

Capillary Electrophoresis in Quality Control

PART I: Application for Therapeutic Proteins

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Abstract

A decade ago, Capillary Electrophoresis (CE) was considered a novelty. Today it is commonly used in quality control worldwide, providing automated, high-resolution methods, with online detection. In the first part of this series of articles, we will illustrate an overview of CE in a modern quality control laboratory. The complete series of articles will address in depth the following: Applications in QC; CE-SDS (sodium dodecyl sulfate) method development and robustness; Application of ICH Q2 (R1) to CE and finally successful CE method lifecycle management, i.e. training, transfer and method replacement.

Electrophoretic techniques such as SDS-PAGE and IEF gels have traditionally been part of release testing in the biopharmaceutical industry. However, these slab gel methods are inconvenient, use toxic reagents, exhibit high intra- and inter- gel effective mobility variability and are generally not reproducible due to inconsistency in staining/destaining steps used for analyte detection. As a result, many biotech companies like Genentech, Amgen, Pfizer, and Eli Lilly, to name a few, have sought out CE methods such as capillary electrophoresis sodium dodecyl sulfate (CE-SDS), capillary isoelectric focusing (CIEF) and capillary zone electrophoresis (CZE) as practical replacements of the slab gel format.¹ With the addition of user-friendly instrumentation, software providing tools to enable compliance with CFR 21 Part 11, and assay kits designed specifically for protein and monoclonal antibody analysis, CE has become a viable replacement in quality control laboratories for setting and justification of release specifications for therapeutic proteins. These specifications are an integral part of the release of a commercial drug substance/drug product and generally contain methods to measure safety, identity, purity and composition of a drug substance and drug product.

This article describes the advantages of three CE applications for the analysis of protein-based pharmaceuticals. The first application illustrates the application and advantages of a generic and quantitative CE-SDS assay with UV and or laserinduced fluorescence (LIF) detection to assess the size heterogeneity of protein products. The second section focuses on the use of capillary isoelectric focusing (CIEF) to determine charge

heterogeneity of protein products. Finally, given the growing importance of glycosylation characterization, we will review the application of capillary zone electrophoresis (CZE), coupled with LIF as well as MS detection for complex glycoprotein analysis.

CE-SDS for Analysis of Therapeutic Proteins

For updated information please see: [IgG Purity/Heterogeneity and SDS-MW Assays with HighSpeed Separation Method and High Throughput Tray Setup](#)

Historically, SDS-PAGE has been applied routinely to quality control systems for monitoring of manufacturing consistency and apparent protein molecular weight. CE-SDS has emerged as a robust replacement for SDS-PAGE. This assay is used to determine the apparent molecular weight of proteins and to evaluate the size heterogeneity, purity and manufacturing consistency of biologics.²⁻⁶ In contrast to SDS-PAGE, CE-SDS offers direct on-column UV or fluorescence detection, automation, enhanced resolution and reproducibility, accurate quantification of proteins, and determination of molecular weight.^{7,8} Currently, linear or slightly branched polymers such as linear polyacrylamide, polyethylene oxide,⁹ polyethylene glycol, dextran, and pullulan are used as sieving matrices for CE-SDS.^{7,10,11} In comparison to cross-linked polyacrylamide gel matrices, these polymers add a great deal of flexibility to CE-SDS since they are water-soluble and replaceable between CE analyses, resulting in enhanced overall precision and robustness.¹²

Results regarding the performance of a commercial rMAb CE-SDS based method were recently reported by eleven independent biopharmaceutical companies and a regulatory authority. In this study, the reduced rMAb standard contained a mixture of light chain (LC), non-glycosylated heavy chain (NGHC) and heavy chain (HC) components. The highly resolving nature of the CE-SDS separation was demonstrated by showing baseline resolution between HC and NGHC species present at low levels (less than 5%). The relative standard deviation (RSD%) for the relative migration time of those components was ~ 2%. Moreover, the percentage of peak area quantitation of the rMAb sample components was comparable across all organizations in the study with RSD% values of less than 9%.¹

It is important to indicate that an advantage of CE-SDS over other size-based separation methods such as highperformance size-exclusion chromatography (HPSEC) and SDS-PAGE is improved resolution of closely related size-variants. CE-SDS can be run in a variety of modes, including reduced and non-reduced sample preparation, each offering their own advantages to a QC system. With respect to the non-reduced sample preparation, CE-SDS is highly capable of resolving protein fragments in the size range of 10-100kD while allowing for concurrent detection of the intact antibody and any aggregates that may be present. This broad MW separation efficiency makes CE-SDS the ideal tool for monitoring the intact protein-to-fragment ratio for release and stability of monoclonal antibodies and proteins alike.

Figure 1 (bottom) illustrates an example electropherogram of a native rhuMab CE-SDS separation visualizing intact antibody and common antibody fragments. Additionally, as seen in Figure 1 (top), CE-SDS of reduced monoclonal antibodies offers a simple solution for reliable and accurate quantitation of the non-glycosylated heavy chain, which is generally well separated from the glycosylated heavy chain variant.^{2,3}

Traditional sample preparation conditions including heat treatment at elevated temperatures (e.g. 90° C) are employed to form SDS-protein complexes prior to electrophoretic analysis. In the case of non-reduced rMAbs, this could lead to sample preparation artifacts in the form of thermally induced fragmentation attributed to disulfide reduction and exchange reactions.^{2,6} It was also reported that high pH conditions during heat treatment also enhanced the fragmentation of SDSrMAb complexes.⁵ These artifacts significantly alter the true representation of the size heterogeneity of a protein and may increase the variability of quantitative CE-SDS methodologies of non-reduced samples. It is therefore crucial to not only optimize capillary electrophoresis conditions, but to invest a significant amount of effort into defining the correct sample preparation conditions.

Many applications of CE-SDS (reduced and non-reduced) apply UV detection, as shown in Figure 1, which is equivalent to SDS-Comassie staining sensitivity. However, certain companies including Genentech, have chosen a laser induced fluorescence (LIF) detection strategy for their CE-SDS based

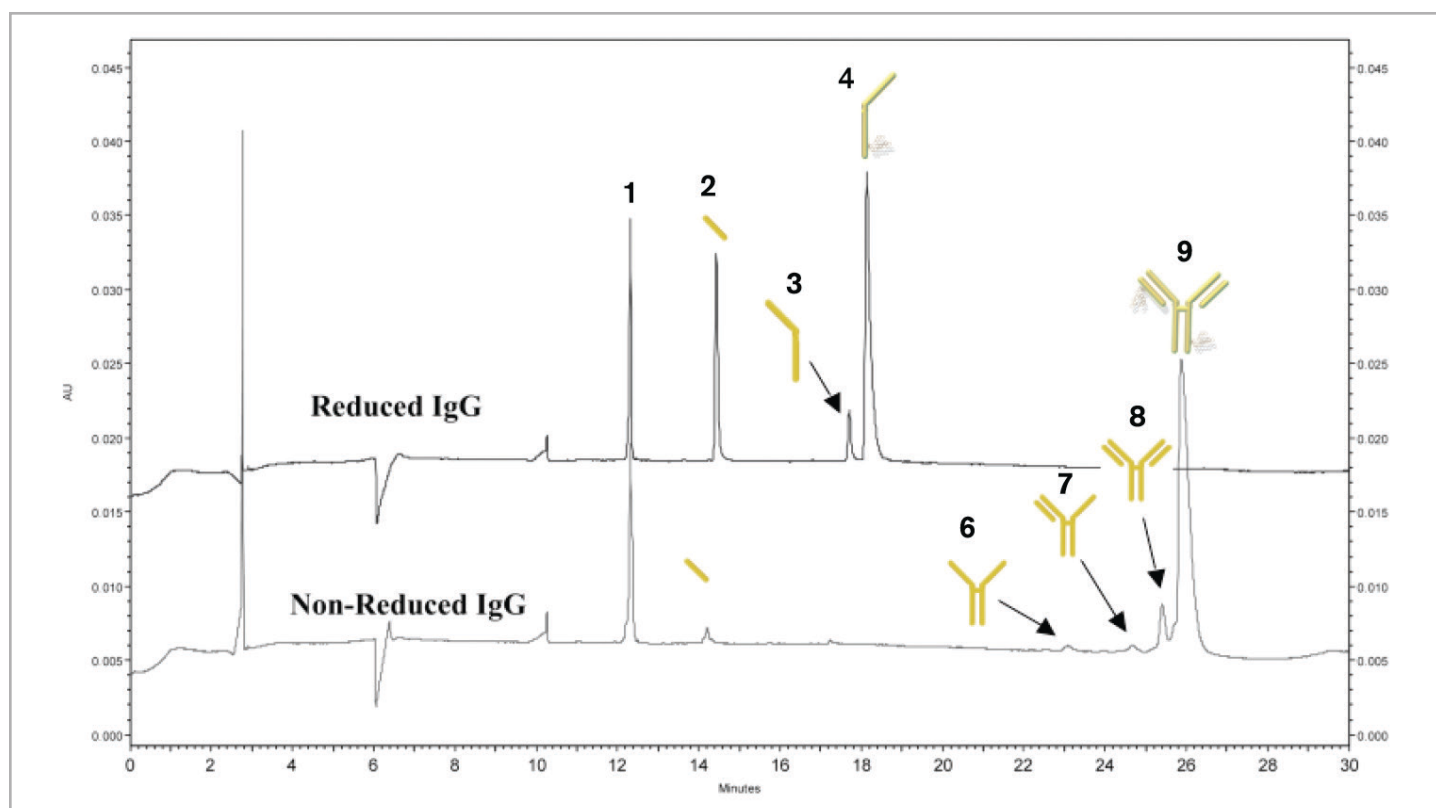


Figure 1. Analysis of both reduced (top) and non-reduced (bottom) IgG suitability standard. Peak identification: 1: Internal standard (10 kDa); 2: Light chain (L); 3: Non-glycosylated (NG) Heavy chain (H); 4: Heavy chain (H); 6: Heavy chain (2H); 7: 2 heavy 1 light chain (2H1L); 8: NG HC; 9: IgG monomer. (Reprinted from Reference 23, with permission)

assays.⁶ Although it adds additional sample preparation and the potential to introduce sample artifacts, fluorophore labeling and subsequent LIF detection does offer two significant advantages. First, LIF detection offers sensitivity on par with silver-stained SDS-PAGE.^{2,6} Figures 2A and 2B show example data for a CE-SDS-LIF analysis in both reduced and non-reduced mode. The analysis of trace-level rMAb variants and process impurities at levels as low as 50 ppm were reported using CE-SDS with LIF

detection. The second advantage is that while the capillary is filled with a viscous solution, gel-interference during the on-column detection will to some degree affect the baseline noise, and thus, the integration of minor protein variants. Labeling of proteins with 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA.SE) can significantly decrease gel interference during online detection while adding up to a 100-fold increase in sensitivity.

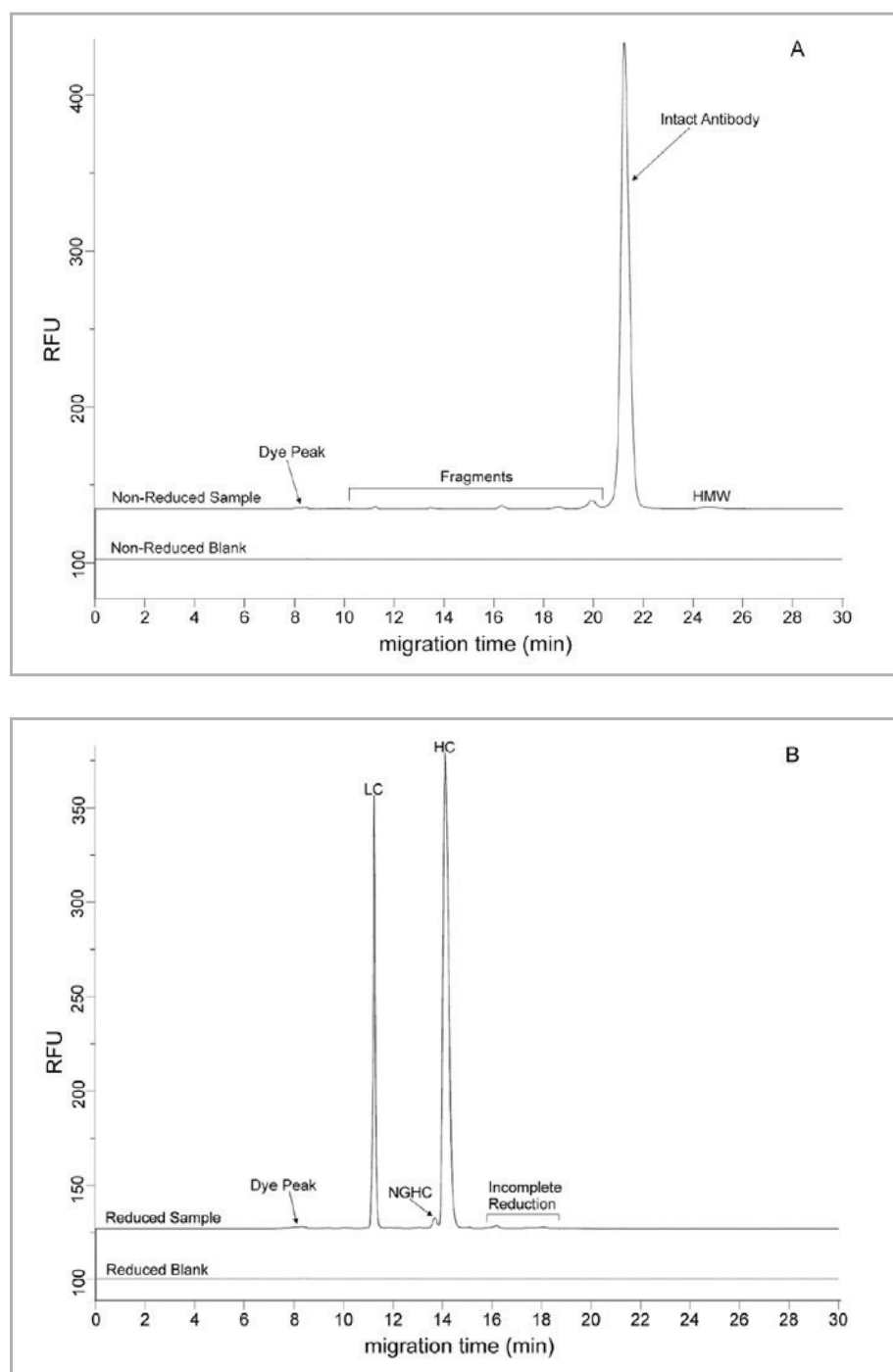


Figure 2. CE-SDS separations of (Figure 2A,top) non-reduced and (Figure 2B, bottom) reduced preparations of a 5-TAMRA SE-labeled rMAb sample. Separation conditions were as follows: SCIEX PA 800 Series instrument equipped with LIF detection; 50- μ m ID, 375- μ m OD uncoated fused-silica capillary effective length 21.2 cm, total length 31.2 cm; both anode and cathode buffers were SCIEX CE-SDS gel solution. Samples were injected at a constant electric field of 160 V/cm for 20 s and electrophoresed at 480 V/cm (32.5 μ A) and 40°C. (Reprinted from Reference 3, with permission).

Capillary Isoelectric Focusing for Monitoring Charged Variants

Regulatory agencies routinely require assessment of charge heterogeneity for biotechnology products. Charged variants of recombinant protein products often include post-translational modifications such as sialylation, phosphorylation, deamidation and addition of C-terminal lysine residues. Such variants are often monitored by widely used ion-exchange chromatography (IEC) techniques. However, the development of an IEC method is typically extensive and product-specific. IEC assays require the optimization of complex separation parameters including column, mobile phase composition, pH, salt, temperature, and gradient. CE techniques such as CZE and CIEF offer the advantages of faster analysis time and development of generic methods for multiple products, which is desirable in today's fast paced therapeutic protein arena.

In CZE, analytes are separated from each other based on the differences in their electrophoretic mobilities. Therefore, this technique also introduced a size-based element to the separation. Alternatively, the ability for CIEF to separate solely based on charge may have an added advantage in isolating charge variants of recombinant monoclonal antibodies (MAbs).

CIEF separation of a MAb sample illustrates the powerful separation efficiency of modern CIEF technology (Figure 3).

CIEF (both by on-column detection and image detection) as a tool for protein purity analysis is quickly becoming the method of choice for many biopharmaceutical companies due mainly to its fast method development and highly reproducible nature.¹³ CIEF is usually carried out in commercial CE instruments employing on-column UV absorbance detection. Following the focusing of an analyte in a capillary, protein zones are detected using chemical, electrophoretic or hydrodynamic mobilization strategies. Convenient protein and rhuMab chemistry kits are commercially available facilitating not only method development but also integration into quality control.

Figure 4 gives an example of the reproducibility obtained using conventional CIEF technology for charge variant analysis. As summarized in Table 1, RSDs using this technology are generally less than 1% for the main peak and 2-5% for acidic and basic variants, demonstrating equivalent, if not superior, reproducibility to ion exchange chromatography methods.¹⁴ Additionally, CIEF can be used as an identity assay on the basis of highly reproducible measurements of the apparent pI of the main isoform peak (See Table 1).

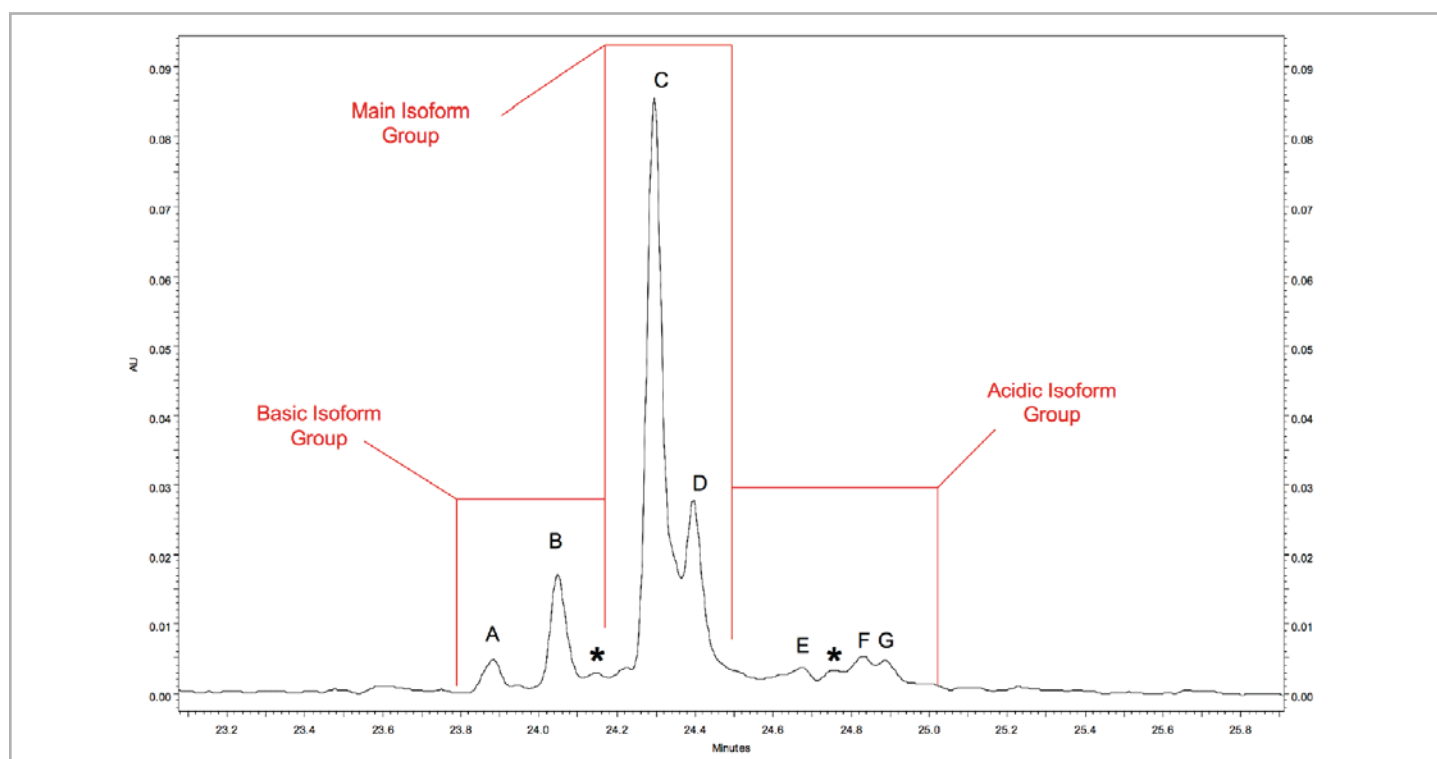


Figure 3. The three isoform groups and seven signature isoform peaks used for the intermediate precision analysis of a MAb CIEF separation. Peaks lettered A through G were used to characterize variation in the estimated pI values and isoform group percent compositions. Peaks denoted with an asterisk were only used to estimate variation of the isoform group percent compositions (as shown in Table 1) (Reprinted from Reference 14, with permission).

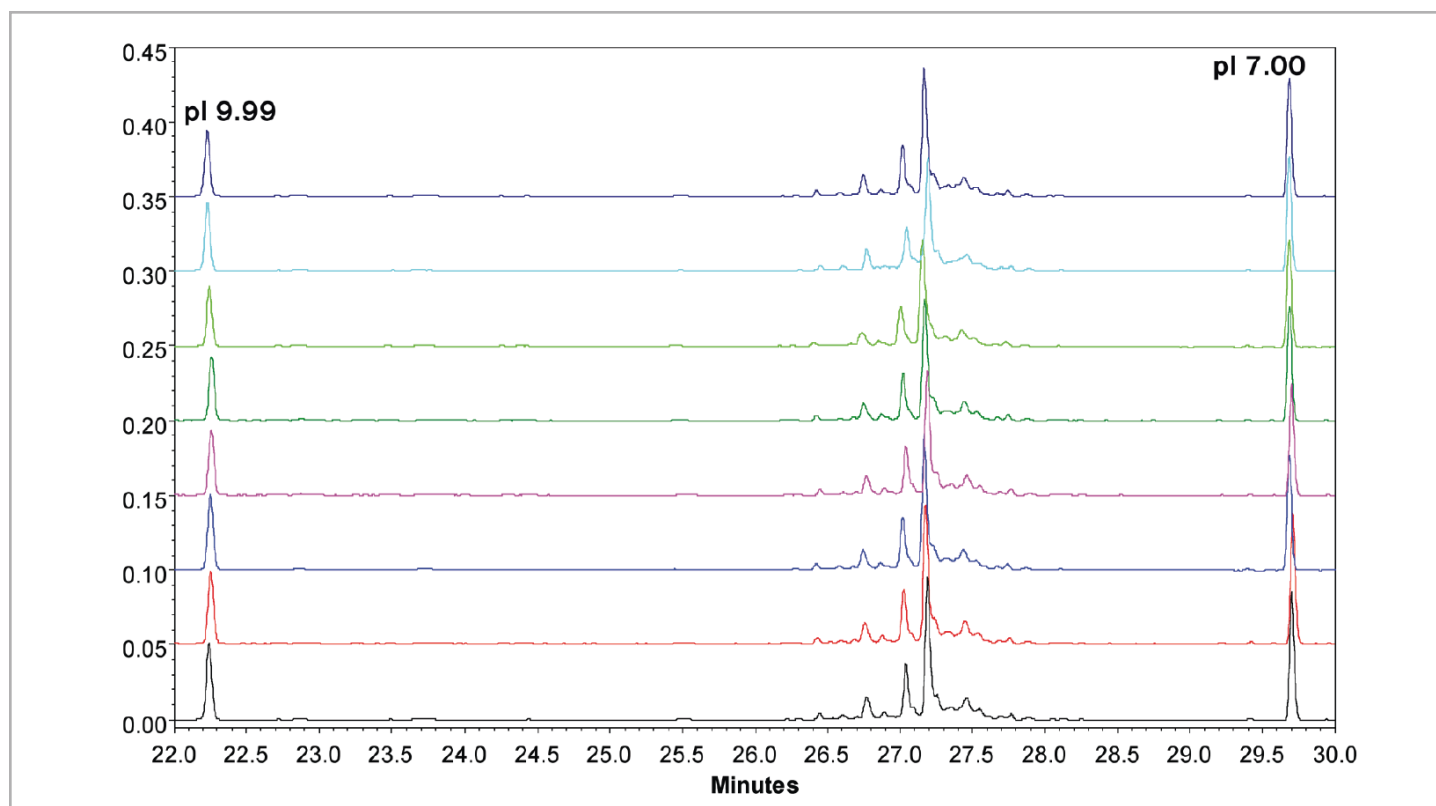


Figure 4. CIEF intermediate precision data for separation of a MAb. The detector traces are aligned to the peaks of the pI 9.99 and 7.00 synthetic peptide pI markers. Separation parameters are as follows: Focusing was performed for 15 min at 25 kV; mobilization using 350mM acetic acid for 20 min at 30 kV. The sample solution contained 2.5M urea, 1.8% w/v Pharmalyte pI 3–10 Commercial Ampholyte, 1.7mM iminodiacetic acid (IDA), 42mM arginine and 10 mM each of the pI 7.00 and 9.99 synthetic peptide markers, all diluted into SCIEX' CIEF polymer solution. Quantitative data is shown in Table 1. (Reprinted from Reference 14, with permission).

Peaks	Average			Standard deviation			%CV		
	mAb 1	mAb 2	mAb 3	mAb 1	mAb 2	mAb 3	mAb 1 (%)	mAb 2 (%)	mAb 3 (%)
Estimated pI/									
A	9.65	8.31	7.79	0.00507	0.00500	0.00493	0.05	0.06	0.06
B	9.58	8.18	7.59	0.00577	0.00577	0.00351	0.06	0.07	0.05
C	9.48	8.13	7.46	0.00640	0.00583	0.00458	0.07	0.07	0.06
D	9.44	8.07	7.43	0.00812	0.00569	0.00289	0.09	0.07	0.04
E	9.33	8.01	7.38	0.00700	0.00583	0.00351	0.08	0.07	0.05
F	9.27	7.90	7.28	0.00645	0.00583	0.00507	0.07	0.07	0.07
G	9.24	7.78	7.12	0.00781	0.00400	0.00458	0.08	0.05	0.06
Isoform group percent composition									
Basic	13.94%	30.97%	18.03%	0.42%	0.67%	0.28%	3.04	2.17	1.53
Main	71.97%	45.01%	68.42%	0.46%	0.45%	0.42%	0.64	0.99	0.62
Acidic	14.09%	24.02%	13.55%	0.34%	0.60%	0.34%	2.38	2.50	2.49

Table 1: Summary of CIEF separation inter-day reproducibility data analysis for 3 MAb molecules (MAb 1, MAb 2 and MAb 3) (Reprinted from Reference 14, with permission).

CZE-LIF for N-Linked Glycans Analysis

For updated information please see: [Fast Glycan Labeling and Analysis: High-Resolution Separation and Identification in Minutes](#)

Glycosylation, one of the many post-translational modifications present on therapeutic proteins, has received considerable attention in the field of proteomics due to its reported role in protein function. In the biotechnology industry, it is well known that carbohydrates factor significantly into a glycoprotein's activity and efficacy and therefore must be carefully monitored.^{15,16} Even minor changes in glycan distribution can have a notable effect on the activity of a biopharmaceutical product.^{17,18} Therefore, advances are continually being made in the biopharmaceutical industry to minimize glycan heterogeneity and improve analytical assays for identification and quantification of minor changes across products and/or batches.¹⁹ However, the task of glycoprotein characterization remains an analytical challenge

due to the vast heterogeneity of these species. Capillary Zone electrophoresis (CZE) is an excellent tool for profiling glycosylation due to the advantages of automation, short analysis times and the ability to separate and quantify isomeric species. The coupling of CE with on-column LIF detection has the advantage of highly sensitive analysis. The primary assay used for profiling rMAb glycosylation involves: 1) Releasing N-linked oligosaccharides with the endoglycosidase PNGase-F; 2) Labeling with a charged fluorophore; and 3) Sample analysis using CZE-LIF.

Common labeling reagents include 8-aminopyrene-1,3,6-trisulfonate (APTS), 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) or analogous negatively charged labels or alternatively, 2-aminopyridine (2-AP) and 2-aminobenzamide (2-AB) which are net positively charged labels used for LIF labeling of glycans. This strategy offers the advantage of low-picomole sensitivity attainable for LIF-labeled carbohydrates.^{20,12,22,25}

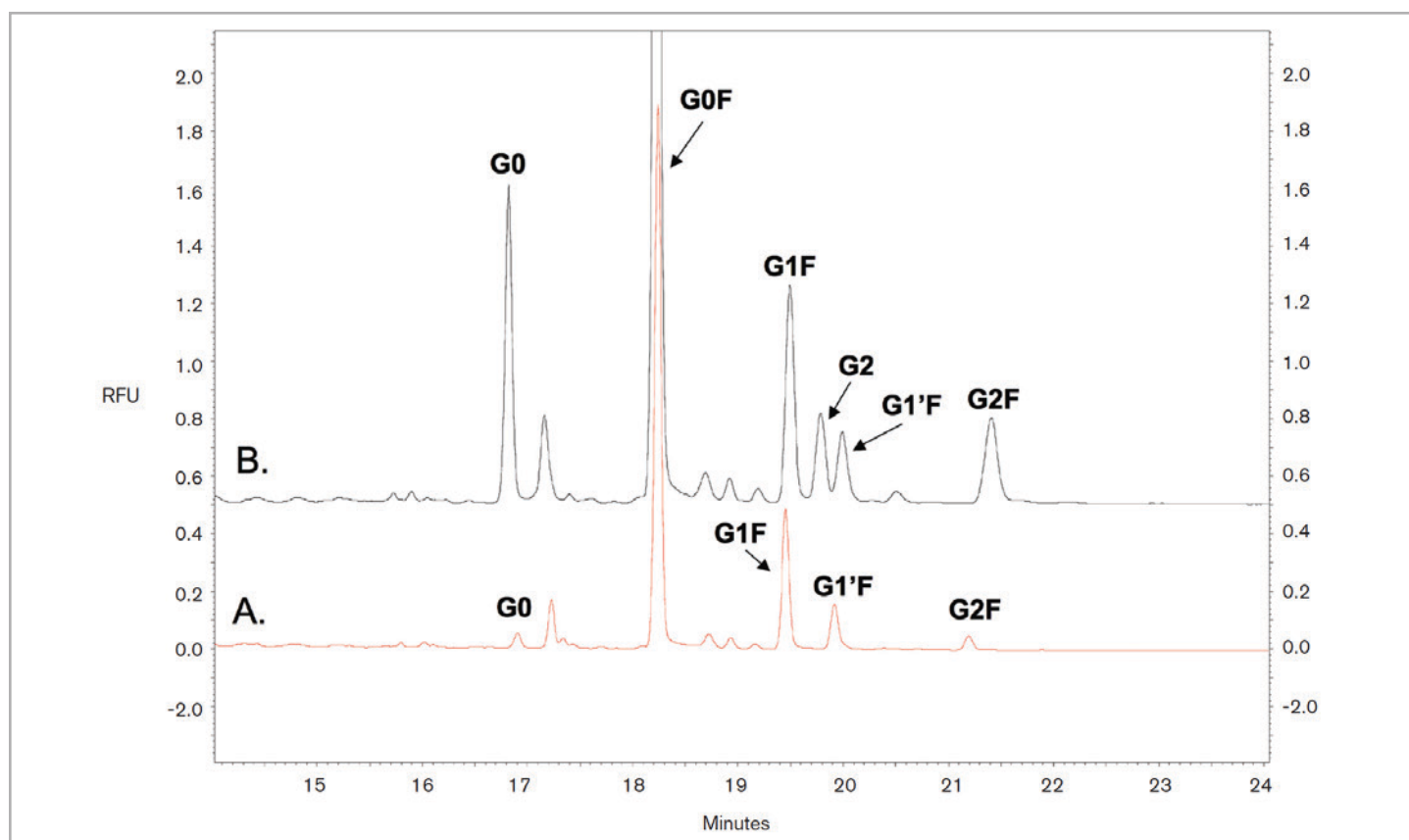


Figure 5. Separation of oligosaccharides associated with a recombinant therapeutic MAb. Oligosaccharides were cleaved from a therapeutic MAb, APTS labeled, and separated by CE using the SCIEX Glycan separation buffer. A number of oligosaccharide species were resolved from one another (A). In order to identify and help illustrate resolution between co-migrating glycan species, we spiked the MAb sample with standards. Relative to the oligosaccharide standards, we were able to quantifiably identify G0, G0F, G1F, G1'F, and G2F. G2 standard was also spiked into the mixture to indicate the location in the separation at which this oligosaccharide species would reside. Further experimentation is being performed to identify the additional species present in the electropherogram. Separation conditions were the same except that injection for the MAb alone was 0.5 psi and that for the MAb + glycan standards was 1.5 psi. (Reprinted from Reference 24, with permission).

SCIEX provides a Carbohydrate Labeling and Analysis Kit that includes the necessary reagents for characterizing glycan populations associated with therapeutic proteins. This solution utilizes an APTS derivatization strategy and separation gel buffer with a separation efficiency allowing for routine separation of the G1 isomers. Figure 5 shows an example trace for N-Glycans released from an APTS labeled rMAb. In this profile, the major glycan species G0 G1, G1' and G2, with and without fucose, in addition to several unidentified minor peaks can be easily resolved at ~24 minutes.

One disadvantage of CE-based methods, which is especially apparent in glycan analysis, is the lack of routine detection methods that can provide direct structural information on migrating species. Global process transfer for commercial products is a reality for many biopharmaceuticals and as a result, the ability to assess minor glycosylation differences using a validated method is critical. In the glycan assay, characterization can be achieved using one of two methods: 1) co-migration with commercially available standards, and 2) exoglycosidase treatment followed by co-elution with standards, such as shown in Figure 5. These methods can be particularly labor-intensive and do not provide any direct information on glycan structure.

Recently, efforts have been undertaken to couple CE to Mass Spectrometry (MS), and Glycan analysis,²⁵ with its MS-compatible ammonium acetate or aqueous epsilonamino-caproic acid (EACA) separation buffers, is an ideal application

for this somewhat novel technology. By coupling CE with MS or even MS/MS in an online fashion, complex carbohydrate structures can be resolved in as simple as three steps:

1) Release of carbohydrate from protein backbone 2) Labeling of the glycan entities for concurrent LIF analysis 3) Structural analysis via online MS/MS analysis.²²

The ability to perform complex CE-Glycan characterization in an online fashion opens new doors for the use of CE not only as part of QC release, but also as part of process characterization and validation. The clock for CE-MS in a quality control environment has already started with a validated CE-MS assay for glycoprotein analysis, as presented by Paula Domann from LGC, UK at CE in Pharmaceutical Analysis, 2008.

Conclusions

Capillary electrophoresis (CE) methods for the analysis of therapeutic proteins are routinely used in quality control lot-release testing. This article was meant to introduce the reader to the advantages of CE-SDS, CIEF and CZE-Glycan for the analysis of protein therapeutics. As has been shown, properly optimized CE methods are robust and reproducible, averaging migration time RSDs of less than 2% and peak area reproducibility for main peak of < 5%. In the second part of this series of articles, we will focus on optimization and robustness testing for the most common QC CE technology: CE-SDS in reduced and non-reduced mode.

Acknowledgements

Alpine Analytical Academy

Dr. Chantal Felten, founder of Alpine Analytical Academy, consults both large and small biopharmaceutical companies in Quality Control systems. Over the last 14 years, she has been actively involved in developing applications for various CE instruments, including the coupling of CE to Mass Spectrometry and teaches several professional development courses on the principles of CE, method validation and specifications for CASSS. With expertise in both HPLC and CE, she has a joint Ph.D. in physical chemistry from Prof. Heinz Engelhardt and Prof. Barry Karger.

Prior to founding the Alpine Analytical Academy, Dr. Felten was the QC CE expert at Genentech where she was responsible for CZE, CE-SDS and CIEF method validation, transfer and training.

At Genentech, she led the Early Stage (Phase I/II) and the Late Stage (Phase III to post commercial) validation functions, managed commercial method transfers and trouble-shooting, and supported IND/BLA filings with technical oversight over numerous method development validations for both commercial and clinical stage products.

About Alpine Analytical Academy:

Alpine Analytical Academy specializes in CE application quality control systems, and offers webinars, customized training solutions and consulting services related to applications of analytical technology in the biopharmaceutical industry. Alpine Analytical Academy focuses on offering practical solutions in method development, validation and setting of specification needs.

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