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Short Communication

Quantitative assessment of mAb Fc glycosylation of CQA importance by capillary electrophoresis

The attached carbohydrates at the highly conserved asparagine-linked glycosylation site in the C_H2 domain of the fragment crystallizable (Fc) region of monoclonal antibody therapeutics can play an essential role in their mechanism of action, including ADCC, CDC, anti-inflammatory functions, and serum half-life. Thus, this particular glycosylation represents one of the important critical quality attributes (CQA) of therapeutic monoclonal antibodies, which should be closely monitored and controlled during all stages of biopharmaceutical manufacturing. To study Fc glycosylation related quantitative critical quality attributes, the N-glycan pool of adalimumab (Humira®) was spiked with increasing amounts of mannose-5 oligosaccharide, a glycan with high CQA importance. The method enabled precise quantitative CQA assessment with high detection sensitivity.

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Biotherapeutics / Capillary electrophoresis / Critical quality attributes / Glycosylation / Quantification
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The biological activity of therapeutic monoclonal antibodies is highly influenced by their protein structure and the associated N-linked carbohydrates at the conserved asparagine linked glycosylation site (Asn 297, in most instances) in the C_H2 domain of the fragment crystallizable (Fc) region [1, 2]. Efficacy, stability, half-life and immunogenicity are all dependent on this particular N-glycosylation of therapeutic antibodies [3]. Albeit, this post-translational modification (PTM) apparently does not influence target recognition, it reportedly affects the effector functions of this class of therapeutic proteins [4]. Therefore, if effector function is the main mode of action of a monoclonal antibody therapeutic, microheterogeneity of this Fc glycosylation such as core fucosylation (for antibody-dependent cell-mediated cytotoxicity, ADCC), galactosylation (for complement-dependent cytotoxicity, CDC) and sialylation (for anti-inflammatory function) [5] should be closely monitored in each phase of product development [6]. On the other hand, albeit not important for the above listed functions, the presence of high mannose type glycans should be scrutinized as it greatly affects serum half-life via interaction with mannose-binding lectins (MBL) in the liver [7].

Adalimumab (Humira®) is a recombinant human IgG1 type monoclonal anti-tumor necrosis factor-alpha (TNF-α) antibody, originally developed to treat rheumatoid arthritis (RA) [8, 9]. It was approved by the FDA in 2002 as the first fully human mAb therapeutics on the market. In addition to its high efficacy in RA by binding TNF-α, adalimumab found to be effective in other autoimmune diseases such as Crohn's disease and Wegener's granulomatosis [10] via enhancement of complement system activation (CDC) that is greatly dependent on the galactosylation level of the Fc glycosylation [11]. The serum half-life of the product is influenced by the presence of high mannose type sugars at that PTM site [12]. Therefore, accurate and quantitative carbohydrate microheterogeneity analysis of this conserved N-glycosylation site of adalimumab is necessary to gain important information about the product, i.e., whether it meets the glycosylation aspects of critical quality attributes (CQA).

Due to its exceptional resolving power and high separation efficiency, CE is one of the most powerful bioanalytical techniques that can be readily utilized for the characterization of therapeutic proteins, including their bound carbohydrates [13]. To accomplish these tasks, CE can be applied in various separation modes including zone electrophoresis

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; **APTS**, 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt; **CDC**, complement-dependent cytotoxicity; **CGE-LIF**, capillary gel electrophoresis with laser induced fluorescence detection; **CQA**, critical quality attributes; **DP**, degree of polymerization; **Fc**, fragment crystallizable; **PNGase F**, Peptide N-Glycosidase F; **RA**, rheumatoid arthritis

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(CZE), gel electrophoresis (CGE), isoelectric focusing (cIEF) and isotachophoresis (cITP), all offering great opportunities for mAb characterization at various levels and most of them have already been implemented in the biopharmaceutical industry [14]. Capillary gel electrophoresis with laser induced fluorescent detection (CGE-LIF) is a high sensitivity glycoanalytical technique to analyze fluorophore (e.g., aminopyrenetrisulfonate - APTS) labeled oligosaccharides [15]. In addition, CGE-LIF with the recently introduced triple internal standard based automated GU calculation method [16] is capable of instant structural assignment for the separated glycans. In this paper we applied capillary gel electrophoresis with laser induced fluorescent detection to precisely measure the presence and concentration of the mannose-5 structure over a wide concentration range in the adalimumab (Humira®) glycan pool with the goal to establish a method that fulfills the quantitative CQA analysis requirement.

Chemicals: The high mannose type oligosaccharide standard (Man5), sodium cyanoborohydride (1M in THF), acetonitrile and all other chemicals were from Sigma-Aldrich (St Louis, MO). The PNGase F enzyme was from Asparia Glycomics (San Sebastián, Spain). Adalimumab (Humira®) was a kind gift from the Medical School at University of Debrecen.

Sample Preparation: The Fast Glycan Sample Preparation and Analysis (SCIEX, Brea, CA) kit was used for sample preparation and fluorophore labeling. Briefly, after denaturing 100 μg of adalimumab, the asparagine linked glycans were removed by PNGase F digestion. Then, the released glycans were captured on magnetic beads and labeled using 8-aminopyrene-1,3,6-trisulfonate (APTS) followed by another magnetic bead-based purification step [17] prior to CGE-LIF analysis.

Instrumentation: The PA 800 Plus Pharmaceutical Analysis System (SCIEX) was equipped with a solid state laser based fluorescent detector (λ_{ex} = 488 nm/λ_{em} = 520 nm) and used for all capillary gel electrophoresis analyses. 20 and 50 cm effective length (30 and 60 cm total length; 50 μm I.D.) bare fused silica capillary columns, filled with the HR-NCHO gel-buffer system, were employed for all separations with the application of 30 kV electric potential at 30°C separation temperature. A three stage injection sequence was applied as follows: (i) pre-injection of water for 3.0 s at 5.0 psi, (ii) 2.0 kV/2.0 s sample injection and (iii) 2.0 kV/2.0 s bracketing standard (DP2 + DP15). The 32 Karat, version 10.1 software package (SCIEX) was employed for data acquisition and analysis.

Figure 1 depicts the CGE-LIF separation of the APTS labeled N-linked oligosaccharides of adalimumab. The identified N-linked glycans, depicted by the numbers above the peaks, are listed in Table 1 along with their structural interpretations, GU values and Area% distributions. One of the major glycoforms of adalimumab was the core fucosyated biantennary oligosaccharide with no terminal galactosylation (FA2, Peak 3 in Fig. 1) representing 72% of all the glycans, followed by its monogalactosylated forms at the 6 (12%) and 3 (4.5%) arms (Peaks 7 and 8, respectively). High mannose structure was represented by the mannose-5 peak

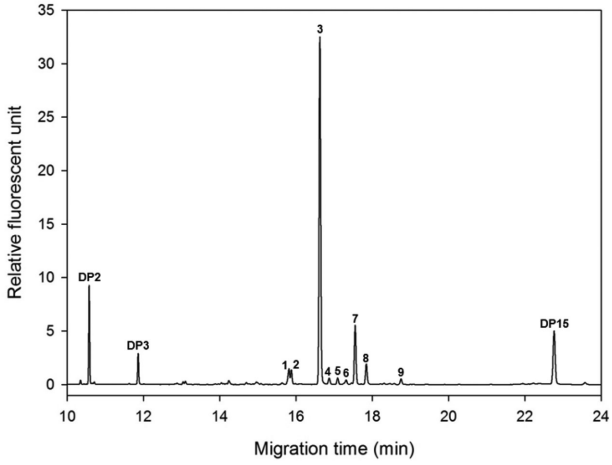


Figure 1. CGE-LIF analysis of APTS labeled adalimumab (Humira®) N-glycans. Peak numbers correspond to the structures listed in Table 1. Conditions: 50 cm effective (60 cm total) length, 50 μm ID bare fused silica capillary with HR-NCHO separation matrix; Separation temperature: 30°C; Separation voltage: 30 kV; Injection: pre-injection of water for 3.0 s at 5.0 psi, followed by 2.0 kV/2.0 s sample injection and 2.0 kV/2.0 s bracketing standard (DP2 and DP15).

Table 1. Identified N-glycan structures of adalimumab (Humira®) with the corresponding GU values (measured at 30°C) and Area%

Peak	Structure	ID	GU value	%Area
1		A2	6.76	3.61
2		Man5	6.82	2.47
3		FA2	7.67	72.24
4		A3	7.94	1.33
5		FA1(6)G1	8.21	1.25
6		FA3	8.47	0.99
7		FA2(6)G1	8.74	12.54
8		FA2(3)G1	9.08	4.45
9		FA2G2	10.11	1.12

(Man5, Peak 2) with 2.47%. Since the mechanism of action of adalimumab in rheumatoid arthritis (RA) is TNF-α binding and not based on CDC or ADCC functions [18], for this particular method development it was not necessary to monitor its galactosyation or core fucosylation levels. However,

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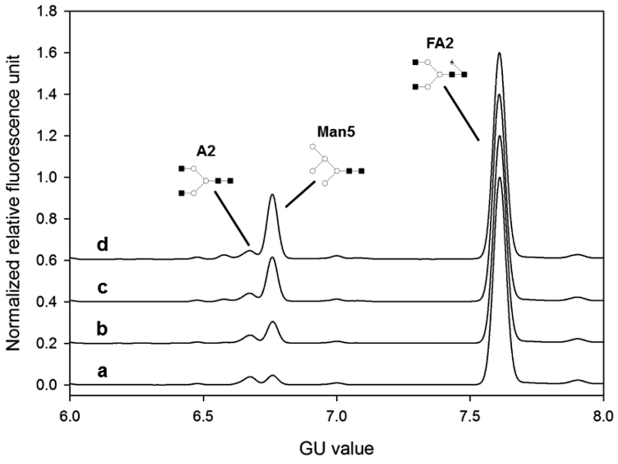


Figure 2. Relevant sections of the CGE-LIF analysis traces of interest of the APTS labeled adalimumab (Humira®) N-glycans spiked with increasing amounts of mannose-5 oligosaccharide (Man5). Traces a, b, c and d show the results with zero, 5.0 ng, 10 ng and 15 ng of Man5 added to the released adalimumab N-glycan pool prior to APTS labeling, respectively. Separation conditions were the same as in Fig. 1.

quantitative analysis of the mannose-5 structure (Man5) was of high importance due to its role in clearance.

To address the quantitative aspects of N-glycan analysis by CGE-LIF and the respective CQA, the PNGase F released adalimumab N-glycan pool was spiked with increasing amounts of mannose-5 (Man5) standard before the APTS labeling reaction step. Please note that the presence of high mannose type glycans on the Fc domain of monoclonal antibody therapeutics highly affect their serum half-time, thus, represents one of the important CQAs. The spiked traces of adalimumab glycans were aligned using the three internal standards of DP2, DP3 and DP15 and the comparative profiles are shown as the function of glucose unit (GU) values in Fig. 2. Using this approach, the %RSD of GU values at the X axis was below 0.05%, i.e., featuring very high precision. In order to obtain adequate resolution between the mannose-5 (Man5, Peak 2 in Fig. 1) and the complex A2 (Peak 1 in Fig. 1) glycans, a 50 cm effective length (60 cm total) was used. The RFU values were normalized to the highest peak in the electropherograms, which was the FA2 (Peak 3 in Fig. 1). These normalization steps on both axes resulted in easy run-to-run comparability that enabled accurate peak area calculation from the electropherograms, supporting quantitative CQA assessment.

To establish accurate and reproducible correlation between the Man5 concentrations and the corresponding peak areas, the electrokinetic injection parameters as well as the effect of sample concentration and volume were all examined. First, the injection voltages and injection times were varied between 1.0 to 3.0 kV and 1.0 to 3.0 s, respectively, from 50 and 100 μL (0.5 ng/μL concentration) sample volumes. A combined injection parameter was established by multiplying the injection voltage and time, referred to as injection coefficient, which was used during the concentration calculation

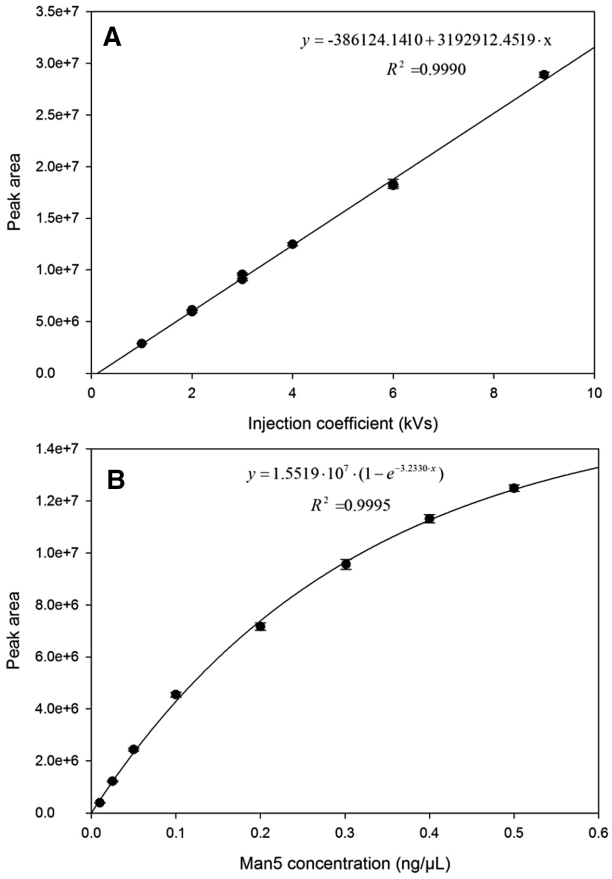


Figure 3. Correlation between the injection coefficient (i.e., combined injection parameters) and measured Man5 peak areas (A) as well as the peak areas of Man5 as a function of its concentration (B). Separation parameters were the same as in Fig. 1, except 30 cm total length (20 cm effective length) BFS capillary was used.

study. The peak areas obtained by using the various injection parameters and different sample volumes are shown in Supporting Information Table 1. Excellent correlation was found between the injection coefficient and the resulting peak areas using the different injection parameters as delineated in Fig. 3A.

The effect of sample concentration on the resulting Man5 peak area was also investigated. The mannose-5 stock solution (0.5 ng/μL) was serially diluted up to 50× with HPLC grade water and analyzed using 2.0 kV and 2.0 s sample injection parameters. Please note that while this range of concentration allowed to obtain good signal intensity at lower concentrations it did not saturate the peaks at the higher concentrations. The obtained RFU and peak area values at the different dilution levels are listed in Supporting Information Table 2. An exponential function was used to establish correlation between the sample amounts and peak areas in the concentration range used as shown in Fig. 3B.

Based on the results obtained by applying different injection parameters, various Man5 concentrations and altered sample volumes, the concentration of the Man5 in the

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adalimumab sample was precisely calculated. The CQA interest of mannose-5 content in the original adalimumab sample was determined as 2.94 ng.

CGE-LIF analysis of the Fc N-glycans of CQA importance in the monoclonal antibody therapeutic of adalimumab provided adequate qualitative and quantitative carbohydrate identification with special focus on the extent of mannose-5 structure (Man5) present. This N-glycosylation related quantitative CQA was addressed by spiking the Fc glycan pool of adalimumab with Man5 oligosaccharide at different concentrations. Accurate quantitation was possible with the use of electrokinetic injection allowing high precision prediction between the relationship of peak areas and the concentrations of the N-glycan of interest in the sample. By demonstrating that the actual concentration of the mannose-5 carbohydrate of CQA interest can be accurately measured in a wide concentration range, the method reported in this paper can facilitate the required quantitative assessment for other carbohydrates of interest.

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The authors have declared no conflict of interest.

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