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Research paper

Glycosylation profiles of therapeutic antibody pharmaceuticals

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ABSTRACT

Recombinant antibodies specific for human targets are often used as therapeutics and represent a major class of drug products. Their therapeutic efficacy depends on the formation of antibody complexes resulting in the elimination of a target molecule or the modulation of specific signalling pathways. The physiological effects of antibody therapeutics are known to depend on the structural characteristics of the antibody molecule, specifically on the glycosylation which is the result of posttranslational modifications. Hence, production of therapeutic antibodies with a defined and consistent glycoform profile is needed which still remains a considerable challenge to the biopharmaceutical industry.

To provide an insight into the industries capability to control their manufacturing process and to provide antibodies of highest quality, we conducted a market surveillance study and compared major oligosaccharide profiles of a number of monoclonal antibody pharmaceuticals sampled on the Swiss market.

Product lot-to-lot variability was found to be generally low, suggesting that a majority of manufacturers have implemented high quality standards in their production processes. However, proportions of G0, G1 and G2 core-fucosylated chains derived from different products varied considerably and showed a bias towards the immature agalactosidated G0 form. Interestingly, differences in glycosylation caused by the production cell type seem to be of less importance compared with process related parameters such as cell growth.

1. Introduction

Immunoglobulins are serum proteins that play a central role in the humoral immune response by binding and inactivating antigens or triggering inflammatory or other cellular responses. The antibody core structure is composed of two identical light chains and heavy chains. They form a Y-shaped structure consisting of two identical antigen-binding domains (Fab) and an antibody crystallisable domain (Fc) with the heavy chain constant domains. All antibodies contain N-linked carbohydrate structures at conserved positions in the constant regions of the heavy chains. IgG, the most abundant in serum, has a single N-linked biantennary moiety at Asn297 following the consensus sequence N-X-S/T (X ≠ P), which is located in both heavy chains in the Fc region of the molecule [1]. In addition, IgG may possess Fab-associated carbohydrates with considerably varying structures [2].

Recombinant immunoglobulins are emerging as powerful pharmaceuticals for the therapy of a wide variety of diseases such as cancer or rheumatoid disorders. Several therapeutic and diagnostic monoclonal antibodies have been approved for market authorisation, and hundreds are in clinical trials or under development. Candidates in the production pipelines may be recruited by selecting or modifying epitope specificities or enhancing Fc effector functions [3]. All currently licensed antibody therapeutics are of the IgG class and have been manufactured using recombinant DNA technology in mammalian cell lines such as Chinese hamster ovary (CHO), mouse NS0 or SP2/0 myeloma cells. Fc glycosylation is essential to the structural integrity of the molecule [4,5], and alterations in glycosylation patterns due to differences in production conditions have been reported to affect susceptibility to proteolytic degradation, clearance rate *in vivo*, Fcγ receptor binding and activation, antibody-dependent cellular cytotoxicity (ADCC) and C1q component binding mediated complement activation [6–9]. Alterations in glycosylation could therefore compromise effector functions including bioactivity, clinical efficacy, pharmacokinetics, safety, stability and antigenicity [10,11]. Hence, glycosylation of recombinant IgG antibody molecules should optimally encompass naturally occurring oligosaccharides structures to avoid unexpected consequences. The challenge is to define expression systems that allow production of antibodies with defined structural and functional properties and Fc glycosylation patterns similar to native human IgG antibodies.

For the regulatory purpose [12,13], manufacturers are required to describe the degree of heterogeneity of their products in relation to the manufacturing process and should demonstrate comparability with clinical or preclinical batches, respectively. The structural characterisation of the molecule including the determination of the position and the nature of oligosaccharide subunits is of utmost

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importance for understanding the structure–function relationship and constitutes a precondition to establish product consistency. Whenever changes in glycosylation occur (e.g. after process changes) they may affect biological, immunochemical or physiochemical properties of the active ingredient and comparability should be confirmed.

We conducted a market surveillance study which provides an on-site comparison of major oligosaccharide glycosylation of therapeutic monoclonal antibody preparations currently licensed for the Swiss market. The glycosylation profiling data are evaluated regarding effects caused by the usage of different production cell lines and production conditions.

2. Materials and methods

2.1. Antibodies and mAb pharmaceutical preparations

Native IgG_1 fractions purified from human and mouse myeloma preparations were purchased from Calbiochem. Redimune® NF-Liquid, a polyvalent human normal immunoglobulin (IgG ⩾ 96%), was obtained from CSL Behring. Formulated drug products and purified cell culture supernatant bulk materials of 16 licensed antibody pharmaceuticals (A–P) were obtained from their respective manufacturers.

2.2. Enzymatic release and fluorescent derivatisation of N-linked oligosaccharides

Before cleavage, samples were desalted by centrifugation through a 30 kDa cut-off membrane (Microcon, Millipore), followed by one wash with peptide-N-glycosidase F (PNGase F) incubation buffer (20 mM sodium phosphate, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.02 (w/v) sodium azide, pH 7.5). Oligosaccharides were released from the protein backbone with PNGase F for 15 h at 37 °C. Proteins were precipitated by heating to 95 °C for 5 min. After centrifugation, oligosaccharide solutions were dried in a SpeedVac. Twenty micro litres labelling reagent (500 µl 15% HAc was added to 5 mg 8-aminopyrene-1,3,6-trisulfonic acid (APTS) labelling dye) and 5 µl 1 M sodium cyanoborohydride/tetrahydrofuran (THF) were added to the pellets and incubated at 55 °C for 2 h. To stop the reaction, 500 µl water was added to the samples.

2.3. Capillary electrophoresis of APTS-labelled oligosaccharides

Cleaved and labelled oligosaccharides have the same charge and were separated based on their size and/or structure using a ProteomeLab PA 800 system (Beckman Coulter) equipped with an argon-laser fluorescence detector (Excitation: 488 nm, Emission: 520 nm). Separations were performed using a eCap N-CHO coated capillary (50 µm i.d., 40 cm effective length, 50 cm total length) in Carbohydrate Separation Buffer (Beckman Coulter). Sample solutions were introduced to the capillary by pressure (0.5 psi for 6 s). Analysis was performed for 15 min at 30 kV. Peak areas were corrected for labelling efficiency using an internal standard (IS), and relative amounts of G0, G1 (1–6), G1 (1–3) and G2 glycans were calculated.

3. Results and discussion

3.1. Characterisation of the mAb glycoforms

Human native IgG is composed of predominately N-linked Fc complex biantennary type oligosaccharides with heterogeneity in core fucosylation, terminal sialylation and galactosylation. This

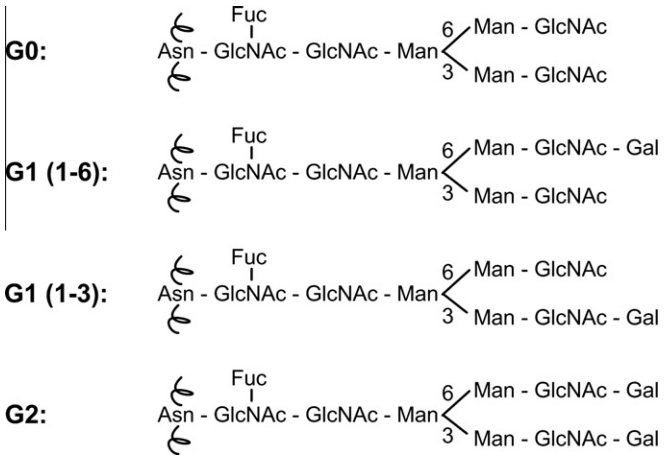

Fig. 1. List of the major N-linked oligosaccharides in IgG antibodies.

neutral (asialo) oligosaccharide structure is characterised by a fucose moiety attached to the innermost N-acetylglucosamine (GlcNAc) residue which in turn is covalently bound to the immunoglobulin asparagine (Asn) residue (Fig. 1). The G0 form lacks both galactose (Gal) residues at the ends of the biantennary chains. G1 (1–6) and G1 (1–3) are biantennary positional isomers carrying one Gal residue attached to the mannose GlcNAc branch. In G2, both branches carry a Gal residue. Recombinant IgGs show

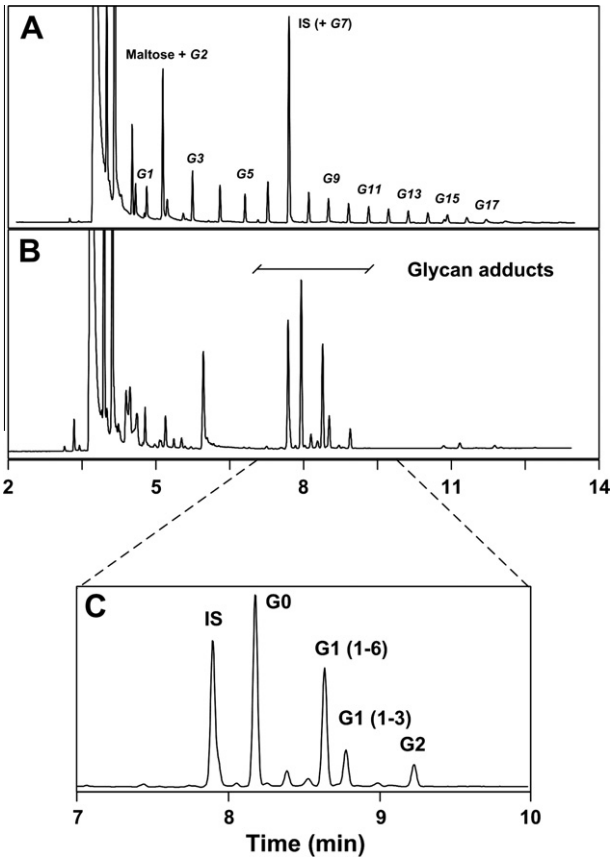

Fig. 2. Representative electropherograms of (A) APTS-labelled glucose ladder standard (*G1–G17*) spiked with maltose and maltoheptaose (IS). (B and C) PNGase F released N-linked glycan adducts of human IgG spiked with maltose and maltoheptaose (IS) and separated by capillary electrophoresis. Identification of relevant IgG oligosaccharides is described in Section 3.

similar glycosylation profiles but, depending on the product and manufacturing process used, can exhibit some degree of heterogeneity [4,7]. To assess such differences, N-linked oligosaccharides were released enzymatically from IgGs using PNGase F, labelled with APTS and detected and quantified by laser-induced fluorescence capillary electrophoreses (LIF-CE) [14]. The method applied was based on a method used by one of the manufacturers of a monoclonal antibody preparation. It was validated according to ICH-guidelines and showed acceptable selectivity, accuracy, linearity, robustness and precision (intermediate precision $\leqslant$3.5% relative standard deviation (RSD)). With the aim to directly compare between recombinant antibody pharmaceutical products, minor peaks were disregarded and quantification of overall glycosylation was assessed solely on the distribution of the major G0, G1 (1–6)/G1 (1–3) and G2 N-glycan structures. Fig. 2 shows representative electropherograms of PNGase F digested APTS labelled human IgG_1 glycans (Fig. 2B and C) and a glucose ladder (Fig. 2A). Released N-glycans were identified by peak alignments to the glucose ladder spiked with maltose and maltoheptoase (used as internal standard). Ladder numbers (*G1*, *G2*, ..., *G17*) illustrate the degree of polymerisation of the glucose ladder size marker. The internal standard was used to correct differences in recovery such as labelling efficiency.

3.2. Oligosaccharides distribution in native human and mouse IgG

To establish a standard which represents a physiological oligosaccharide distribution, three different IgG_1 sources were compared. We tested IgG_1 derived from human and mouse myeloma in parallel with fractionated human native IgG (Redimune®). Redimune® was prepared from a pool of thousands of blood donors and contained at least 96% IgG.

Oligosaccharide analysis of three Redimune® lots (Table 1, lot A–C) revealed rather equally distributed glycan-profiles showing negligible lot-to-lot variations (SD). Twenty-eight percentage of the N-glycans was found to be agalactosidated (G0), 29.5% carry one Gal residue at position (1–6), 20.4% at position (1–3) and 22.1% showed full biantennary glycosylation (G2). Human myeloma derived IgG_1 showed a similar but not identical glycan distribution. Non-galactosidated G0 and G1 (1–6) levels were increased (34.1% and 39.1%), G1 (1–3) and G2 levels (16.6% and 10.2%) decreased compared with levels found in Redimune®, suggesting that glycosylation in a pathological model system may not be considered as a reference system. Differences were even more pronounced in the mouse myeloma derived antibodies. Highly elevated levels of immature G0 forms (79.9%) in conjunction with a significant reduction of G1 and G2 forms may reflect the pathological disease state of the cells and, in addition, species specific differences [15] between mouse and human cells. As a conclusion, Redimune® was chosen as a physiological reference system of human origin representing fully matured oligosaccharide populations.

3.3. Analysis of N-linked oligosaccharides in therapeutic antibody pharmaceuticals

Monoclonals are being developed by many biopharmaceutical companies and production conditions vary considerably between manufacturers. This includes construction of the IgG molecule, the choice of the production cell line or cell culture conditions. To assess comparability among different therapeutic antibody preparations, we initiated a market surveillance study and analysed oligosaccharide distribution (glycoform profiles) of 16 commercially available products currently approved in Switzerland.

Table 1
Oligosaccharide distribution (%) in native human and mouse IgG samples.

Source	Assays	G0 (SD)	G1 (1–6) (SD)	G1 (1–3) (SD)	G2 (SD)
Mouse myeloma (1 lot)	5	79.9 (1.8)	9.7 (1.4)	10.1 (0.3)	0.3 (0.6)
Human myeloma (1 lot)	9	34.1 (0.6)	39.1 (1.1)	16.6 (0.7)	10.2 (0.4)
Fractionated human plasma pools (Redimune), lot A	5	28.2 (0.3)	30.6 (0.2)	19.4 (0.3)	21.8 (0.2)
Fractionated human plasma pools (Redimune), lot B	6	26.9 (1.1)	29.2 (1.3)	21.6 (3.8)	22.3 (1.6)
Fractionated human plasma pools (Redimune), lot C	7	28.9 (0.7)	28.7 (0.6)	20.1 (0.9)	22.3 (0.2)
Fractionated human plasma pools (Redimune), Mean	18	28.0 (1.0)	29.5 (1.0)	20.4 (1.1)	22.1 (0.3)

SD, standard deviation.

Table 2
Analysis of N-linked oligosaccharides from various therapeutic antibodies (anonymised).

Therapeutic antibody	Description	Production cell type	Lots	G0 ± SD (%)	G1 (1–6) ± SD (%)	G1 (1–3) ± SD (%)	G2 ± SD (%)
A	Human	Chinese hamster ovary (CHO)	2	28.8/–	31.8/–	9.9/–	29.5/–
B	Human	Chinese hamster ovary (CHO)	4	50.5/2.5	27.2/1.0	9.9/0.5	12.4/1.4
C	Human	Chinese hamster ovary (CHO)	3	81.6/2.9	13.4/1.9	4.0/0.6	1.0/0.4
D	Mouse/human	Chinese hamster ovary (CHO)	3	56.5/3.8	28.7/2.3	9.0/0.9	5.8/1.0
E	Mouse/human	Chinese hamster ovary (CHO)	3	55.0/8.1	30.3/5.1	9.4/1.5	5.3/1.5
F	Mouse/human	Mouse myeloma Sp2/0	4	98.5/0.4	1.5/0.3	0/–	0/–
G	Mouse/human	Mouse myeloma (NSO)	3	46.7/1.1	32.5/0.6	10.8/0.3	10.0/0.4
H	Mouse/human	Mouse myeloma Sp2/0	3	59.5/0.3	26.3/0.2	9.4/0.2	4.8/0.3
I	Mouse/human	Chinese hamster ovary (CHO)	5	83.9/5.3	11.5/3.6	3.7/1.2	0.9/0.5
J	Mouse/human	Mouse myeloma (NSO)	3	51.8/3.0	29.7/1.7	9.0/0.4	9.5/1.0
K	Mouse/human	Mouse myeloma Sp2/0	3	42.2/4.5	35.5/2.4	11.7/0.6	10.6/1.8
L	Mouse/human	Mouse myeloma (NSO)	3	71.6/1.4	13.4/0.8	10.4/0.7	4.6/0.1
M	Mouse/human	Chinese hamster ovary (CHO)	4	50.7/15.1	32.2/8.9	9.8/2.5	7.3/3.8
N	Mouse/human	Chinese hamster ovary (CHO)	4	69.0/2.4	21.7/1.6	7.0/0.5	2.3/0.3
O	Various[a]	Chinese hamster ovary (CHO)	3	51.8/1.4	31.7/1.0	10.3/0.3	6.2/0.1
P	Various[a]	Chinese hamster ovary (CHO)	3	78.0/1.6	10.7/1.0	9.7/0.6	1.6/0.2
Redimune[b]	Human IgG	–	3	28.0/1.0	29.5/1.0	20.4/1.1	22.1/0.3

SD, standard deviation.
[a] Groups consisted in IgG_4 subclass and of various origin, respectively.
[b] Polyclonal human IgG was used as standard.

For confidentiality reasons, all preparations were anonymised and presented as letters (A–P). Table 2 provides an overview of N-glycans derived from various monoclonal antibody preparations including 3 fully human IgG_1, 11 mouse–human chimeric or humanized IgG_1, 1 IgG_4, and 1 mAb preparation derived from alternate animal species. Human polyclonal IgG (Redimune®) was used as a reference.

Currently only three major cell types are in use for the production of monoclonal antibodies. From the products selected for analysis, a majority of 10 were produced in CHO cells, three in mouse myeloma Sp2/0 and three in NS0 cells.

Depending on availability, 2–5 lots of final product and final bulk material were analysed. To achieve reproducible assay conditions, equal amounts of sample material underwent buffer-exchange before digestion with PNGase F, labelling and LIF-CE-detection [14]. For each lot, at least four repeating measurements were performed. Most recombinant IgG exhibited a rather low standard deviation (Table 2), indicating a considerably high level of product consistency. Interestingly, Redimune®, which consists of a pool of native IgG, showed the lowest lot-to-lot variability. For products E, I and M, significant standard deviations were observed which could not be explained by the variability of the analytical method but could be due to product process variations.

In contrast to Redimune®, which showed a equally distributed glycan profile, G0 levels were significantly elevated in a majority of therapeutic antibodies preparations (Table 2 and Fig. 3A and B). Except for product A, the non-galactosidated G0 form was elevated by a factor of 1.5 up to 3.5 compared to Redimune®. Further, G1 (1–3) and G2 but not G1 (1–6) overall glycan levels decreased by a factor of 2.4 (G1 (1–3)) and 3.2 (G2). G1 (1–6) levels remained with a factor of 1.2 on comparable levels. The predominance of immature G0 forms and the presence of a negative bias towards bisecting G1 (1–3) and biantennary (G2) forms (Figs. 3 and 4) suggest either a cell type or manufacturing process dependent

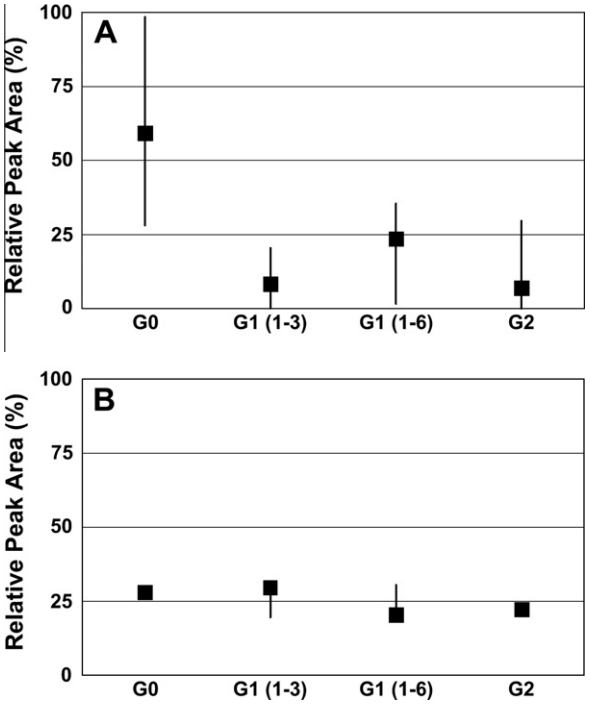

Fig. 3. Oligosaccharide distribution of therapeutic antibody products compared with human native IgG (Redimune®). (A) For therapeutic antibody products A–P. (B) For human native polyclonal IgG fraction (Redimune®). Squares represent mean values; error lines indicate minimum and maximum values.

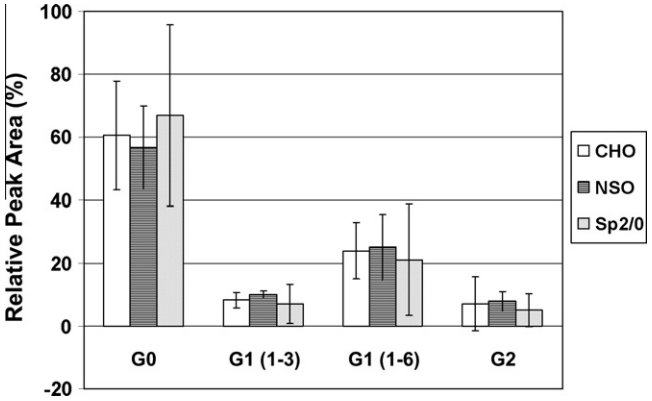

Fig. 4. Cell type specific oligosaccharide distribution of therapeutic antibody products. Bars represent mean values generated from products made in either CHO ($n = 10$), NS0 ($n = 3$) or Sp2/0 ($n = 3$), respectively. Error lines indicate standard deviations.

decrease in glycosyltransferase expression levels or altered specific enzyme activities [15–17].

To uncover whether differences in G0, G1 (1–6)/G1 (1–3) and G2 levels observed in the entire therapeutic antibody population is influenced by the cell type, oligosaccharide profiles of 10 monoclonal antibodies produced in CHO, 3 in NS0 and Sp2/0 cells, respectively, were grouped and compared (Fig. 4). Although expression of glycosyltransferases and, hence, protein glycosylation is known to be cell type specific [15], and glycosylation may be dependent on the culture conditions chosen [18], we found rather evenly distributed glycan levels among all three cell lines. Considering relatively large standard deviations within each group, mean values were comparable, resulting in a similar picture that was observed with the whole antibody product population (Fig. 3A). Taken into account that, due to a limited availability, our data are based on a minimum of lot numbers, we conclude that the impact of the cell line is not the utmost important factor which directs product-to-product heterogeneity and that differences in glycosylation patterns between CHO, NS0 and Sp2/0 cell lines are negligible.

To explain the unevenly distributed glycan levels, we took advantage of the product specific marketing authorisation application dossiers. The screening of the quality documentations indicated a link between fermentation time and accumulation of G1 and G2 forms. At the beginning of the fermentation process when cells expand and protein expression is ongoing, agalacto (G0) oligosaccharide structures dominate. After a certain time, cells move into stationary-phase, cell division decreases and glycosylation leads to more mono- (G1) and digalacto (G2) forms. For instance, the fermentation process of product F (98.5% G0) was designed to keep the cells in a constant growth by applying a continuous feed and harvest mode. In contrast, product A (28.8% G0) was produced by applying a prolonged fermentation time, thus enabling the production of a rather uniform and physiological glycosylated antibody.

In the past few years, a number of cases have been reported where safety or efficacy was affected. For example, galactose-α-1,3-galactose, which is not found on human native IgG [19] but on the Fab portion of cetuximab [20] has been implicated in hypersensitivity reactions and anaphylactic shock [21]. In addition, cell line derived monoclonal antibodies may bear glycolylneuraminic acid (Neu5Gc) normally not found on human IgG [22]. The formation of antibodies in patients against Neu5Gc have been implicated in accelerated clearance of the drug [23]. Moreover, recombinant antibodies may contain high levels of high mannose glycans generally not found in human IgG [24] causing increased serum clearance in patients [25]. Finally, non-fucosylated

glycoforms have been clearly shown to increase binding to Fcγ RIIIa receptors [17,26]. Clearly, a screening strategy for the detection of undesirable oligosaccharide structures would be helpful for the prevention of future adverse events.

4. Conclusion

As glycosylation patterns of IgGs have been shown to both cause and reflect specific physiological and pathological conditions [7,10,11], approval to market requires the demonstration of reproducibility of its safety and efficacy as well as of manufacturing consistency. Assessment of reproducibility of the glycosylation status is of paramount importance to ensure maintenance not only of potency, but also of its pharmacokinetic properties. Hence, regulatory agencies have increasingly requested to determine the oligosaccharide distributions in quantitative terms [12,13]. The characterisation of IgG glycosylation is generally performed at the stage of the development of the manufacturing process. However, in spite of the pivotal role of IgG glycosylation in many physiological and pathological processes, glycosylation monitoring is often neglected when it comes to setting the release specifications for both active ingredients and final products, respectively.

In this report, glycosylation analysis of various therapeutic antibody pharmaceuticals by LIF-CE indicates that N-linked carbohydrate structures were predominantly of core-fucosylated asialo biantennary types with varying degrees of galactosylation. Minor lot-to-lot variations within a given product demonstrate that most manufacturing processes are tightly controlled and ensure a high degree of product consistency. However, differences of glycosylation profiles amongst various manufacturers are considerable and a clear bias towards immature agalactosidated and unequally distributed G1-forms compared with native IgG were observed. Taken into account that alterations in glycosylation may affect product safety and efficacy, care should be taken when it comes to the design and the implementation of the manufacturing process.

As biopharmaceutical companies constantly improve antibody-based therapeutics, it is anticipated that a combination of optimising cell culture conditions and glycosylation engineering may lead to antibodies with defined functional and structural properties.

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