CE Pharm 2016 troubleshooting workshop report: cIEF and materials for CZE

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Introduction

Over the past decade, Capillary Electrophoresis (CE) techniques have steadily integrated into the analytical workflow within the biopharmaceutical industry, complementing and often replacing the traditional gel based electrophoretic techniques. This is perhaps unsurprising, given the many advantages of the capillary format, and readers are referred to these reviews for a comprehensive overview of the benefits of CE (**1 - 7**). With the advent of new instrumentation, commercial reagents and assay kits, CE methods are well positioned to replace most of the traditional slab gel based electrophoretic assays (SDS-PAGE, western blotting, IEF). Despite these advances, CE troubleshooting remains a prominent topic, and user forums, such as the CE Pharm conference, provide an expedient way to bridge the expertise gap between new and experienced users.

In 2016, the CASSS CE Pharm conference organized and conducted its annual workshop on troubleshooting CE assays. This 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. To focus the discussion, the workshop this year concentrated on troubleshooting issues related to capillary isoelectric focusing, or cIEF, a routine assay used in biopharmaceutical companies for charge heterogeneity analysis of biotherapeutic molecules.

Troubleshooting cIEF

The principles behind separation in cIEF parallel those of traditional slab gel IEF. The capillary is filled with an ampholyte mixture containing the protein sample of interest, pI markers, and additives important for stability such as methylcellulose, urea and gradient blockers. Following the application of high voltage across the capillary, the electric field drives migration of the

ampholytes according to their net charge, and eventually settles (“focuses”) into zones corresponding to their pKa. Likewise, the protein analyte will migrate through these different zones until it reaches a zone with a pKa corresponding to its isoelectric point (pI). Depending on the instrument format, detection of the separated protein species is done by either mobilizing the focused zones past a UV detector, or alternatively, by using whole capillary imaging **(8 - 20)**. Pioneers in the field are Stellan Hjertén **(e.g. 11 - 13)** and Pier Giorgio Righetti **(e.g. 2, 8 - 10)**.

Since ampholytes are central to effecting separation, the workshop kicked off the discussion around some implicit assumptions underlying the determination of pI by cIEF:

1. Broad range ampholytes form continuous linear pH gradients and the total number of ampholytes is uniformly distributed throughout the range.
2. Ampholytes are manufactured by very precise controlled synthesis.
3. Resolution is inversely proportional to the ΔpI slope, so decreasing slope will help to resolve previously unresolved isoforms.
4. All ampholyte pI ranges (low, middle, and high pH range) have about the same quality and effectiveness.

In other words, a common assumption is that the experimental pI (or apparent pI) is an absolute physical measurement (within the confines of experimental error). In reality though, the pI is affected by parameters such as temperature, media composition (dielectric constant) and ionic strength (influences the dissociation of ionizable functionality). Additionally, the pH gradient generated from an ampholyte mixture is more complex than one may expect based on a typical standard curve derived from internal pI markers. The chemical synthesis process for ampholytes yields a heterogeneous pool of products, which are subsequently fractionated to yield the various ranges of ampholytes and buffer capacity. Several ampholyte related questions were compiled by the workshop organizers based on their own experiences as well as input from conference attendees. Since this was not the focus of the workshop, the discussion points summarized below did not delve into the complexities of this critical reagent. Readers are encouraged to refer to the work of Righetti et al. (**21**) for an in-depth discussion on ampholytes.

What are the differences between Servalyt and Pharmalyte?
The chemical manufacturing process for Servalyt (Serva) and Pharmalyte (Pharmacia) are different. For Servalyt the reaction process continues after bottling and this contributes to the greater background noise of Servalyt with age (more than 5 years since the date of manufacture). Pharmalytes are generated using a different chemistry and don’t have the same issue. It was also suggested that sometimes, nonspecific interactions between the ampholyte and the protein analyte are significant – for example there were anecdotal observations that acidic range Servalytes are better for resolving fusion proteins.

Are there alternative ways to make pH gradients other than the use of these ampholytes?
Use of amphoteric compounds is the most effective way to generate gradients. Because the ampholytes separate into discrete zones, a large number of ampholytes with overlapping zones provides the appearance of a continuous gradient (**21**). There are opportunities to customize the

pH gradient using the commercially available ampholytes. For example, one can combine ampholytes of different ranges, or from different manufacturers, keeping in mind that lot-to-lot variability would be magnified when mixing ampholytes from multiple manufacturers. Some users indicated lot-to-lot performance is more consistent for narrow range than broad range ampholytes. Changing the temperature of the ampholyte mix can also shift the pKa's of the ampholytes, thus altering the gradient and potentially improving resolution, although the effects are less predictable. Ampholytes require an aqueous environment to prevent precipitation but are otherwise compatible with many other additives in the sample mixture.

There is a new ampholyte recently introduced on the market from Advanced Electrophoresis Solutions (ceinfinite.com). So far, there is very little experience with this from the users.

The remainder of the troubleshooting workshop focused on user examples, which could be broadly categorized into electropherogram spikes, peak splitting and integration of complex peak patterns.

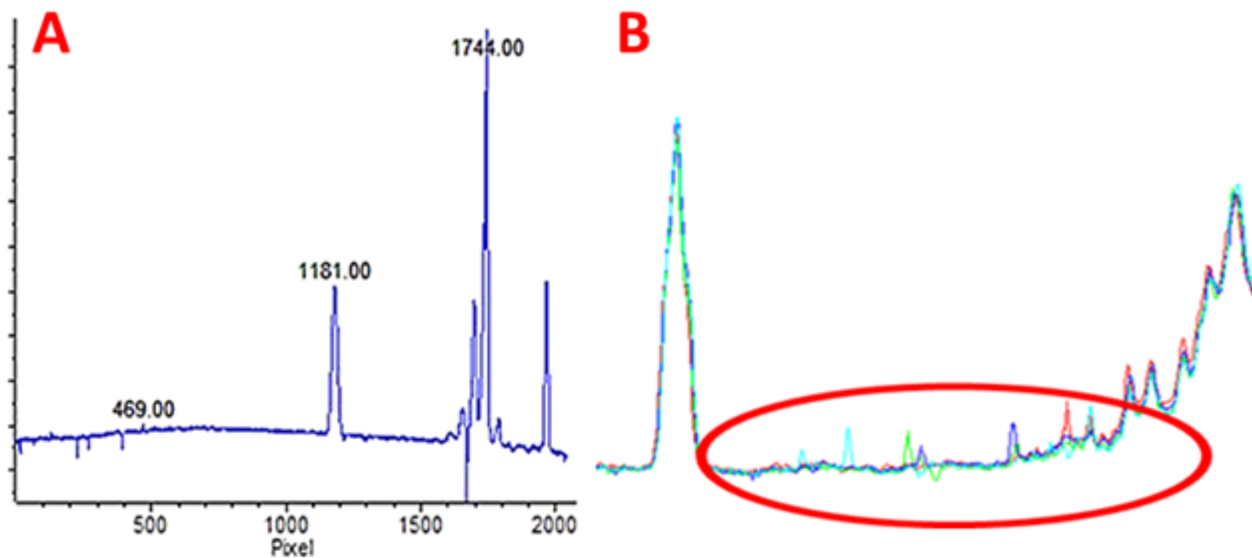

Figure 1: Two examples of spikes in the electropherogram during capillary isoelectric focusing of a protein
A: Air bubbles in the sample
B: (Zoomed) Spikes due to suboptimal conditions and/or protein precipitation

Spikes in the electropherogram

One common troubleshooting issue is the presence of unexplained spikes (positive or negative) in cIEF profiles, as illustrated in Figure 1. It was noted in this example that the spikes appear occasionally, can be random and at different positions and there is nothing in the current trace to suggest errors with the electrophoretic conditions. Spikes in the electropherogram compromise charge heterogeneity assessments, since the spikes could be mistaken and included with peak integration for minor charge variants of the analyte; conversely, minor charge variants could be mistaken as method artefact (i.e., protein signal interpreted as noise).

Additionally they can impede peak integration. The detection method in this example was obtained from whole capillary imaging, and attendees noted spikes are usually caused by air bubbles in the capillary, dust in the optical path, or imperfections (e.g. scratches) on the capillary. Since there is no consistent pattern to the appearance of the spike, it is very unlikely that it is due to a scratch or some other type of physical imperfection either on the capillary or the detector. A blank subtraction is typically performed in the whole capillary imaging format, so minor scratches are usually not an issue since these spikes would be “zeroed out” during blank subtraction. However, air bubble formation or dust particles on the capillary may shift slightly due to vibration and could lead to unpredictable spikes in e-grams.

Examination of the intermediate capillary images taken during separation will help identify whether it is due to dirt or air bubbles (spike will be present at the same location in every image for the former). Centrifuging samples is usually enough to degas and prevent air bubbles, and also serves to settle reagent particulates to the bottom of the sample vial. Likewise, dusting off the capillary and meticulous maintenance of the instrument should alleviate issues of imaging fouling due to particles. Figure 1B shows a complex pI profile of a molecule having multiple peaks, some of which show poor reproducibility between runs and were characterized as spikes. Regardless of the nature of the protein, complex charge heterogeneity profiles such as the one in Figure 1B can sometimes arise due to suboptimal separation conditions and protein precipitation. Additives such as glycerol, urea and methyl urea typically counteract the tendency of proteins to precipitate when they are at or near their pI. While urea concentrations above 3M may result in a loss of resolution, it may also mitigate e-gram spikes due to light scattering from protein precipitates. Another suggestion was to first use orthogonal techniques to explore the aggregation propensity of the protein under the sample buffer conditions used for cIEF. Finally, there was a note to be careful of solubility concerns due to the number of additives and their concentrations, especially if the samples are at low temperature (10°C). It may be that the spikes are caused not by the protein analyte, but by buffer components precipitating out of solution.

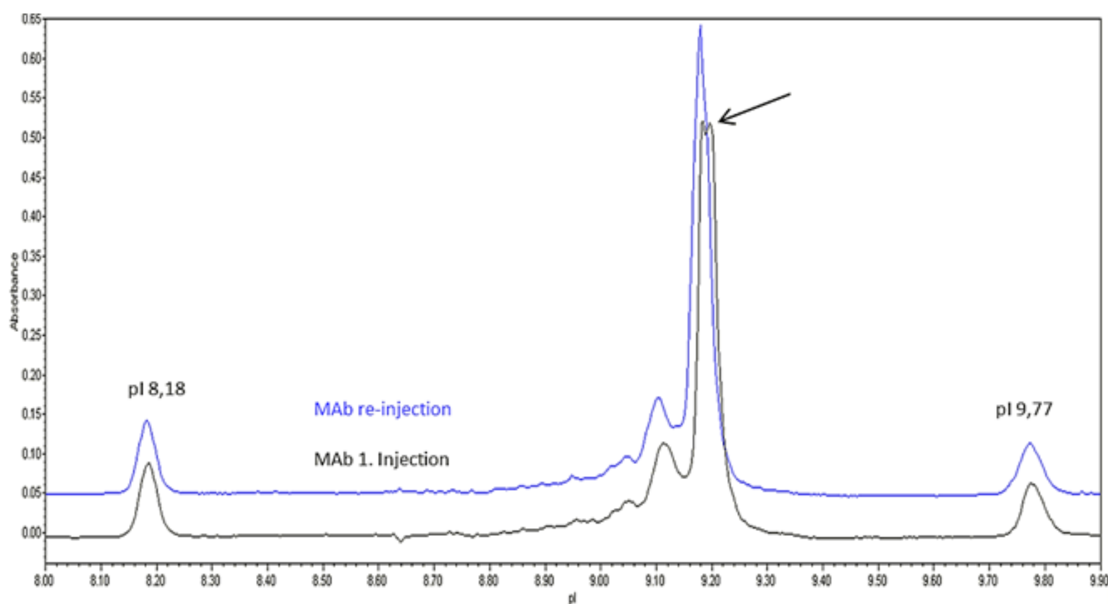

Figure 2: Capillary isoelectric focusing with peak splitting in the first but not the second injection. Sample: Pharmalyte pH 3-10, 1 % methylcellulose, protein concentration 0.3 mg/ml. Focusing: Temperature microinjector: 8 °C, pre-focussing: 1 min at 1500 V, focussing: 7 min at 3000 V.

Peak splitting

Figure 2 details a user example where the initial injection of the sample shows an unexpected split in the main peak, which was not reproduced upon re-injection (blue lane) under the same system parameters. It was noted by the submitter that the product stability, sample matrix and instrument hardware had been ruled out as potential causes of this issue. Attendees suggested, as a general practice, to first check whether this problem could be a result of incomplete focusing. This can be done by checking the e-gram snapshots during the focusing period to confirm whether the anodic and cathodic fronts have merged and the peak pattern is stable during the last snapshots. An examination of snapshots at time points beyond the targeted focusing time is also helpful in confirming the robustness of the experimental conditions in establishing stable focused zones. In this particular case, the other minor peaks are consistent, so incomplete focusing is unlikely to be the cause. Technical experts from the instrument vendor stated the signal intensity was too high and suggested diluting the sample as a fix. Too high a protein concentration could have two effects. First, the protein could saturate the detector and lead to unreliable peak shapes for the most concentrated regions (i.e. at the main peak apex region). Second, a high concentration of the sample in one particular zone of the capillary could lead to a breakdown of the gradient since the sample would exceed the buffering capacity of the ampholytes in the region. This is especially prominent at the basic ranges, where carrier ampholytes have wider amphoteric zones than ampholytes at more neutral pI values (i.e pI 5-8). The height of the main peak in Figure 2 was ~ 0.6 AU, whereas the recommended peak height is 0.2 - 0.4 AU for simple profiles (i.e. one predominant charge variant) and 0.1 AU for complex profiles with many peaks of similar intensity. It was noted that it is sometimes informative to look at the pixel position of the peaks during troubleshooting. The reason is that minor shifts in the pixel position (due to minor differences in the pI gradient) from run to run could cause some runs to focus in a portion of the capillary affected by imperfections/dust, whereas other runs may remain unaffected. Converting the pixel positions into pI units may obscure these differences.

Repeatability

Users also discussed instances of poor reproducibility from repeat injections of the same sample. That is, when the overall peak patterns are similar between injections, but the relative intensity of peaks were variable. This was more frequent for fusion proteins, where the peak patterns are typically more complex than those of antibody therapeutics. Low reproducibility can be caused by protein precipitation during focusing, especially if minor spikes are observed (see, for example, Figure 1B). Thus, low repeatability can be an indication that further optimization of solubility might be beneficial. Users are also encouraged to understand the characteristics of their molecules prior to embarking on DOE experiments to optimize method conditions. For example users should check for aggregation propensity when the protein analyte is at high concentrations and kept at or near its pI. If precipitation is a concern, the user can potentially

mitigate the issue by decreasing protein concentration, increasing ampholyte concentration and/or inclusion of various buffer additives (e.g., urea, PEG, glycerol, formamide).

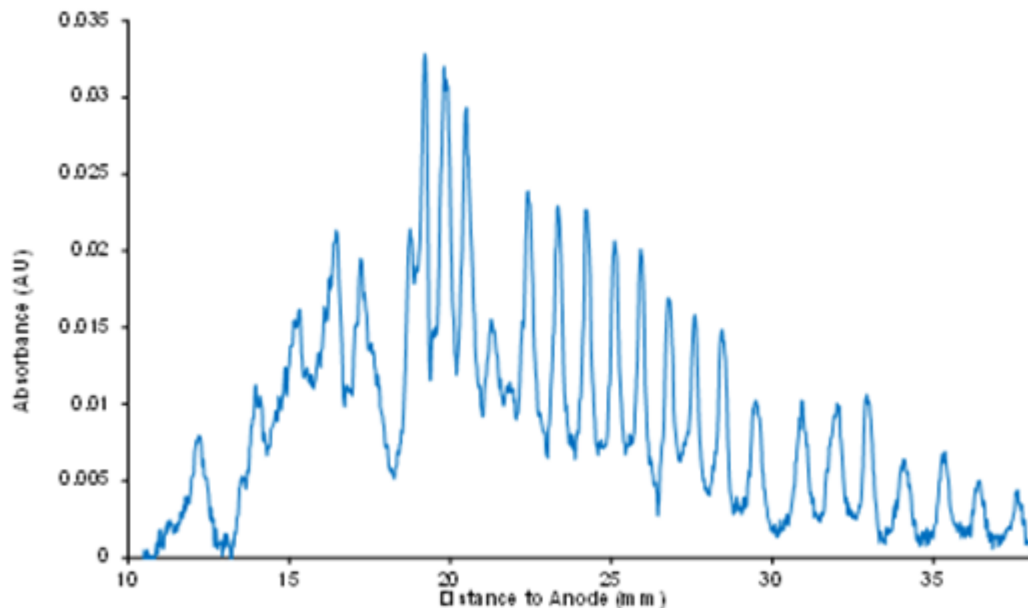

Figure 3: Complex peak patterns can make distinction between acidic, basic and main peaks difficult

Peak pattern

Although not a method troubleshooting topic per se, an ICIEF user submitted a question regarding peak integration and definition for complex profiles (Figure 3). This is a pertinent issue since there is an intrinsic level of charge heterogeneity among many, if not all, biopharmaceuticals. A common practice is to identify the most abundant charge variant peak as the "Main peak" and to group other peaks relative to the main peak, e.g. peaks with a lower pI are grouped into an "acidic region" and higher pIs into a "basic region". This naming convention obviously breaks down for very complex peak profiles or when there is no distinct "main peak". Most attendees agree that the peak definitions can be flexible and suit the needs of the program e.g. ADCs using lysine conjugation have multiple charge variants that reflect different degrees of drug conjugation: grouping these peaks into acidic/main/basic regions would be neither easy nor informative. Complex charge variant profiles on biopharmaceuticals typically result from a high degree of post-translational modification (e.g. sialyation, or small molecules cross-linked to lysine residues). It is important to get an understanding of the nature of the charge variants in order to have a meaningful naming convention for the peaks. To facilitate identification of charge variants, some suggested options are enzymatic treatment (e.g. deglycosidases and carboxypeptidases) of the drug substance, or preparative fractionation followed by MS identification. Likewise, monitoring the change in peak patterns in forced degradation samples can be informative in deciding if there is a "main" species in the unstressed material. No matter the naming convention, the peak pattern should be reproducible and consistent with reference standard. In addition the convention should be clearly communicated and consistently adopted.

Troubleshooting CZE - Material quality

A final topic that was raised was the issue of lot-to-lot consistency of reagents from different vendors, using the specific example of HPMC. HPMC is a common BGE component in CZE separations and while CZE is not an isoelectric focusing technique, it is a CE method that has been traditionally used as an identity method and more recently to assess charge heterogeneity in biopharmaceuticals. Some users had observed that certain lots of HPMC resulted in loss of protein signal from the e-grams whereas others did not. Troubleshooting studies in their labs suggested these lots of HPMC were ineffective in preventing protein adhesion the capillary wall. When BSA was passed through the capillary beforehand, this mitigated the problem seen for the troublesome HPMC lots. Interestingly several labs had observed the same issue, and came to the same conclusions independently. Some attendees pointed to this as an example of how vendors should enforce stricter quality controls and release testing to ensure consistency of their reagents. However, others noted that this may not be a sustainable solution to the problem. Because of the variability of biopharmaceuticals, subtle lot-to-lot variability in a reagent may have a dramatic effect on some drug molecules but not on others. So it may not be commercially viable for the vendor to implement additional release tests without evidence of a broad systemic issue. In addition, the use of HPMC within the CE techniques comprises a negligible market segment for the producer. Instead many users expressed interest in exploring mechanisms for sharing information (e.g. online forums) when these types of reagent issues come up. Certainly, in the case of the HPMC example, it would have helped to alert analytical groups in different companies, potentially reducing the duplication of efforts in troubleshooting.

Concluding Remarks

Capillary isoelectric focusing is a powerful alternative to traditional slab-gel based methods for the assessment of charge heterogeneity in the biopharmaceutical industry. 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.

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