

# Comparative characterization of the Fc domain N-glycosylation in monoclonal antibody and fusion protein therapeutics by CGE-LIF and UPLC-FL

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## Introduction

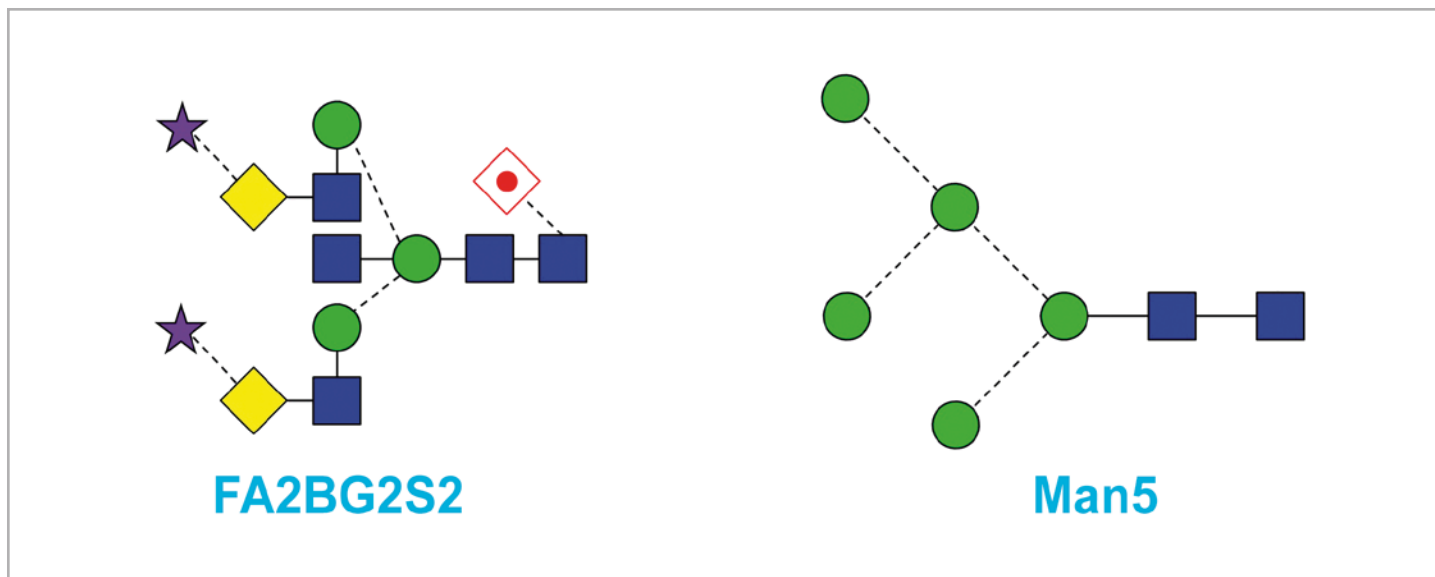
Comprehensive characterization of the N-linked carbohydrates of therapeutic antibodies and Fc-fusion proteins provides essential information about this critically important quality attribute.<sup>1</sup> Considering the fact that in mid-2018, the top ten pharmaceutical products were protein therapeutics (<https://public.tableau.com/views/Top10drugsby2018sales/Dashboard1?:showVizHome=no&:embed=true>), a full analysis of their N-glycosylation represents a growing area of interest.

Monoclonal antibodies and Fc-fusion proteins possess one conserved N-glycosylation site at Asn 297 of the CH2 region of the Fc (fragment crystallizable) domain of the molecule. This particular glycosylation influences some of the important biological activities of the molecule such as effector function, anti-inflammatory properties and serum half-life.<sup>2</sup> Sugar compositional variability depends on the type of the host cell line and the expression conditions used. Figure 1 shows a typical complex core fucosylated, sialylated, biantennary glycan with bisecting GlcNAc (FA2BG2S2, left panel) and a high mannose structure (Man5, right panel), both potentially present in the Fc N-glycan pool. Among the monosaccharide building blocks, core-fucosylation is responsible for antibody dependent cellular cytotoxicity (ADCC) in an inverse relationship (i.e., lowering the core fucosylation level exponentially increases ADCC function). Terminal sialylation plays an important role in the anti-inflammatory properties of the product. Antennary galactosylation is influencing complement dependent cytotoxicity (CDC), while the presence of high mannose type oligosaccharides at this conserved site promotes faster clearance, i.e., shorter serum half-life. In case of Fc-fusion proteins, such as etanercept (Enbrel®), the fused protein part that is TNF $\alpha$  receptor in this case, also possesses N-linked carbohydrate moieties.<sup>3</sup> Precise structural determination of these glycans is of high importance during the entire product development process, from clone

selection to lot release for both the innovator molecules as well as their biosimilar versions.

N-linked glycosylation processing starts by co-translationally linking a GlcNAc2Man9Glc3 oligosaccharide to the polypeptide backbone during the polypeptide synthesis.<sup>4</sup> This oligosaccharide is processed in the Endoplasmic Reticulum and the Golgi apparatus by various glycosidases and glycosyltransferases until the final structure is formed that also supports proper folding of the glycoprotein. However, many factors can alter this process during the production of therapeutic glycoproteins such as the expression levels of the glycosyltransferase / glycosidase enzymes in the host cell line, the availability of the monosaccharide building blocks and the bioprocessing environment including temperature, oxygen/carbon dioxide level and pH, just to mention the most important ones. All of these parameters may influence the final oligosaccharide decoration on the therapeutic glycoprotein molecule, resulting in a large number of possible structural variants that should be analyzed and identified in a robust and reproducible manner.

Approaches to comprehensively analyze complex carbohydrates comprise numerous bioanalytical and biochemical methods including mass spectrometry, liquid chromatography, capillary electrophoresis, NMR and exoglycosidase array based sequencing.<sup>5</sup> In some instances, hyphenation of those like LC-MS or CE-MS is also applied. The most frequently used high performance liquid phase separation methods for carbohydrate analysis are ultrahigh pressure liquid chromatography (UPLC) and capillary electrophoresis (CE). Both methods require derivatization of the released sugars prior to analysis to support optical detection (commonly fluorescent). In case of capillary electrophoresis, a charged fluorophore should be used to support the electromigration process.<sup>6</sup> One of the most frequently used fluorophore for carbohydrate labeling in CE is aminopyrenetrisulfonate (APTS) via a simple reductive



**Figure 1.** Typical complex core fucosylated, sialylated, biantennary glycan with bisecting GlcNAc (FA2BG2S2, left) and a high mannose (Man5, right) structures.

amination reaction. This tag features excellent fluorescent characteristics and provides uniform labeling at the reductive end of the sugars only, i.e., one fluorophore per oligosaccharide. The same reductive amination based approach is used for UPLC analysis most of the time, however, in this instance the charge on the fluorophore is not of high importance. In most cases 2-aminobenazmide (2-AB) label is used for chromatography based analysis of carbohydrates.

In this technical note, the separation of fluorophore labeled N-linked carbohydrates, released from the monoclonal antibody therapeutics of adalimumab (Humira®) and the Fc-Fusion protein of etanercept (Enbrel®) is compared with the use of capillary gel electrophoresis with laser induced fluorescence (CGE-LIF) detection and UPLC with fluorescence (FL) detection. In both instances, standard industry sample preparation and separation parameters were used for the analysis.

## Experimental

**Carbohydrate release and labeling:** For CGE-LIF, carbohydrate release and APTS labeling was accomplished using the Fast Glycan Sample Preparation and Analysis kit (SCIEX, Brea, CA).<sup>7</sup> For UPLC, 2-AB labeling followed the method published earlier in details by Szekrenyes et al.<sup>8</sup>

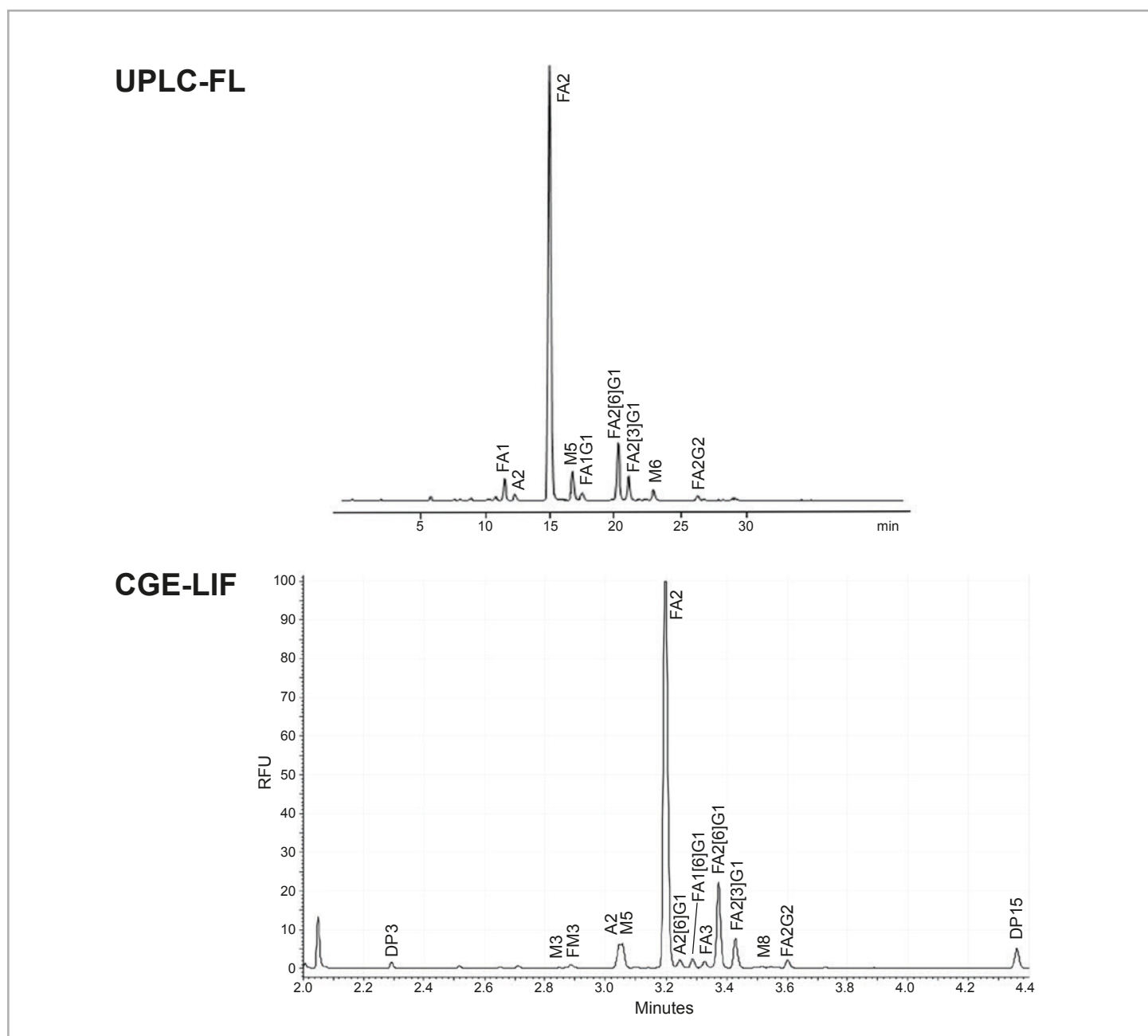
(SCIEX) was equipped with a solid state laser based fluorescent detector ( $\lambda_{ex}=488$  nm/ $\lambda_{em}=520$  nm) and used in all capillary electrophoresis separations. A 20 cm effective length (30 cm total length; 50  $\mu$ m I.D.) bare fused silica capillary was filled with the gel-buffer system of the Fast Glycan kit (HR-NCHO) and used during CGE-LIF analysis. The applied electric field strength was 1000 V/cm and the separation temperature was 25° C for adalimumab and 30° C for etanercept analysis. Pre-injection of water (3.0 sec /5.0 psi) was followed by 2.0 kV/2.0 sec sample injection and finally introducing the bracketing standard (2.0 kV/2.0 sec). The 32Karat, version 10.1 software package (SCIEX) was employed for data acquisition and analysis.

**UPLC-FL:** Ultra-high performance liquid chromatography analyses of the 2-AB labeled N-glycans were performed on an AQUITY H-Class system (Waters, Milford, MA) equipped with a fluorescence detector (excitation 360 nm, emission 428 nm) using a HILIC BEH columns (2.1 x 150 mm). The columns were fully equilibrated to the starting conditions (60° C; 78% of acetonitrile, 22% 100 mM of ammonium formate, pH 4.5) before each run. A 60-minute (separation and column regeneration) UHPLC-HILIC method was used in gradient elution mode as specified in.<sup>8</sup> The Empower 2/3 software package was employed for data acquisition and analysis.

## Results and Discussion

First the N-linked carbohydrate profile of adalimumab (Humira®) was analyzed by using industry standard UPLC and capillary electrophoresis separation parameters. This monoclonal antibody therapeutic was produced in a CHO cell line, so no alpha galactosylated structures were expected in the glycan pool. Figure 2 compares the CGE-LIF (lower trace) and UPLC-FL (upper trace) data. To obtain comparable separation

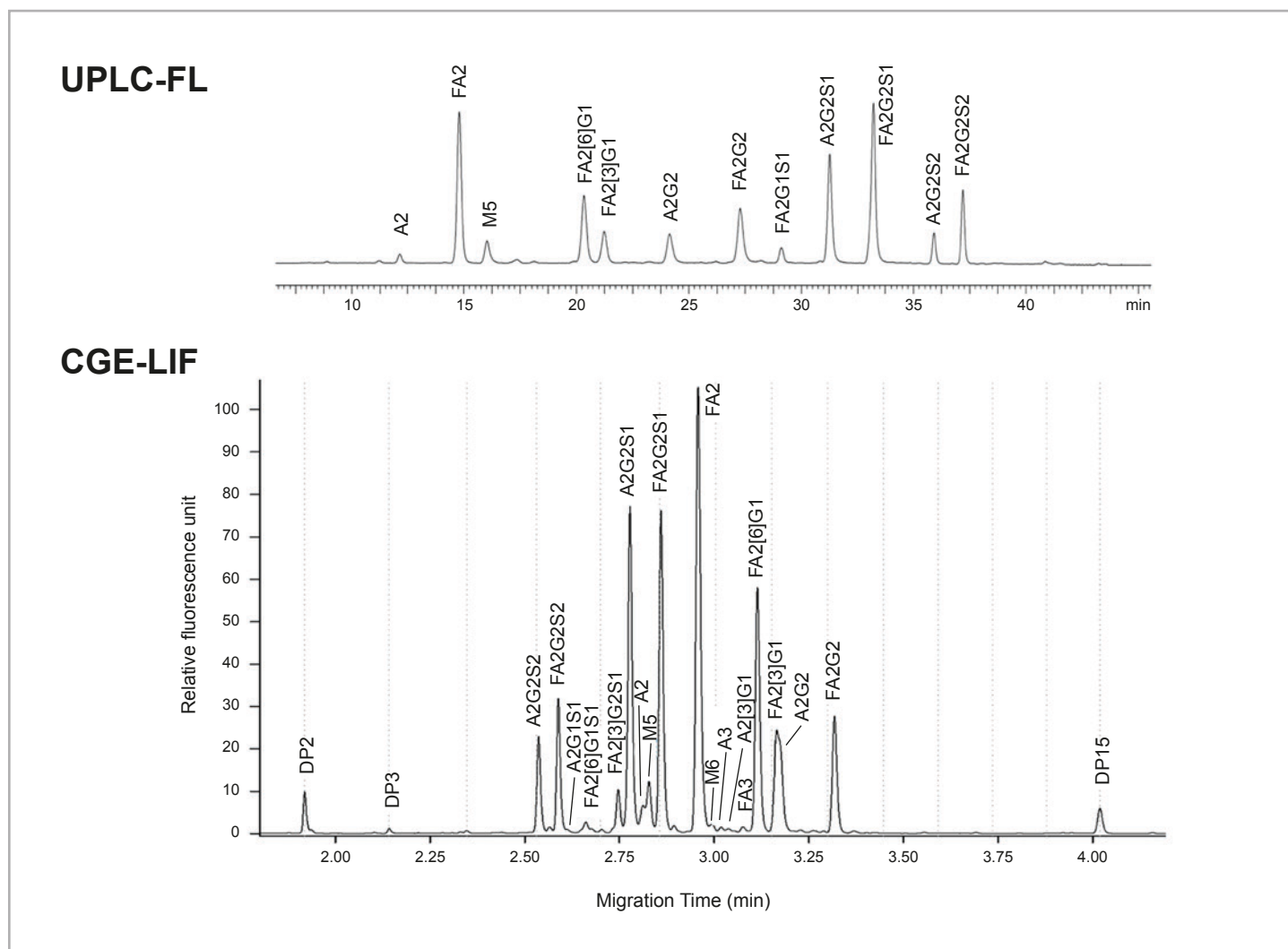
performance, the standard UPLC method required 30 minutes separation time in contrast to the CGE analysis that was accomplished in 4 minutes. In both instances, nine major glycans were identified as depicted above each peak using the annotation suggested by Harvey et al.<sup>9</sup> While most of the annotated peaks were the same with the two separation methods, some extra peaks were identified in the CE trace resulting in 12 structures in contrast to the 9 with UPLC.



**Figure 2.** N-linked carbohydrate profiling of adalimumab (Humira®) analyzed by using standard UPLC (upper trace) and capillary gel electrophoresis (lower trace) separation parameters. Conditions were as described under Experimental.

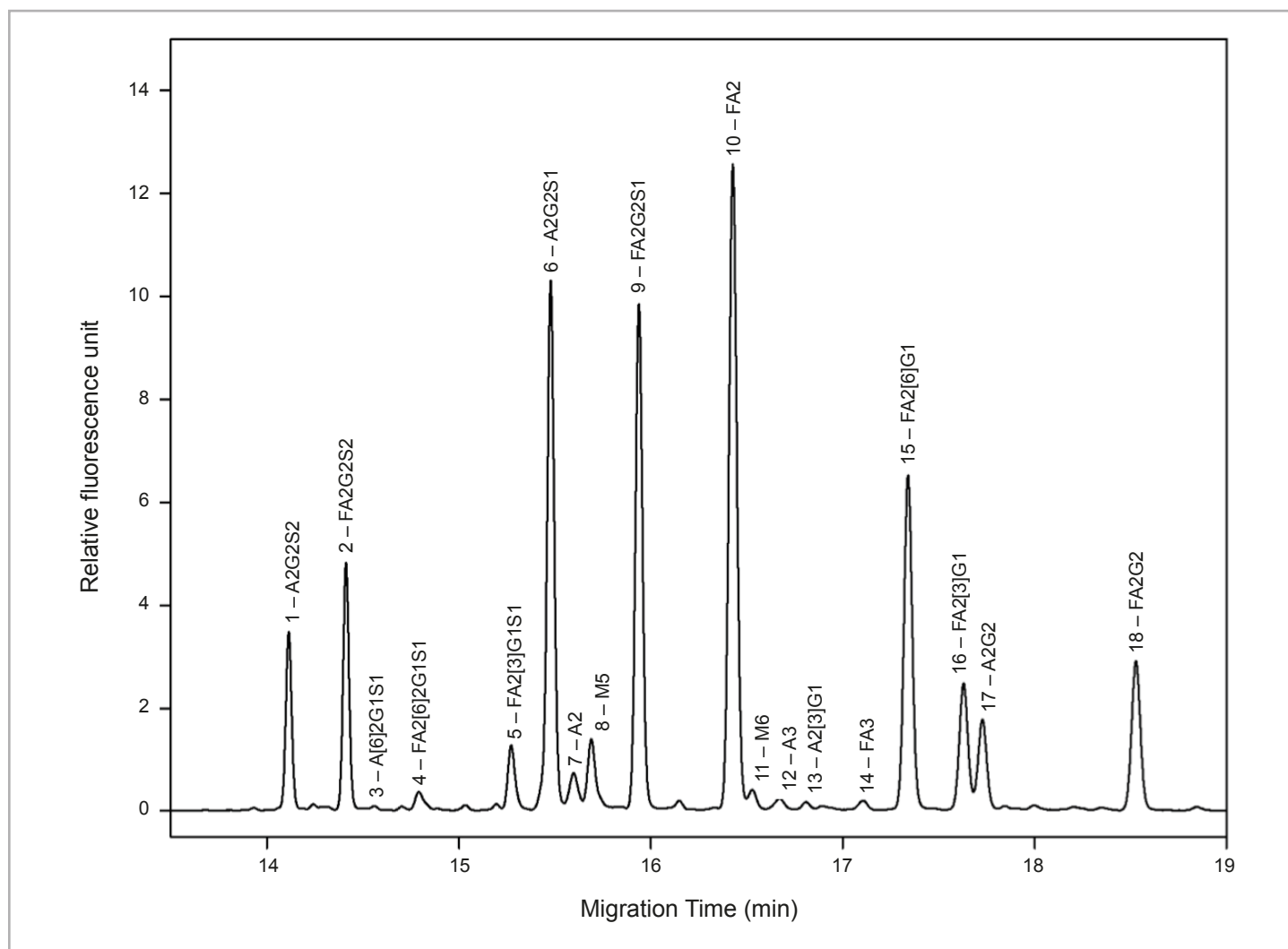
Figure 3 compares the CGE-LIF and UPLC-FL analysis of APTS and 2-AB labeled etanercept N-glycans, respectively. Etanercept has three N-linked glycosylation sites, one at the Fc domain and two at the TNF $\alpha$  receptor domain of the molecule. The upper panel shows the standard UPLC separation that took almost 40 minutes with the identification of 12 glycan structures as

depicted above the peaks. The CGE trace is delineated in the lower panel featuring the identification of 18 glycan structures. Please note that in the case of this CGE analysis, the separation temperature was increased to 30 $^{\circ}$  C to assure the best resolution<sup>10</sup> that resulted in even faster separation (<3.5 min) of the structures in the released N-glycan pool.



**Figure 3.** N-linked carbohydrate profiling of etanercept (Enbrel<sup>®</sup>) analyzed by using standard UPLC (upper trace) and capillary gel electrophoresis (lower trace) separation parameters. Conditions were as described under Experimental.

If higher resolution is required, a longer capillary can be used. Figure 4 shows the separation of the APTS labeled etanercept N-glycans using a 50 cm capillary (effective length) featuring the same 18 peaks with higher resolution. Please note the baseline separation between the M5 and A2 structures (peaks 7 and 8, respectively).



**Figure 4.** N-linked carbohydrate profiling of etanercept (Enbrel®) analyzed by capillary gel electrophoresis using a 50 cm capillary (total length 60 cm). Other conditions were as described under Experimental.

## Conclusions

Separation of the asparagine linked oligosaccharides released from adalimumab (Humira®, a monoclonal antibody therapeutics) and etanercept (Enbrel®, an Fc-Fusion protein) was compared with the use of capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF) and ultrahigh performance liquid chromatography (UPLC-FL) with fluorescent detection after

endoglycosidase release (PNGase F) and fluorophore labeling (APTS and 2AB, respectively). In both instances, standard industry settings were applied for the analysis. In case of CGE-LIF, the separation time was approximately 5-10 times faster, still resulting in similar or even better performance than that of UPLC-FL.



## References

1. Szigeti, M., et al., Quantitative assessment of mAb Fc glycosylation of CQA importance by capillary electrophoresis. *Electrophoresis*, 2018. 39: p. In Press.
2. Natsume, A., R. Niwa, and M. Satoh, Improving effector functions of antibodies for cancer treatment: Enhancing ADCC and CDC. *Drug Des Devel Ther*, 2009. 3: p. 7-16.
3. Houel, S., et al., N- and O-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-flight mass spectrometry equipped with electron-transfer dissociation functionality. *Anal Chem*, 2014. 86(1): p. 576-84.
4. Ajit Varki, R.D.C., Jeffrey D Esko, Hudson H Freeze, Pamela Stanley, Carolyn R Bertozzi, Gerald W Hart, and Marilyn E Etzler., *Essentials of Glycobiology*. 2009. 2nd edition.
5. Guttman, A., Bioanalytical Tools for the Characterization of Biologics and Biosimilars. *LC.GC Magazine*, 2012. 30: p. 412-421.
6. Guttman, A., High-resolution carbohydrate profiling by capillary gel electrophoresis. *Nature*, 1996. 380(6573): p. 461-2.
7. Guttman, A., Fast Glycan Labeling and Analysis Kit Application Guide. SCIEX 2017. Part Number B94499PTO(RUO-IDV-05-4092-B): p. 1-40.
8. Szekrenyes, A., et al., Multi-site N-Glycan mapping study 2: UHPLC. *Electrophoresis*, 2018. 39: p. In Press.
9. Harvey, D.J., et al., Symbol nomenclature for representing glycan structures: Extension to cover different carbohydrate types. *Proteomics*, 2011. 11(22): p. 4291-5.
10. Szigeti, M. and Guttman, High Resolution Glycan Analysis by Temperature Gradient Capillary Electrophoresis, *Anal Chem*, 2017 89: p. 2201-2204.

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