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N-LINKED GLYCOSYLATION AND CHARGE VARIANT ANALYSIS OF BIOSIMILAR ANTI HER2 ANTIBODY IN TREATMENT OF BREAST CANCER

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ABSTRACT

Glycosylation moiety and charge variants both are playing important role in biological activity of Anti HER2 antibody. The binding affinity of the mAb molecule always depends upon the glycosylation pattern. However the variation in glycan profile may affect the biological activity which further promotes the subsequent mechanism in ADCC as well as CDC activities, the terminal galactosylation and afucosylation of glycan residues effect on CDC and ADCC activity. Therefore, the evaluation of glycan profile was carried out by Rapid PNGaseF digestion followed by UHPLC analysis. Mostly G0, G0F, Man5, G1Fa, G1Fb, G2 and G2F residue were found in the Anti-HER2 molecule. Further, each glycan was identified based on its retention time of standard glycans library and a comparison was made with respect to originator HERCLON®. Based on the results the molecule containing predominant residues was identified and also demonstrated their role *in vivo* biological activity. The charge variant analysis was equally important to demonstrate the quality of the Anti-HER2 antibody. Acidic variant, main (HC-K0) and basic variants (K1, K2) were determined by cation exchange chromatography (CEX) and percentage of all variants were evaluated based on separation on Protein Pak SP column in UHPLC system. The comparisons was carried out based on response of reference peaks under identical condition and the percentage of acidic, main and basic variants was

determined. Our results showed that the high degree of similarity in glycan profiling and charge variants of biosimilar Anti-HER2 antibody with respect to the innovator product may ensure in comparable biological activity.

KEYWORDS: Biosimilar, N-glycosylation, Charge variants, Anti-HER2 antibody.

INTRODUCTION

Breast cancer is the most common cancer affecting the women throughout the world. HER2 is a member of a family of receptors that interact with each other and various ligands to stimulate various intracellular signal transduction pathways involved in cell growth control.[1] HER2 or human epidermal growth factor receptor II refers to the HER2 gene and its associated protein. The protein helps to control the growth of healthy cells. But if the HER2 gene is amplified, or the protein is over expressed, the cells can grow uncontrollably and lead to cancer. HERCLON® is the first humanized monoclonal antibody to be approved for therapeutic use and the first oncogene-targeted treatment with proven survival benefit in women with HER2-positive metastatic breast cancer. The approval of biosimilar Anti-HER2 is not as an alternative but it is necessity to increase health coverage, improves the patient's life quality with reduced treatment cost.

The HER2 protein is an important therapeutic target in breast carcinoma for several reasons. First reason is, the HER2 gene is overexpressed in 20-25% of invasive breast cancer.[2] Receptor overexpression is generally due to gene amplification, with one study reporting up to a 25-fold increase in HER2 copy number.[3] Second reason is the elevated HER2 level strongly correlate with the pathogenesis and prognosis of breast cancer.[2] Third, the level of HER2 gene amplification in human carcinoma cell is much higher than in normal adult tissues; thus, HER2 targeted drugs targeting the HER2 protein could reduce the pathogenicity occur by HER2 overexpression. Fourth the overexpressed HER2 protein is found both in metastatic sites and primary tumor[4], indicates that Anti-HER2 therapy could be effective in all disease locations.

Overexpression, or amplification, of the human epidermal growth factor receptor (EGFR) 2 protein (HER2), which is correlated with poor clinical outcome in patients with breast cancer, is believed to result from gene amplification.[5]

The HER2 gene (also known as neu and c-erbB-2) encodes a 185kd transmembrane/kinase receptor, designated pl85HER2, that has partial homology with the other members of the EGFR family. Antibodies directed against HER2 can inhibit the growth of tumor xenografts and transformed cells that express high concentrations of this receptor.[6][7][8] This protein is located on the cell's surface, where it interacts with growth factors. When the HER2 protein is overexpressed, the cells divide, grow, and multiply at a faster rate than normal, contributing to the development of cancer.

The monoclonal antibody Anti-HER2 is adjuvant treatment specifically for patients with HER2-positive early stage breast cancer. The results revealed that the 10 year survival for HER2 positive breast cancer patients who received chemotherapy without Anti-HER2 was 75%, whereas for those who also had Anti-HER2, it was 84%.

Mechanism of Anti-HER2 antibody

Several mechanisms following binding of the antibody to the extracellular domain (ECD) of the HER2 receptor; these mechanisms include antibody-dependent cell-mediated cytotoxicity (ADCC), inhibition of cleavage of the ECD of the HER2 receptor (preventing formation of a residual truncated but constitutively active form),[9] inhibition of ligand-independent HER2 receptor dimerization, inhibition of downstream signal transduction pathways, induction of cell-cycle arrest, induction of apoptosis, inhibition of angiogenesis, and interference with DNA repair.[10][11] Natural killer (NK) cells are a belongs to population of lymphocytes ($CD56^{+}/CD3^{-}$) that were first identified by their mechanism to lyse tumor cells without prior immunization.

Anti HER2 antibodies enhances killing of breast cancer targets by different mechanisms: Antibodies binds to HER2 and reduces signaling in phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, which increased cyclin dependent kinase-inhibiting protein ($p27^{Kip1}$) levels by decreasing cyclin dependent kinase (CDK2) mediated phosphorylation of $p27^{Kip1}$and interaction with CDK2, results in G1 cell cycle arrest and apoptosis.[12] Anti-HER2 antibody blocks HER2 signaling via disruption of the phosphatidylinositol-3 kinase PI3K /Akt signaling pathway which prevents the proliferation of cancerous cells.[13]

Glycosylation

The most common form of post-translational modification is glycosylation. It is an enzyme mediated process by which oligosaccharide side chains are covalently attached to either side chain of asparagines (N-linked) or serine/threonine (O-linked). Glycosylation pattern have significantly impact on pharmacokinetics and pharmacodynamics. The oligosaccharide moiety of proteins is essential for signalling, recognition and interaction between and within cells and proteins. It plays an important role in defining the conformation and folding of the protein.[14] The mammalian expression system producing therapeutic protein exhibit a different glycosylation profile and can vary from batch to batch.[15]

ADCC involves an interaction between the constant region (Fc) of an antibody and leukocyte (Fcγ) receptors (FcγRs). The genotype of the FcγRIIIa-158 correlates with response and progression-free survival (PFS) in patients receiving Anti-HER2 based therapy for metastatic breast cancer, suggesting that ADCC is important in the antitumor activity of Anti-HER2.[16] Principal immune cell type, natural killer cells involved in ADCC express the Fc gamma receptor, to which the Fc domain of Anti-HER2 binds, activating natural killer cell mediated lysis. The IgGl Fc portion of the immunoglobulin can be recognized and bound by the Fcγ receptor (FcRγ) located on the surface of effector cells such as macrophages, monocytes and natural killer cells. This would trigger the activation of the effector cells and destruction of the opsonized target cells through the release of cytoplasmic granules that contain perform and granzymes from the effector cells.[17] The interaction of the Fc region with Fcγ receptor is mainly dependent on the three dimensional conformation of Fc domain. Altering the glycosylation therefore lead to conformational changes which results either enhance or reduce the binding affinity between Fc region and Fcγ receptor.[18] The presence of GlcNAc and removal of fucose can enhance ADCC by increasing the affinity of Fc region with Fcγ receptor, increasing level of terminal sialylation has opposite effect it reducing the ADCC.[19] Degree of galactosylation does not affect ADCC but it escalate interaction between Fc region and C1q, therefore plays an important role for activating CDC (Complement Dependent Cytotoxicity)[20] while expose galactose may increase the clearance of antibody.[21] Core fucosylation inhibiting binding to Fc_{γ}RIIIa which results in decreasing ADCC activity.[22] similarly increase in the mannose content increase the clearance of antibody, prone to elicit immunogenicity and enhances the FcRIIIa binding thus increases ADCC but substantially reduces C1q binding results in decease CDC activity.[23]

Charge Variants

Charge variants is also one of the main critical attribute because it plays an important role on recognition of binding sites and target receptors through affecting electrostatic and hydrophobic interactions with charged groups of cells and tissues.[24] Charge variants can alter the binding to protein or membrane targets thus affecting the tissue distribution, penetration and pharmacokinetics of monoclonal antibody.[25]

Charge variants are generally originates from the incomplete cyclization of N-terminal glutamic acid of the heavy chain or light chain to form pyroglutamic acid, as well as from the oxidation of methionine residues, deamidation or isomerization of asparagine and aspartic residues, incomplete removal of C-terminal lysines.[26] Acidic variants mainly forms due to oxidations, deamidations, and glycation within the antibody which affects its distribution and clearance, however acidic variants are prone to elicit immunogenicity and basic variants are mainly forms due to presence of Lys C terminal and aminations that could affect their tissue uptake and clearance. Modification of N-terminal and C-terminal are not expected to have effect on antibody stability, structure and functions because these regions are highly exposed and these regions are not part of any ligand binding sites. C-terminal Lys did not affect the thermal stability of IgG1 antibody.[27] Similarly oxidation of methionine residues in the Fc region did not affect antigen binding and potency of recombinant antibody.[28] The impact on the biological function is mainly dependent on the sites and the level of modifications.

Glycosylation and charge variants are the critical quality attributes that can impact the development of Biosimilar Anti-HER2 antibody. Glycosylation patterns generally changes with different expression system, culture condition, processes and scale up, these variations can have a profound effect on biological activities, so close control of glycosylation is required to ensure the higher similarity with innovator. Although batch to batch heterogenecity is present in antibody.[29] Several techniques are used to elucidate the glycan structures of mAbs. Therefore an appropriate characterization is essential to establish its limit. MALDI-TOF MS is often used as a first step because its capacity to generate rapidly information about the nature and diversity of glycans and recombinant glycoproteins.[30] As a limitation, MALDI-TOF MS analysis does not able to characterize between isobaric glycans.[31] Normal phase UHPLC coupled to fluorescent labelling can overcome this issue. Normal Phase UHPLC of fluorophore labeled glycans provides greater sensitivity. In UHPLC the hydrophilic interaction liquid chromatography (HILIC) mode is predominantly used to

assign the structure of glycan. Intact mAb Mass check and High mannose test standards were analyzed with samples under identical conditions and determined the presence of glycan residues in the samples. Similarly the percentage of charge variants were identified and compared with the different lots of HERCLON® standards under the same conditions. The goal of this work is to present a sensitive and reproducible analytical platform for comparing glycan profiling and charge variants of biosimilar Anti-HER2 antibody with different lots of originator product (HERCLON®).

MATERIALS AND METHODS

Reagents

Sodium di-hydrogen phosphate, disodium hydrogen phosphate and sodium chloride were purchased from Merck, Germany. HPLC grade formic acid and acetonitrile (ACN) was obtained from Rankem. All aqueous solutions were prepared using ultrapure (Milli-Q) water. Intact mAb Mass check standard, RapiFlour-MS High Mannose test standard and Dimethylformamide (DMF) were procured from Waters, USA. Biosimilar Anti-HER2 antibody was obtained from Serum Institute of India Pvt. Ltd, Pune. Three different lots of HERCLON® from F.Hoffmann, La Roche Ltd. Basel, Switzerland, were used for the comparability studies.

Preparation of samples

Anti-HER2 Antibody was purified from the harvested medium using different chromatographic steps. Anti-HER2 antibody and different lots of originator HERCLON® were obtained in solution form at 2mg/mL concentration in formulation buffer. The Intact mAb Mass check standard was reconstituted in Milli-Q water to a concentration of 2mg/mL and High Mannose test standard was dissolved sequentially in 9µL of Milli-Q water, 10µL of dimethylformamide (DMF) and 21µL of Acetonitrile (ACN) to achieve a sample load of 25 pmoles.

Glycan analysis

N-Glycan profile of Anti-HER2 molecule was performed using Rapi Flour-MS N-Glycan kit according to protocol (Waters, USA). Briefly, the Anti-HER2 antibody and three different lots of HERCLON® were solubilized and digested by GlycoWorks Rapid PNGase F (Waters, USA). After deglycosylation the glycan moiety containing protein mixture was treated with Rapi FlourMS, the unbound MS tag was removed and glycans were seperated using HILIC microelution plates. Total 10µL of standards and sample were injected and analyzed in UPLC

H-class Bio system in ACQUITY UPLC® Glycan BEH Amide column, (2.1 X 150mm, 130Å, 1.7µm; Waters; USA). A gradient of 50 mM ammonium formate pH 4.4 as mobile phase A and acetonitrile (ACN) as mobile phase B was used to elute all the glycan residues in the sample. Equilibration of column was carried out with 20% of mobile phase A and 80% of mobile phase B at 45°C, different glycan moieties were separated out of column with a 3 min linear decrease from 80% to 73% of mobile phase B, further a linear decrease in gradient from 73% to 63% of mobile phase B for 32 min at a flow rate of 0.5mL/min, then mobile phase A was raised to 100% in 1.5 min at 0.2mL/min flow rate, maintaining this condition for 3 min, then linear gradient was kept to 20% mobile phase A and 80% mobile phase B for 3.5 min at 0.2mL/min, then linear increase in flowrate was carried out from 0.2mL/min to 0.5 mL/min for 5 min followed by equilibration for 7 min. Elution of glycans was monitored with fluorescence detection with excitation at 265 nm and emission at 425 nm. However, Intact mAb Mass check standard (Waters, USA) and Rapi Flour-MS High Mannose test standard (Waters, USA) obtained in the kit were used under identical condition in order to identify various glycan residues present in the sample. The results were reported based on the relative percentage area of each glycan residues are expressed as the average of the percentage of total peak area ± standard deviations (RA±SD). CV% was calculated for each isoform for all the samples.

Charged species variants

Ultra-High performance analytical cation exchange chromatography was performed on ACQUITY UPLC H-class Bio system equipped with a strong cation exchange Protein –Pak™ Hi Res SP column (4.6 X 100mm, 7µm; Waters; USA) to detect charged species of Anti-HER2 antibody. Equilibration of column was carried out with 100% mobile phase A 10mM Phosphate buffer pH 6.8 at a flow rate of 0.5mL/min. Mobile phase B was 10mM Phosphate buffer pH 6.8 containing 200mM NaCl, the column temperature was set at 35°C and total 20µg of Anti-HER2 antibody was injected for analysis. Charge species variants of Anti-HER2 antibody were seperated out of column with 8 min linear gradient 0% to 10% of mobile phase B, then the %B was raised in linear gradient fashion from 10% to 22% in 29 min, maintaining 100% phase B for 3 min followed by 100% mobile phase A for 5 min. The chromatographic separation was monitored at 214 nm with UV detection and data analysis was performed using Empower® software (Waters, USA). At least three run replicates were obtained for three lots of HERCLON® and for Anti-HER2 antibody. CV% was calculated for

each variant for all the samples. Relative abundance of each variant in samples was expressed as the average of percentage of total peak area ± standard deviations (RA±SD).

RESULTS AND DISCUSSION

N-Linked Glycosylation Profile

N-linked glycans were released from Anti-HER2 antibody and different lots of originator with Rapid PNGase F and were fluorescently labelled with RapiFlour-MS, samples were clean-up prior to analyses using GlycoWorks HILIC µElution plate and subjected to UHPLC analysis. Separation by HILIC and detection of RapiFlour-MS labelled glycans by UHPLC allowed identifying almost 100% of the glycan composition in all the samples, including four major glycans (G0, G1Fa, G1Fb and G2F) and other minor glycan variants. The glycan identification was done by comparing the elution patterns of the analyzed originator HERCLON® Lot#101 with commercially available Intact mAb Mass check glycan standards as shown in Fig.1. Four major peaks were observed in the chromatogram, G0F, G1Fa, G1Fb and G2F, contributing to about 86% of the total glycan variants. The rest of the glycan variants represented less than 14% of the total glycosylation.

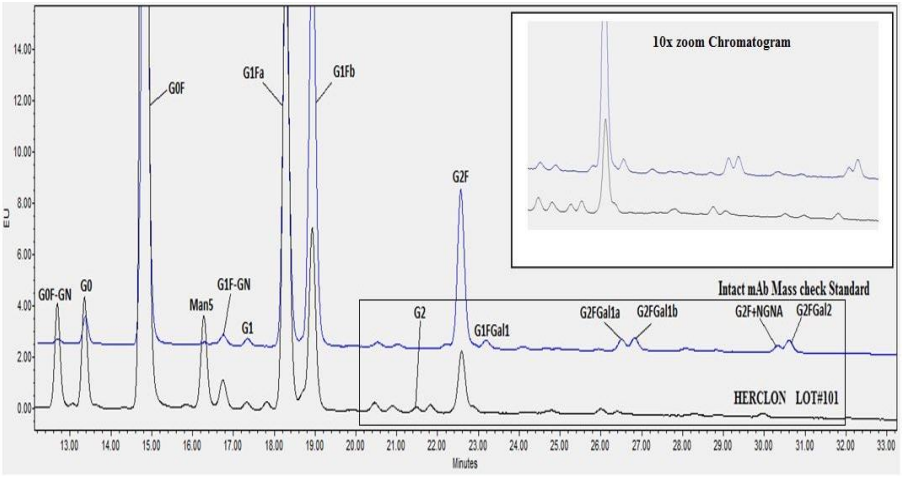

Fig. 1: Comparison of the glycan profile of the originator HERCLON® Lot#101 with respect to the Intact mAb Mass Check Standard by UHPLC method. Different types of glycans eluted from the samples were assigned with respect to the known glycan standards analyzed under same conditions with 10x zoom chromatogram to show low abundance N-glycans.

Abbreviations

UHPLC, ultra high performance liquid chromatography; G0F-GN, asialo-agalacto-fucosylated without one N-acetylglucosamine biantennary; G0, asialo-agalacto-biantennary;

G0F, asialo-agalacto-fucosylated biantennary; Man5, oligomannose 5; G1F-GN, asialo-monogalactosylated fucosylated without one N-acetylglucosamine biantennary; G1Fa/G1Fb, asialo-monogalactosylated fucosylated biantennary isoforms; G2, asialo-galactosylated biantennary; G2F, asialo-galactosylated fucosylated biantennary; G1FGal1, asialo-monogalactosylated fucosylated biantennary with one galactose attached to either end; G2FGal1a/G2FGal1b, asialo-galactosylated fucosylated biantennary isoforms with one galactose attached to either end; G2F+NGNA, asialo-galactosylated fucosylated biantennary with N-glycolylneuraminic acid; G2FGal2, asialo-galactosylated fucosylated biantennary with two galactose attached to end.

Glycans with an additional α-1,6 galactose i.e. G2FGal1a {G2F(1,6)+α Gal}, α-1,3 galactose G2FGal1b {G2F(1,3)+α-Gal},G2F+NGNA and G2FGal2 {G2F+$(\alpha\text{-Gal})_2$} were also observed in Intact mAb Mass check standard in less abundant form. The levels of High mannose glycans present in the antibody plays an important role in clearance from the blood which ultimately affects the half life thus alter the pharmacokinetic profile of an antibody. High mannose content exhibit fast clearance from the blood[23] results in reduces the half life of mAb, so very close control on mannosylation are required during development of biosimilar.

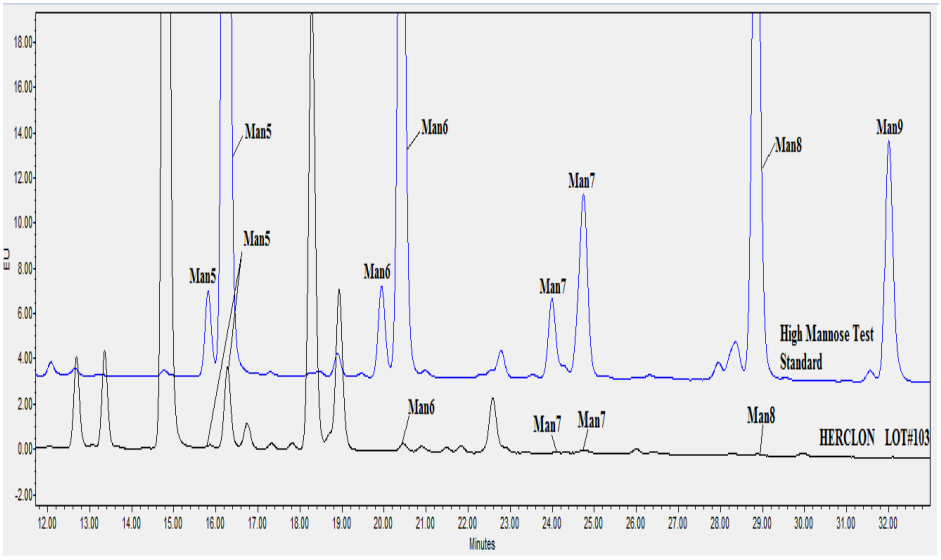

Fig. 2: Comparison of the N-glycan profile of the originator HERCLON® Lot#103 with respect to the High Mannose test standard by UHPLC method. Mannose assignments were done by comparison with High Mannose test standard analyzed under same conditions.

Abbreviations

Man5, oligomannose 5; Man6, oligomannose 6; Man7, oligomannose 7; Man8, oligomannose 8; Man9, oligomannose 9.

To ensure the proper control on mannosylation, identification and quantification of different oligomannose; High Mannose test standard was used to assigned the Mannose residues. Relative abundances of each mannose residues were calculated as shown in Table 1. and comparison was carried out between HERCLON® Lot#103 with respect to the High Mannose test standard as shown in Fig.2. Man5, Man6, Man7, Man8 and Man9 are the oligomannose residues obtained during analysis of High Mannose test standard and the relative peak intensities detected in HERCLON Lot#103 were low due to less abundance of oligomannose present in the originator as shown in Fig.3 with 10X magnification.

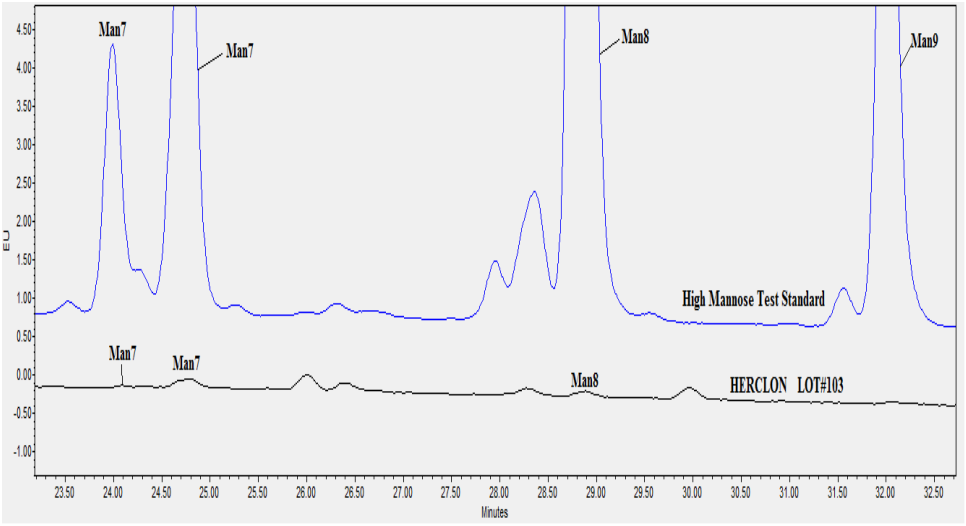

Fig. 3: Chromatogram scaled with a 10x zoom to show low abundance mannose in originator HERCLON® Lot#103 with respect to the High Mannose test standard.

N-glycan profile comparison was carried out between the biosimilar Anti-HER2 antibody and different lots of originator HERCLON®. The glycan patterns as shown in Fig.4 revealed that Anti-HER2 antibody and the different lots of originator comprised of the same principal glycoforms. Less abundant glycoforms such as G2FGal1a and G2F+NGNA were not present in HERCLON® lots and Anti-HER2 antibody as shown in Fig.5, while relative abundance of G2FGal1b residues in all samples was quite similar. G2FGal2 was not present in originator while sample contain very less abundance 0.05% glycan residue. Similarly small amount of 0.11% oligomannose (Man9) was present in sample whereas absent in HERCLON® lots, which probably will not expect to alter the PK profile.

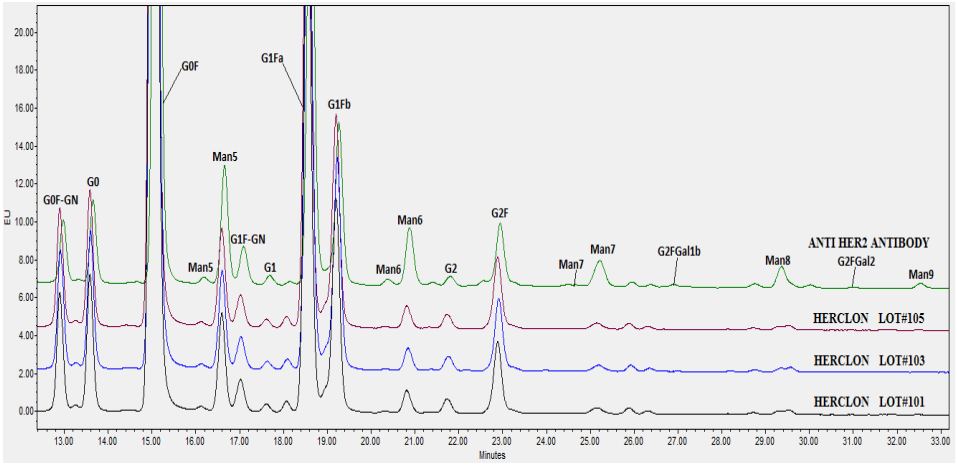

Fig. 4: Comparison of N-Glycan profile of the biosimilar Anti-HER2 antibody with respect to the three different lots of the originator HERCLON® Lot#101, HERCLON® Lot#103 and HERCLON® Lot#105 analyzed under identical conditions.

In general CV% values were calculated for each isoform and found to be lower than 10% for major glycan but for the less abundant glycans such as G2FGal1b, G2FGal2 CV% could reach between 10-11%. Quantification of glycan abundances was done and used to compare between the tested sample, whereas relative abundance (RA %) and RT of each glycan isoform were included in Table 1. The total mannosylation was 4.88% ±0.03 for Anti-HER2 antibody and 3.70% ±0.14, 4.08% ±0.09, 4.58% ±0.12 for HERCLON LOT#101, HERCLON LOT#103 and HERCLON LOT#105 respectively; present similarity in terms of relative abundances may expect comparable PK profile and similar immunogenicity response.

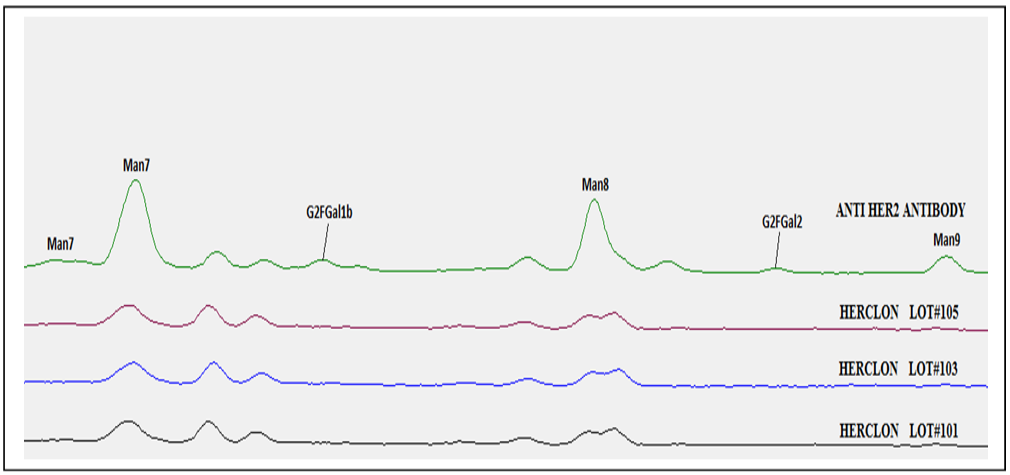

Fig. 5: Chromatogram scaled with a 10x zoom to show low abundance N-glycan variants in different lots of the originator HERCLON® with biosimilar Anti-HER2 antibody.

The mean relative abundance of total galactosylation for HERCLON LOT#101, LOT#103, and LOT #105 were 30.65%, 30.04% and 29.87%, for Anti-HER2 antibody was 34.51% respectively which was within pre-specified limits of 80-125% compared with originator. Galactosylation level plays an important role in CDC activity, CDC increases with increase in galactose content. Comparable galactosylation level may expect similar binding affinity and functional property of Anti-HER2 antibody. The degree of Afucosylation contributing their role in ADCC activity; mean relative afucosylated glycoforms was 8.80% for Anti-HER2 antibody and 7.45%, 8.07% and 8.56% for HERCLON LOT#101, LOT#103 and LOT#105 respectively which may exhibit comparable pharmacodynamics profile with similar $Fc_{\gamma}RIIIa$ binding affinity.

Table 1: The percentage relative abundance of different N-Glycan species variants obtained from biosimilar Anti-HER2 antibody and different lots of the originator HERCLON®.

GLYCAN OXFORD NOTATION	GLYCAN VARIANTS	RT (MIN)	HERCLON LOT#101		HERCLON LOT#103		HERCLON LOT#105		ANTI-HER2 ANTIBODY	
		(AVERAGE ± SD)	RA (%)	CV (%)	RA (%)	CV (%)	RA (%)	CV (%)	RA (%)	CV (%)
FA1	G0F-GN	12.95±0.05	2.99±0.03	1.16	2.85±0.08	2.81	2.94±0.12	4.12	2.26±0.03	1.17
A2	G0	13.57±0.10	3.32±0.05	1.57	3.42±0.09	2.64	3.50±0.15	4.32	3.02±0.06	1.82
FA2	G0F	14.99±0.19	59.59±0.17	0.29	60.91±0.48	0.78	59.31±0.33	0.55	56.44±0.16	0.29
M5	Man5	16.57±0.16	3.06±0.04	1.32	2.97±0.10	3.37	3.67±0.12	3.14	3.6±0.01	0.28
FA1G1	G1F-GN	16.93±0.29	1.05±0.03	3.30	1.01±0.04	3.74	1.00±0.03	2.53	1.76±0.02	0.98
A2G1	G1	17.66±0.13	0.24±0.01	4.75	0.25±0.02	6.93	0.24±0.02	8.33	0.30±0.02	5.15
FA2G1	G1Fa	18.57±0.21	17.00±0.01	0.07	16.57±0.19	1.15	16.52±0.39	2.38	18.78±0.07	0.37
FA2G1	G1Fb	19.12±0.14	6.66±0.00	0.09	6.61±0.08	1.17	6.53±0.08	1.24	7.73±0.01	0.15
M6	Man6	20.77±0.18	0.37±0.03	8.61	0.66±0.04	5.35	0.57±0.05	8.34	0.63±0.02	3.32
A2G2	G2	21.73±0.17	0.24±0.00	2.37	0.33±0.02	6.37	0.23±0.02	6.55	0.44±0.02	3.50
FA2G2	G2F	22.84±0.16	2.38±0.01	0.49	2.36±0.15	6.50	2.31±0.01	0.50	3.04±0.03	1.00
M7	Man7	24.99±0.46	0.14±0.01	8.06	0.33±0.02	6.37	0.21±0.02	7.16	0.49±0.01	2.04
FA2G2Ga1	G2FGal1b	26.89±0.08	0.09±0.01	11.11	0.06±0.01	10.19	0.10±0.01	10.00	0.15±0.02	10.41
M8	Man8	29.21±0.48	0.07±0.01	7.87	0.12±0.01	8.33	0.13±0.01	4.33	0.22±0.01	2.66
FA2G2Ga2	G2FGal2	30.77±0.51	0.00	-	0.00	-	0.00	-	0.05±0.01	10.83
M9	Man9	32.40±0.27	0.00	-	0.00	-	0.00	-	0.11±0.01	9.09
	Total Mannose		3.70±0.14	3.91	4.08±0.09	2.09	4.58±0.12	2.52	4.88±0.03	0.54

Notes: The percentage relative abundance of each glycan isoform was calculated as area % ± SD.CV% was calculated for each isoform for all four samples (calculated from RA%). The retention times were in minutes as an average of the RT. Samples were run in triplicates.

Abbreviations: RT, retention time; RA, Relative abundance; SD, standard deviation; CV, coefficient of variation.

This method allows distinguishing between different isobaric structures, such as G1Fa and G1Fb, with the same molecular weight but different oligosaccharide structural distribution which were fully resolved and appeared in the chromatogram at different RT (18.57±0.21 and 19.12±0.14min respectively), moreover this method can able to detect less abundant glycan isoforms comprising less than 1% each one. The observed relative abundances of each glycan species between samples demonstrated that glycan profile and relative abundance between samples were quite similar, thus considerably difference would not expected to affect the biosimilarity of an Anti-HER2 antibody.

Charge Variants Analysis

Considering pharmacokinetics, charge heterogenecity is one of the important quality attributes to be evaluated using appropriate analytical technique. The peaks eluted from CEX-UHPLC method were typically distinguish as three distinct variants. Early and late eluting peaks were designated as acidic and basic variants respectively; the most abundant peak was assign as the main peak (HC-K0). Charge variant analysis by CEX-UHPLC showed correspondence of acidic, main and basic variants between three different lots of HERCLON® and biosimilar Anti-HER2 can be observed in Fig.6. Deamidation of light chain asparagine 30 to aspartate in one or both light chain was contributing for two acidic forms, while lysine variants (HC-K1, HC-K2) were present in the C-terminal of the heavy chain represents the basic variants[32]. All the charge variants were well resolved in a defined run time.

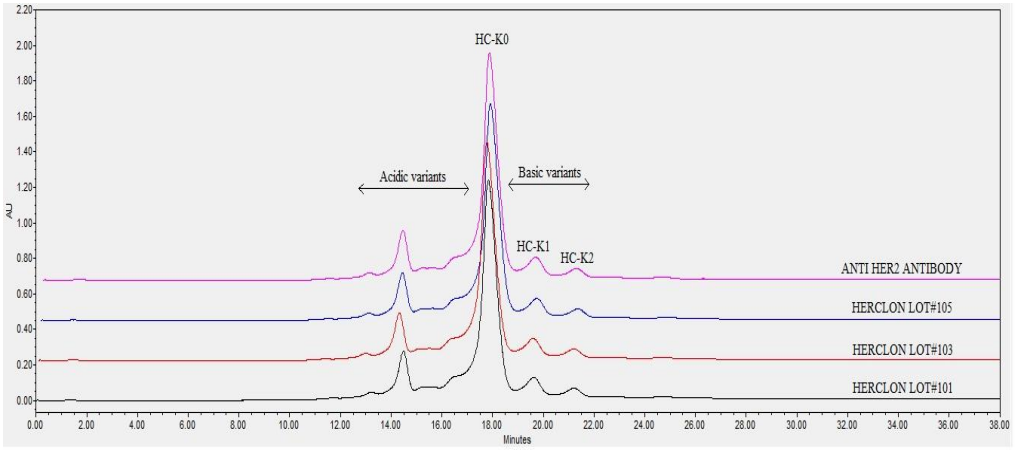

Fig. 6: Charge variant profile of the biosimilar Anti-HER2 antibody with respect to the different lots of originator HERCLON® by UHPLC.

Abbreviations

HC-K0, heavy chain with no terminal lysine; HC-K1, heavy chain with one terminal lysine; HC-K2, heavy chain with two terminal lysine.

Acidic variants affects the distribution and clearance of mAbs and basic isoforms could affect the tissue uptake and their clearance however, carboxypeptidases in human blood are able of remove C-terminal lysine from mAbs within two hours when administered intravenously[33]; therefore basic variants are not expected to have a profound impact on pharmacokinetic of mAb.

Table 2: Summary of the results of charge variants analysis by UHPLC method: comparison between biosimilar Anti-HER2 antibody and different lots of originator HERCLON®.

VARIANTS (%)	HERCLON LOT#101		HERCLON LOT#103		HERCLON LOT#105		ANTI-HER2 ANTIBODY	
	RA(%)	CV(%)	RA(%)	CV(%)	RA(%)	CV(%)	RA(%)	CV(%)
Acidic	22.95±0.14	0.62	21.76±0.45	2.06	21.22±0.51	2.40	21.63±0.07	0.32
Main	65.06±0.25	0.38	67.54±0.25	0.37	67.12±0.61	0.91	67.14±0.04	0.06
Basic	11.98±0.37	3.07	10.71±0.22	2.09	11.67±0.38	3.26	11.25±0.04	0.36

Notes: Samples were run in triplicates.

The results obtained from charge variants analysis as shown in Table 2, revealed that the relative abundances of the acidic, main and basic variants for Anti-HER2 antibody exhibit similar profile compared with the three different lots of originator. The relative abundance (RA%) of each charge variants were calculated as shown in Table 2, CV% was calculated for each isoform for all four samples (calculated from RA%). In general CV% values were lower than 10% and RA% was quite similar when compared between Anti-HER2 antibody and different lots of originator. Overall the results from charge variants analysis revealed that Anti-HER2 antibody and different lots of originator demonstrate comparable charge heterogeneities, thus no differences in functional activity should be expected.

CONCLUSION

Biosimilar development required a detailed characterization to obtain a strong knowledge of its CQAs. Glycosylation and charge variant profile are the two important critical quality attributes that could alter the pharmacokinetic profile and functional properties of mAbs. Therefore, it is very important to have analytical tools that can quantitate, monitor and used as routine method for characterization of glycosylation and charge variants patterns which

could diminishes the uncertainty in biological assays, allow the establishment of in-process control strategies and ensure the batch to batch consistency to obtain desired quality product. In this study, most and the less abundant glycans and distinct charge variants were identified and compared, which reveals the highly similarity between Anti-HER2 antibody and its originator product that may results in comparable pharmacological profile which would be confirm by upcoming nonclinical and clinical studies.

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