Rapid charge heterogeneity analysis of new therapeutic protein modalities

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Introduction

This technical note reports on the use of the SCIEX CZE Rapid Charge Variant Analysis Kit (P/N C44790) for rapid high resolution charge variant analysis of two bispecific antibodies. Comprehensive charge heterogeneity study of these test articles required < 10 minute total CZE analysis time with excellent migration time (0.2 %RSD) and corrected peak area distribution (<3.5% RSD) reproducibility. The method was also applied to forced degradation based stability testing, especially focusing on deamidation, i.e., generating acidic charge variant shifts.

Charge heterogeneity analysis of new therapeutic protein modalities, such as multispecific antibodies, fusion proteins and nanobodies, is of high importance during product development, production, stability and release testing.[1] Therefore, rapid and reproducible high throughput process analytical technology methods are of high value to biopharma. Analysis of charge variants is also important during forced degradation studies for quality assessment.[2] Charge heterogeneity of these new generation biotherapeutics arises from deamidation, methionine oxidation, C-terminal lysine variants, N-terminal pyroglutamate formation and glycosylation, just to list the most frequent ones.[2-4]

Traditionally used methods for protein charge variant analysis are ion exchange chromatography [5] and various forms of isoelectric focusing [6, 7]. However, both chromatographic and isoelectrophoretic methods are relatively slow, requiring up to 1 hour of analysis time. To alleviate this issue, capillary zone electrophoresis (CZE) was introduced a decade ago for charge heterogeneity analysis of protein therapeutics [8]. In CZE, the differential electromigration of the sample components is based on their hydrodynamic volume to charge ratio, i.e., can be readily optimized for high resolution separation of charge variants. CZE also represents a versatile separation platform to analyze product related attributes with efficient sample preparation, also supporting high throughput screening processes. With the use of high concentration zwitterionic buffer components such as epsilon amino caproic acid and specific capillary coating additives like triethylenetetramine [9], the electroosmotic flow can be minimized during capillary electrophoresis. In this way, bare fused silica capillary columns can be readily employed to obtain rapid analysis of the charge variants of therapeutic proteins.

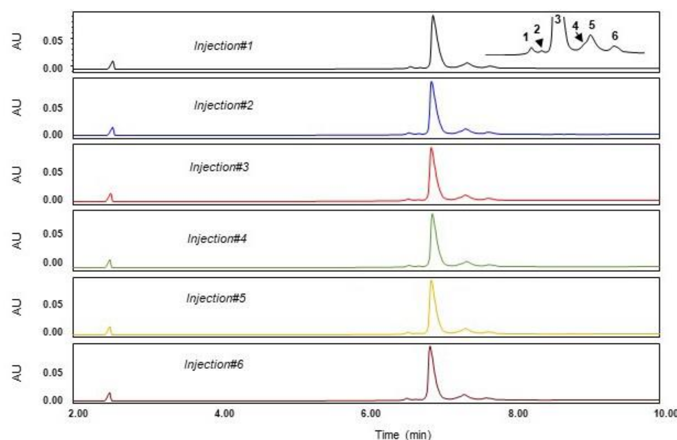

Figure 1. CZE charge heterogeneity analysis and reproducibility study of the bispecific mAb-1 test article. Peaks 1 and 2: fast migrating charge variants, Peak 3: main component, Peaks 4-6: slow migrating charge variants. The inset depicts the enlarged part of the electropherogram with the peaks numbered. Conditions: 30 cm total capillary length (20 cm effective, 50 µm ID), applied electric field strength: 1000 V/cm, Separation temperature: 25ºC. Pressure injection at 5 psi for 10 sec.

Rapid charge variant analysis of two bispecific antibodies was demonstrated revealing highly reproducible separations in less than 10 minutes with excellent migration time and corrected peak area distribution reproducibility. The method was readily applied for forced deamidation monitoring.

Key features

- Rapid analysis times (<10 minutes) of the native forms of new modality protein therapeutics
- Excellent migration time (0.2 %RSD) and corrected peak area (1.55 %RSD) reproducibility
- Easy sample preparation protocol for low concentration samples
- High throughput analysis option for stability testing

Experimental

Chemicals

TRIS base and hydrochloric acid (37%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The bispecific monoclonal antibody test articles of mAb-1 and mAb-2 were from Genentech (South San Francisco, CA) and Amgen (Thousand Oaks, CA), respectively. To perform the charge heterogeneity separation of new modalities, the CZE Rapid Charge Variant Analysis Kit (SCIEX, Brea, CA, part#C44790) was used consisting of CZE Rapid Charge Variant Separation Buffer, Acid Wash/Regenerating Solution, CE Grade Water and Protein Test Mix.

Sample preparation

Test article mAb-1 (30 mg/mL) was diluted with water to 1 mg/mL concentration and analyzed by CZE. Test article mAb-2 had significantly lower concentration of (0.2 mg/mL) thus to avoid matrix related disturbances, it was buffer exchanged prior to CZE analysis by washing the therapeutic protein three times with 100 µL of water through a 10 kDa cut-off centrifugation filter (Millipore, Billerica, MA) at 14 000 x g. The purified protein was recovered in the same volume as the volume dispensed onto the filter in the formulation buffer.

In order to perform forced deamidation mAb-1 sample, Tris-HCl buffer was prepared at 1 M concentration by carefully adding concentrated hydrochloric acid solution to TRIS to adjust the pH at 8.7. The solution of test article mAb-1 was diluted with water and 1 M Tris-HCl buffer (pH 8.7) to obtain a 1 mg/mL protein solution in 100 mM Tris-HCl buffer. The diluted sample solution of the therapeutic protein was incubated in a heating block at 45 ^{o}C and samples were taken for CZE separation after 1, 2 and 3 days of incubation times and buffer exchanged on 10 kDa cut-off filters prior to analysis.

Capillary zone electrophoresis

All separations were carried out in a PA 800 Plus Pharmaceutical Analysis System (SCIEX) using the EZ-CE Pre-Assembled Capillary Cartridge (SCIEX, part# A55625) with 30 cm total capillary length (20 cm effective length, 50 µm ID). The separation voltage was 30 kV at 25°C. Injection: 5 psi for 10 sec for mAb-1 and 20 sec for mAb-2. All data was acquired and processed by the 32Karat ver10.1 software package (SCIEX).

Results and discussion

The charge heterogeneity of two bispecific antibody test articles was assessed by CZE with special attention to migration time and corrected peak area reproducibility. Forced degradation of the samples were also accomplished.

The CZE charge variant analysis of test article mAb-1 was accomplished in less than 8 minutes, featuring the separation of 6 peaks as shown in Figure 1. The inset depicts the enlarged part of the electropherogram with the peaks numbered. Peaks 1 and 2 were faster migrating possible basic species, Peak 3 the main component and Peaks 4-6 were slower migrating, possible acidic species. To verify the acidic and/or basic feature of these components, capillary isoelectric focusing analysis is required, which was outside of the scope of this exercise. The 6 consecutive runs of the test article mAb-1 resulted in the average migration time and corrected peak area % reproducibility of 0.2 %RSD (Table 1) and 1.55 %RSD (Table 2), respectively.

Next the therapeutic bispecific antibody test article mAb-2 was analyzed the same way but, in this instance, sample injection without appropriate preparation did not result in evaluable electropherogram (Figure 2, trace A). Removal of the sample formulation matrix, on the other hand, lead to the separation of 5 components as shown in Figure 2, Trace B in less than 6 minutes. Peak 1 was a faster migrating possibly basic ingredient, Peak 2 was the main component and Peaks 3- 5 were slower migrating possibly acidic species. The migration time and corrected peak area % reproducibility were evaluated similarly to those discussed above and were found as 0.28 %RSD and 1.58 %RSD, respectively.

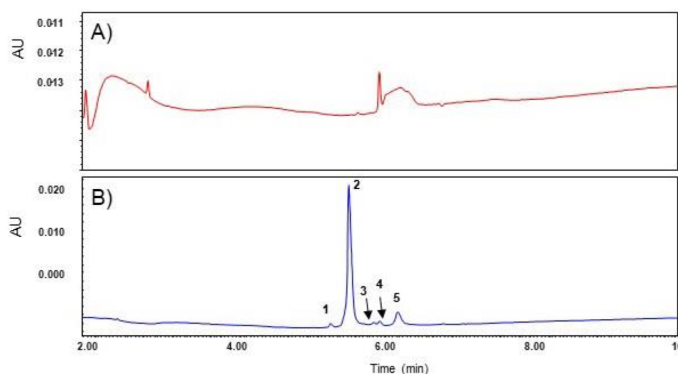

Figure 2. Effect of sample preparation on the charge heterogeneity. Analysis of the mAb-2 test article without (A) and with (B) formulation matrix removal. Separation conditions were the same as in Figure 1, except the injection parameters: 5 psi/20 sec.

Monitoring deamidation of therapeutic proteins provides crucial information about the efficacy of the product. To show the applicability of the rapid capillary zone electrophoresis to address this significant issue, a forced degradation study was performed at pH 8.7 to promote accelerated deamidation using the mAb-1 test article sample. In the time course study, samples were taken at 0 (control), 1-day, 2-day and 3-day time points and analyzed by the CZE method described above. Figure 3 depicts the resulting quantitative data of the separated species. Please note that with increasing incubation time, the shoulder Peak 4 slowly merged into Peak 5, thus, during quantitative evaluation these were considered as a single value (Figure 3, column 4+5). As one can observe, the areas of fast migrating Peak 1 and the main component (Peak 3) decreased, while Peaks 4+5 and 6 increased over the time course of the study. As a first approximation we consider this effect was caused by temperature induced deamidation under high pH conditions, resulting in the increase of the slower migrating, probably acidic components. While this treatment caused deamidation of both Peaks 1 and 3, Peak 2 did not change during this process.

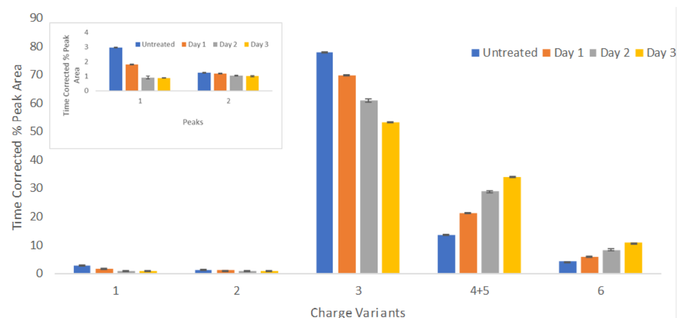

Figure 3. Quantitative CZE analysis of the forced deamidation. Charge variant products of mAb-1 test article in 100 mM Tris buffer (pH 8.7) after 1 (red bar), 2 (gray bar) and 3 (yellow bar) days of treatment in comparison to the untreated (blue bar) sample. Charge variant peaks 1 and 2: slower migrating components, peak 3: main component, Peaks 4+5 and 6: slower migrating components. The inset depicts the enlarged part of the changes in peaks 1 and 2.

Table 1. Migration time reproducibility of the separated charge variants of test article mAb-1 in Figure 1.

Migration time (min)									
Peaks	Injection#1	Injection#2	Injection#3	Injection#4	Injection#5	Injection#6	Average	STDEV	% RSD
1	6.60	6.58	6.58	6.57	6.57	6.55	6.58	0.01	0.22
2	6.73	6.71	6.71	6.70	6.70	6.68	6.71	0.02	0.26
3	6.91	6.89	6.90	6.89	6.88	6.87	6.89	0.01	0.18
4	7.31	7.29	7.30	7.29	7.29	7.27	7.29	0.01	0.19
5	7.37	7.35	7.37	7.35	7.35	7.33	7.35	0.01	0.17
6	7.68	7.66	7.67	7.66	7.65	7.63	7.66	0.02	0.20

Average: 0.20 %

Table 2 Corrected peak area reproducibility values of the separated charge variants of test article mAb-1 in Figure 1.

Time corrected % peak area									
Peaks	Injection#1	Injection#2	Injection#3	Injection#4	Injection#5	Injection#6	Average	STDEV	% RSD
1	2.94	2.94	2.97	3.02	2.96	2.99	2.97	0.03	0.94
2	1.21	1.23	1.22	1.25	1.28	1.29	1.25	0.03	2.35
3	77.74	77.79	77.84	77.90	77.75	77.92	77.82	0.07	0.09
4	3.54	3.37	3.39	3.39	3.65	3.31	3.44	0.12	3.35
5	10.21	10.37	10.30	10.28	10.09	10.32	10.26	0.09	0.88
6	4.36	4.29	4.27	4.16	4.26	4.17	4.25	0.07	1.66

Average: 1.55%

Conclusions

- Rapid charge variant analysis of two new modality protein test articles was demonstrated.
- Highly reproducible separations were obtained for both bispecific antibodies in less than 8 minutes with excellent migration time (0.2% RSD) and corrected peak area distribution (<3.5% RSD) reproducibility.
- The importance of sample preparation by removing the formulation matrix was apparently important for the lower concentration mAb-2 test article.
- Charge variant changes were systematically monitored after forced deamidation of the mAb-1 test article in a 3-day time course revealing significant decrease in the quantity of the main components towards the slower migrating, probably acidic species.
- The CZE technique reported here can be readily applied for high throughput charge heterogeneity analysis of new therapeutic modalities.

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References

1. Singh, S.K., G. Narula, and A.S. Rathore, Should charge variants of monoclonal antibody therapeutics be considered critical quality attributes? Electrophoresis, 2016. 37(17-18): p. 2338-46.

2. Hsu, Y.R., et al., Selective deamidation of recombinant human stem cell factor during in vitro aging: isolation and characterization of the aspartyl and isoaspartyl homodimers and heterodimers. Biochemistry, 1998. 37(8): p. 2251-62.

3. Harris, R.J., Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. J Chromatogr A, 1995. 705(1): p. 129-34.

4. Moorhouse, K.G., et al., Validation of an HPLC method for the analysis of the charge heterogeneity of the recombinant monoclonal antibody IDEC-C2B8 after papain digestion. J Pharm Biomed Anal, 1997. 16(4): p. 593-603.

5. Stoll, D., et al., Characterization of therapeutic antibodies and related products by two-dimensional liquid chromatography coupled with UV absorbance and mass spectrometric detection. J Chromatogr B Analyt Technol Biomed Life Sci, 2016. 1032: p. 51-60.

6. Righetti, P.G., Determination of the isoelectric point of proteins by capillary isoelectric focusing. J Chromatogr A, 2004. 1037(1-2): p. 491-9.

7. Sosic, Z., et al., Application of imaging capillary IEF for characterization and quantitative analysis of recombinant protein charge heterogeneity. Electrophoresis, 2008. 29(21): p. 4368-76.

8. He, Y., et al., Analysis of identity, charge variants, and disulfide isomers of monoclonal antibodies with capillary zone electrophoresis in an uncoated capillary column. Anal Chem, 2010. 82(8): p. 3222-30.

9. Moritz, B., et al., Evaluation of capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies. J Chromatogr B Analyt Technol Biomed Life Sci, 2015. 983-984: p. 101-10.

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