Troubleshooting CE-SDS: baseline disturbances, peak area repeatability and the presence of ghost peaks

CE Pharm 2015 troubleshooting workshop report

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Introduction

Troubleshooting is an integral part of the analytical scientist's bench work, and by implication, an integral part of analytical characterizations performed during the drug development lifecycle. Analytical assays need to perform with a predictable level of consistency and robustness in order for them to be useful in monitoring various aspects of drug quality. Whether or not troubleshooting activities are conducted under the formal frameworks of Corrective and Preventative Action (CAPA) and Failure Mode Effect Analysis (FMEA), these types of investigations are critical to understand and resolve unexpected issues with assay performance.

Capillary Electrophoresis (CE) has gained widespread adoption in biopharmaceutical industry as a powerful analytical technique, not only as a superior alternative to traditional gel electrophoresis (SDS-PAGE, IEF), but in novel applications as well (e.g. glycan analysis, preparative charge-based fractionation). Performing gel electrophoresis in a capillary has the added advantage of direct quantitation without the need for staining/destaining. And because the capillary is rather rugged, elaborate rinsing procedures are possible so that the capillary can be refilled and reused. The CE equivalent of SDS-PAGE is called CE-SDS. The capillary is filled with a relatively concentrated Tris-borate buffer containing a linear polymer gel, which serves to form a replaceable molecular sieving matrix. The high buffer concentration contributes to suppressing the EOF, as at a high ionic strength, the electrical double layer at the capillary wall is dense and the EOF slow. During sample preparation, the protein is denatured by heating in the presence of SDS, much like the traditional SDS-PAGE method. CE-SDS can be

performed under reduced and non-reduced conditions. Reduction (typically with beta-mercaptoethanol or DTT) reduces the disulfide bonds in a protein and allows the separation of disulphide bonded protein species. Commercial assay kits are widely used for the non-reduced and reduced analysis of monoclonal antibodies by conventional CE, though further optimization from standard conditions is often desirable to mitigate sample preparation artifacts (1) .

In late 2015, the CE Pharm conference, organized by CASSS, conducted its annual workshop on troubleshooting CE assays. The CE Pharm conference aims to bring together CE users from academia and industry, along with instrument vendors and regulatory agencies, in order to foster collaborative discussions and advance the application of CE. The troubleshooting workshop solicited real life examples of difficult troubleshooting issues from the attendees. These were submitted anonymously prior to the start of the conference and presented by the organizers for discussion at the workshop. The intent is to allow attendees to identify whether other users are experiencing similar troubleshooting issues, as well as propose possible solutions which attendees can then test when they return to their labs. In order to focus the discussion, the workshop this year concentrated on troubleshooting issues related to capillary gel electrophoresis, or CE-SDS, a routine assay used in biopharmaceutical companies for purity analysis of drug substances. Two troubleshooting themes were identified and discussed: baseline stability and peak stability (ghost peaks).

Baseline stability (noisiness, drift, jumps)

One of the most frequently used CE-SDS assays takes advantage of a set of commercial reagents from Sciex. While this assay has been shown to be robust in an intercompany collaboration (2,3) and is extensively used for the analysis of monoclonal antibodies, multiple users noted issues related to baseline stability (see Figure 1A-C for representative examples, submitted anonymously by CE Pharm attendees, of common baseline stability issues). One of the major ones is the amount of noise and drift in the baseline. CE-SDS is often used to monitor product purity, thus baseline noise is a major issue since it complicates identification and integration of minor peaks. A well-recognized feature is the wave or roll in the baseline just after the migration of IgG. Experts from Sciex were present and provided a detailed explanation for the phenomenon. The baseline features are due to Joule heating and is an intrinsic property of the proprietary gel buffer. When UV detection is used in this method to monitor separation of protein, a likely mechanism is that the refractive properties of the buffer change as a result of heating – hence the baseline drift effects worsen over time. Naturally, changing the coolant temperature housing the capillary would not alleviate this artifact. Multiple experienced scientists offered several suggestions for getting around this issue.

- Use of fluorescent labeling and laser-induced fluorescence (LIF) detection (1,4,5).
- Collect data at multiple wavelengths using DAD. Since the refraction artifact affects multiple wavelengths, one could cancel out the baseline drift through a baseline subtraction between the wavelength of interest (i.e. ~214nm) and a reference wavelength where the analyte of interest has minimal absorbance, using the data collection rate to control the smoothness of the e-grams.

- Perform a short end injection (across the 10 cm side) and thus limit the overall duration of separation. This may be the most straightforward method, although the decrease in the effective separation length also means a decrease in the resolution. In general, the loss of resolution is limited and may be acceptable for the intended use of the assay. Optimization of the sample injection conditions to enhance separation efficiency could also be investigated.

- Reducing the applied field strength (applied voltage per length of capillary). This is also a straight-forward measure, but at the cost of time. However, if combined with short-end injection satisfactory results are generally obtained.

- Sciex did mention they are willing to work with companies to explore reformulating the proprietary buffers, but this would again come at the price of a decrease in separating power. Indeed, some users have said that in order to minimize baseline drift they have diluted the gel buffers by up to 50%, and used coated capillaries to suppress the concomitant increase in EOF.

Aside from limitations due to inherent properties of the gel buffer, many attendees also shared their experience with some other factors uncovered during troubleshooting investigations. Sudden jumps in the baseline may occur due to slight misalignment of the sample trays, which cause pressure changes on the interface block as the capillary moves from one vial to another. Stability of the current and insufficient grounding of wires can also play a role, especially when the data is being collected via analog output to a secondary data analysis program (e.g. Chromeleon, Empower). Users should implement routine maintenance checks on the instrument by making sure the fiber optics cable and UV lamps are intact and properly installed. The CE instrument should also be on a firm, vibration-free surface (one attendee mentioned that a working vortex near the CE instrument caused an increase in the baseline noise). Users should ensure no air bubbles are introduced when pipetting minute amounts of viscous samples or buffers in a microvial. Some companies increased the rinsing procedures to reduce sudden baseline steps. Dried buffer components (e.g. SDS, gel) accumulating on key instrument hardware is often the root cause of many issues, and most users agreed proper care and maintenance of the instrument, as well as rigorous cleaning protocols, reduced the incidence of baseline issues.

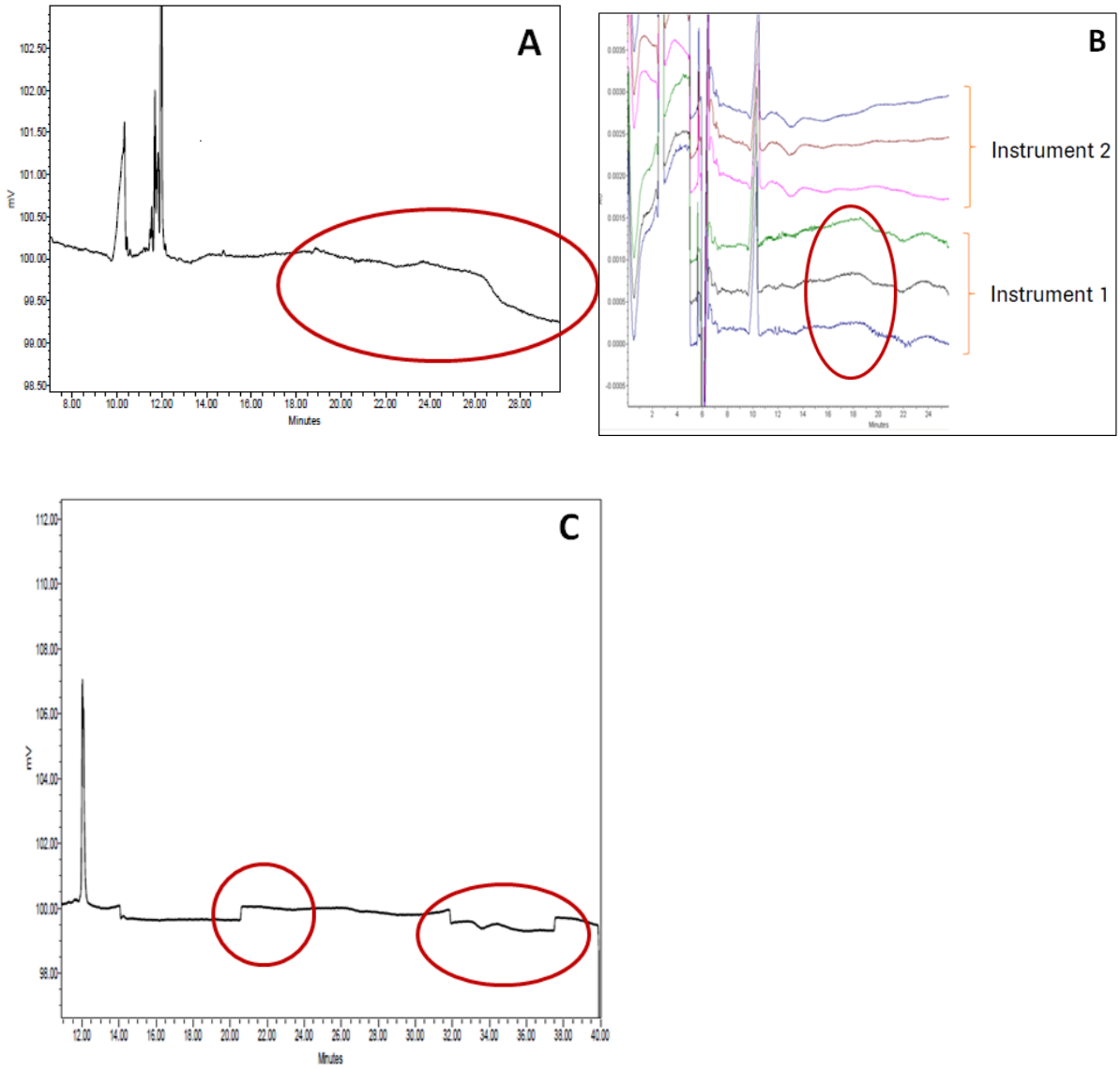

Figure 1. User examples illustrating baseline instability issues. Circled areas in the electrophoretic traces represent telltale examples of the most frequent baseline instability issues such as (A) drift, where the baseline shows wavy fluctuations; (B) noise, where the baseline displays low magnitude high frequency fluctuations; and (C) jumps, where the baseline undergoes sudden changes in value.

Peak stability (peak area repeatability and the presence of ghost peaks)

In this category of submitted examples, attendees observed run-to-run inconsistencies in the intensities, or even the presence, of minor peaks (see Figure 2A-B for representative examples, submitted anonymously by CE Pharm attendees, of ghost peaks and poor peak area repeatability). Since the CE-SDS method involves sample denaturation with SDS and heat, attendees agreed that sample preparation conditions were the most likely culprits for poor reproducibility, either between different sample preps or between replicate injections of the same sample prep. Attendees suggested several practices for improving sample preparation consistency:

- Pipetting small volumes of viscous solutions can be a source of variability and users should take note of the precision of their pipetting steps. Some users pre-mix beta-mercaptoethanol in the sample buffer to reduce variance between the samples.
- Mix by inversion or pipetting instead of vortexing.
- Use a high concentration of denaturation buffer to ensure adequate denaturation
- When performing denaturation under reducing conditions, the amount of beta-mercaptoethanol used should be carefully optimized, since too much beta-mercaptoethanol can give rise to ghost peaks. Use of TCEP can cause baseline distortions and is not recommended.
- Samples should be stored at no lower than 10 ˚C in order to prevent SDS in the sample from precipitating out.
- Sample heating is a key factor and water baths should be used to ensure a consistent temperature. When using water baths, it is also important to make sure the samples are properly sealed and no water gets into the sample.
- Some users have noted that electrokinetic reinjection from the same vial affected sample stability as a result of voltage-induced degradation.
- The kit-based sample buffer of pH 9 is not suitable for every protein particularly with disulfide-linked proteins such as antibodies; a lower-pH sample buffer can be beneficial. Also, some proteins require higher SDS concentrations for adequate denaturation. The general advice is to investigate and optimize the sample preparation for each protein.

In one submitted example, peak instability was associated with certain protein drug molecules and not others. The likely mechanism for this was that the sample reductions conditions were not fully optimized, resulting in occasional disulfide reshuffling and the stochastic appearance of peaks with different molecular weights.

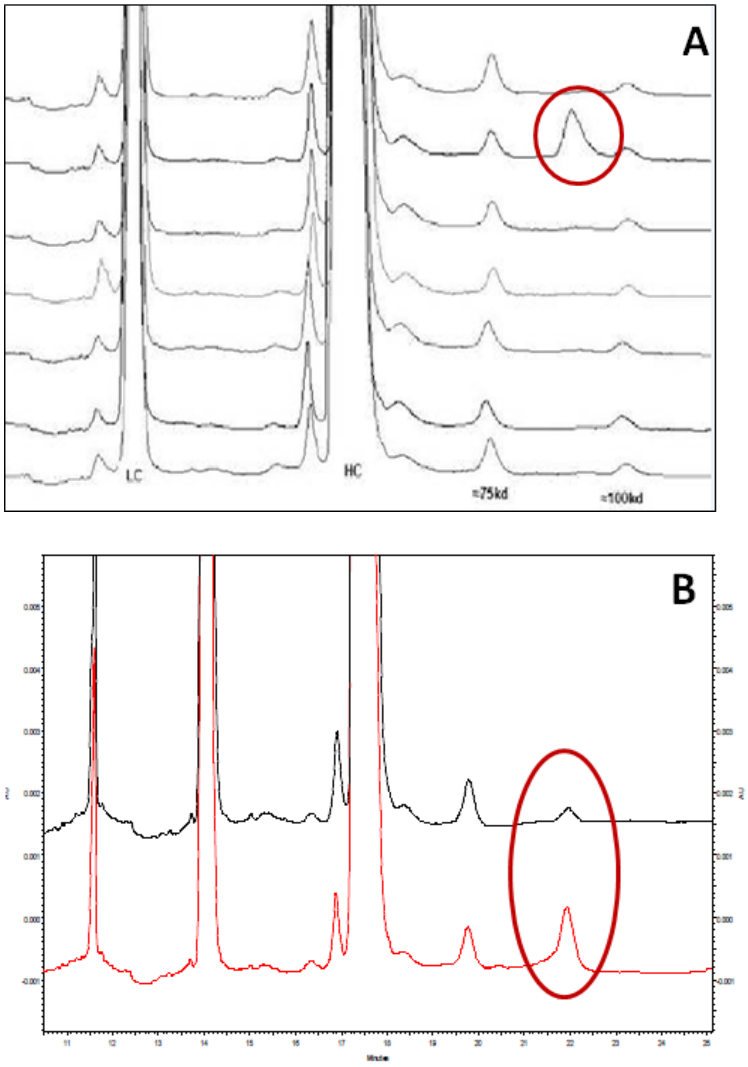

Figure 2. User examples illustrating peak instability issues. Overlaid traces in each panel represent multiple injections of the same sample. Circled areas in the electrophoretic traces highlight telltale examples of the most common peak instability issues such as (A) ghost peaks, where a minor peak appears unpredictably during multiple injections and (B) poor peak area repeatability, where minor peaks show wide fluctuations in their peak area percentage.

Concluding Remark

CE-SDS is a widely adopted methodology within the biopharmaceutical industry. It is increasingly being used in place of traditional slab-gel based methods as an additional assay or for novel applications. The long-term use among a large community has built a knowledge base for troubleshooting, and the CE Pharm workshop is committed to ensuring this knowledge is shared with all users. It is the hope of all participants in the workshop that these discussions lead to improvements in user performance, analytical equipment, and application kits, with the ultimate goal being improved analytical sophistication in the quest to develop and produce safe and efficacious drugs.

Acknowledgments

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