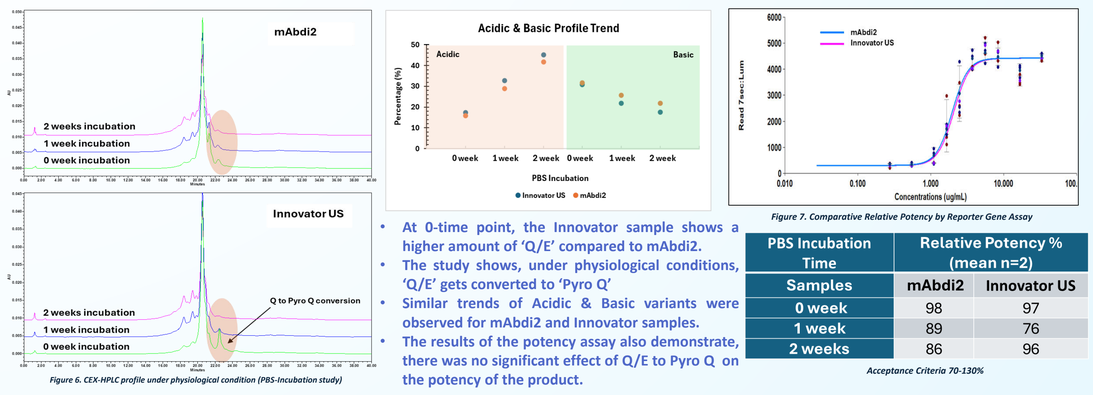


Figure 6. CEX-HPLC profile under physiological condition (PBS-incubation study)

Figure 7. Comparative Relative Potency by Reporter Gene Assay

- **At 0-time point, the Innovator sample shows a higher amount of 'Q/E' compared to mAbdi2.**
- **The study shows, under physiological conditions, 'Q/E' gets converted to 'Pyro Q'**
- **Similar trends of Acidic & Basic variants were observed for mAbdi2 and Innovator samples.**
- **The results of the potency assay also demonstrate, there was no significant effect of Q/E to Pyro Q on the potency of the product.**

PBS Incubation Time	Relative Potency % (mean n=2)	
Samples	mAbdi2	Innovator US
0 week	98	97
1 week	89	76
2 weeks	86	96

Acceptance Criteria 70-130%

Conclusion

In this study, the relative post-translational modifications were evaluated by LC-MS/MS-based workflow between mAbdi2 (Biosimilar candidate) and the Innovator samples with the following observations:

- The Intact mass analysis confirms the similarity in mass and homogeneity in the glycosylation pattern.
- The primary structure of the mAbdi2 was confirmed by peptide mapping sequence coverage.
- Theoretical and Experimental Disulfide linkages were observed with < 5 ppm error margin which confirms the higher-order structure similarity between the samples.
- The comparable Oxidation, Deamidation, and Glycosylation were observed.
- Some minor differences have been observed in terms of relative contents in post-translational variants at N & C terminal (HC), between mAbdi2 and Innovators, as expected for biological products.
- The CpB, QPCT, and PBS incubation study shows, there was no significant impact on relative potency due to variations in the N & C terminal modifications of heavy chain.
- Overall, LC-MS/MS analysis shows a high similarity between the mAbdi2 and Innovator samples.

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