SHIMADZU

WP 039

Characterization of Monoclonal Antibody Biosimilar through C-terminal and Disulfide Bond Peptides Sequencing Analysis on Q-TOF Mass Spectrometer

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1. Overview

- Characterization of C-terminal peptides with and without lysine truncation of mAb biosimilar by using LC-QToF-MS
- Detection of 15 cysteine containing peptides of mAb biosimilar by using LC-QToF-MS with good accuracy (< 2 ppm)

2. Introduction

There is a significant surge in the market of biopharmaceutical products due to their popularity as alternative solution for many chronic diseases. Monoclonal antibody (mAb) is a highly complex biological macromolecule with specific therapeutic effects. It is produced from live cells in extremely complicated culture conditions. Quality control of biopharmaceuticals, especially biosimilars, is a critical step to elucidate any alteration in the primary structure as compared to the reference product (innovator). Peptide mapping and sequencing analysis of C-terminal and disulfide-bonds linked peptides are among the essential attributes for characterization of biosimilars. In this report, characterization of a bevacizumab biosimilar is described, with focusing on peptide mapping and MS/MS sequencing of C-terminal and cysteine-containing peptides of a bevacizumab biosimilar on LCMS-9030, a Q-TOF system.

3. Experimental

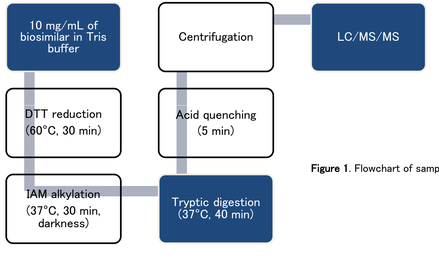

Figure 1. Flowchart of sample preparation

Table 1. Analytical conditions of biosimilar characterization on LCM-9030

Column	Shim-pack GISS C18, 5 µm, 250 × 4.6 mm
Flow rate	0.3 mL/min
Mobile phase	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Elution mode	Gradient elution, 0%B (0 – 5 min) → 50%B (85 min) → 75%B (95 – 100 min) → 0% (103 – 115 min)
Oven temp.	40°C
Injection vol.	20 µL

Interface & temp.	Heated ESI, 300°C
MS mode	MS scan, MS/MS
Block temp.	400°C
DL temp.	250°C
CID gas	Ar (230 kPa)
Nebulizing gas flow	N_2, 3 L/min
Drying gas flow	N_2, 10 L/min
Heating gas flow	Zero air, 10 L/min

4. Results and Discussion

4.1 Detection of C-terminal and disulfide-linked peptides

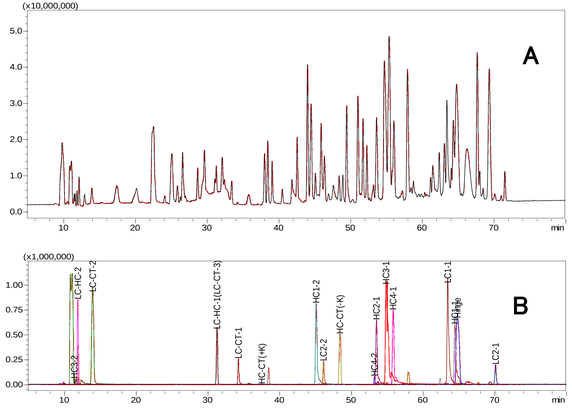

Figure 2. TIC of tryptic digest of bevacizumab biosimilar (A) and diagnosis ions (EICs) for C-terminal and cysteine-containing peptides (B)

Table 2. Detection and confirmation of C-terminal and cysteine-containing peptides of bevacizumab biosimilar

Type & Location		Sequence [1]	RT (min)	m/z, Theor	m/z, Mea.	Charge (+z)	Area %
Inter-chain cysteine containing peptides	LC-HC-1[2]	K.SFNRGEC.- [207, 213] (missed 1)	34.29	869.3570	869.3579	1	4.8
	LC-HC-2	K.SCDK.T [224, 227] (HC)	11.90	509.2024	509.2028	1	5.5
	Hinge	K.THTCPPCPAPELLGGPSVFLFPPKPK.D [228, 253]	64.83	948.8240	948.8255	3	14.0
Intra-chain cysteine containing peptides	LC1-1	R.VTITCSASQDISNYLNWYQQKPGK.A [18, 41]	63.49	915.4498	915.4495	3	12.7
	LC1-2	R.FSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQGTK.V [61, 1	75.33	1151.5285	1151.5271	4	0.02
	LC2-1	K.SGTASVVCLLNNFYPR.E [126, 141]	70.14	870.9405	870.9405	2	1.8
	LC2-2	K.VYACEVTHQGLSSPVTK.S [190, 206]	46.15	606.9733	606.9732	3	1.9
	HC1-1	R.LSCAASGYTFTNYGMNWVR.Q [19, 37]	64.47	714.3242	714.3249	3	6.3
	HC1-2	R.AEDTAVYYCAK.Y [87, 97]	45.14	617.2764	617.2765	2	9.3
	HC2-1	K.STSGGTAALGCLVK.D [139, 152]	53.55	632.8319	632.8331	2	7.0
	HC2-2	K.DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT QTYICNVNHKPSNTK.V [153, 215]	71.17	1343.4687	1343.4664	5	0.23
	HC3-1	R.TPEVTCVVVDVSHEDPEVK.F [261, 279]	54.89	694.6735	694.6739	3	5.8
	HC3-2	K.CK.V [326, 327]	11.43	250.1220	250.1221	1	0.15
	HC4-1	K.NQVSLTCLVK.G [366, 375]	55.87	552.8077	552.8079	2	9.1
	HC4-2	R.WQQGNVFSCSVMHEALHNHYTQK.S [422, 444]	53.26	915.4201	915.4194	3	0.60
C-terminal peptide	LC-CT2	K.SFNRGEC- [207, 213] (missed 1)	31.32	406.6715	406.6714	2	1.9
	LC-CT4	R.GEC- [211, 213]	13.98	365.1125	365.1128	1	13.4
	HC-CT+K	K.SLSLSPGK.- [445, 452]	37.55	394.7293	394.7292	2	0.04
	HC-CT-K	SLSLSPG	48.46	330.6818	330.6824	2	5.5

[1] C represents cysteine with carbamidomethyl modification; [2] Also C-terminal peptide (LC-CT1)

The bevacizumab sample was prepared with addition of reducing agent (DTT) before trypsin digestion. **Figure 2A** shows the TIC of the sample acquired on LCMS-9030. Fifteen cysteine-containing peptides and three C-terminal peptides were detected. A quick detection method of these peptide was set up based on extract ion chromatograms (EICs) of accurate masses (<2 ppm). The results are shown in **Figure 2B** and **Table 2**.

Location	Sequence	RT (min)	m/z (+z), measured		Area %
HC-CT(+K)	SLSLSPGK	37.55	788.4512+	394.7293++	0.76
HC-CT(-K)	SLSLSPG	48.46	660.3563+	330.6818++	99.24

Location	Sequence	RT (min)	m/z (+z), meas.	
LC2-2	VYACEVTHQGLSSPVTK	44.06	938.4646(+2)	625.9842(+3)
	VYACEVTHQGLSSPVTK	46.17	909.9558(+2)	606.9732(+3)

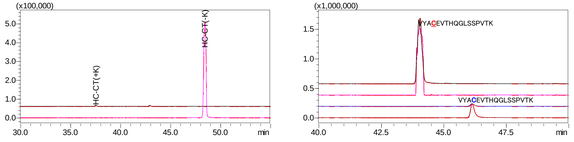

Figure 3. Heavy chain C-terminal peptide with and without lysine and their relative abundances based on peak areas (left), quick confirmation of cysteine-containing peptide (LC2-2) with and without carbamidomethyl modification (right).

C-terminal lysine variants of the HC were observed as shown in **Figure 3 (left),** with both peptides being confirmed by the accurate masses and charge status. The peak areas of EICs indicate the dominance of the clipped lysine (-K) sequence in the sample (1). **Figure 3 (right)** shows a common phenomenon of peptides – carbamidomethyl modification. Both sequences without and with the modification were found in LC2-2 peptides and confirmed firmly by accurate masses and charge numbers of the peptides.

4.2 MS/MS Sequencing of C-terminal and cysteine-containing peptides

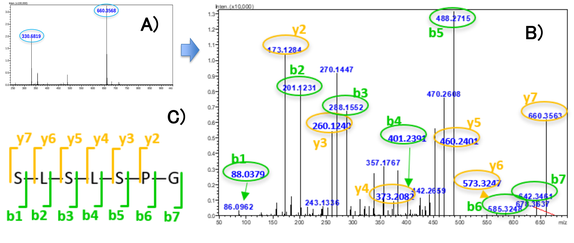

Figure 4. MS detection (A), MS/MS fragmentation of 660.3568+ ion (B) and de novo sequencing of C-terminal peptide (SLSLSPG) of HC (C).

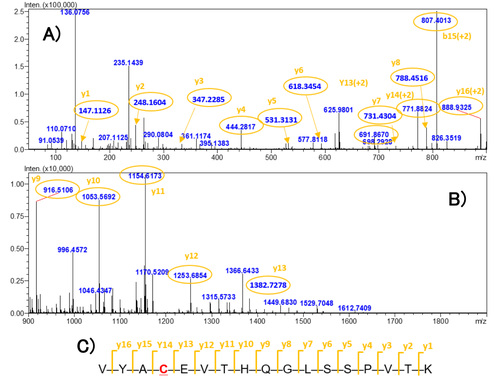

Figure 5. MS/MS spectra of peptide LC2-2 (VYACEVTHQGLSSPVTK, m/z 938.4677++) for m/z range: 50-900 (A) and 901-1900 (B), and annotation of all y ions (y1-y16) (C).

Figure 4 shows an example of de novo sequencing of the main C-terminal peptide (SLSLSPG) with clipped lysine through MS/MS fragmentation and ion annotation. Another representative example of de novo peptide sequencing is displayed in **Figure 5**. The results highlight MS/MS fragmentation of a cysteine-containing peptide LC2-2 in the light chain (VYACEVTHQGLSSPVTK). Both modified (carbamidomethyl) and unmodified ions were found for some peptides in the sample at different retentions (data not shown).

5. Conclusions

Mapping and sequencing of C-terminal and disulfide bond linked peptides are the essential analyses, which are among the critical quality attributes (CQA) to be characterized in biosimilar production. In this study, all the three C-terminal peptides and fifteen cysteine-containing peptides released from disulfide bond linked sequences of bevacizumab biosimilar were successfully detected, identified and sequenced on LCMS-9030 Q-TOF. An easy and reliable approach based on the EICs of accurate mass (<2ppm) and charge status was set up and used in quick peptide identification and mapping.

Reference

(1) L.W. Dick Jr, D. Qiu, D. Mahon, M. Adamo, K. Cheng. C-terminal lysine variants in fully human monoclonal antibodies: Investigation of test methods and possible causes, *Biotech Bioeng*, 100, 1132-1143 (2008)