Journal of Chromatography B, 906 (2012) 63–68

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a rapid capillary zone electrophoresis method for determining charge variants of mAb

Ying Shi[a], Zhen Li[a], Yuanbiao Qiao[b], Jun Lin[c,*]

[a] Department of Biology, Taiyuan Normal University, 030031 Shanxi, China
[b] Graduate Institute of Pharmaceutical Chemistry, Luliang University, 033000 Shanxi, China
[c] Analytical Sciences, Interbio Co. Ltd., 201203 Shanghai, China

ARTICLE INFO

Article history:
Received 26 December 2011
Accepted 16 August 2012
Available online 23 August 2012

Keywords:
Capillary zone electrophoresis
Therapeutic monoclonal antibody
Optimization
Validation
Application

ABSTRACT

This work aimed to develop a rapid capillary zone electrophoresis (CZE) method to provide abundant purity and identity information of monoclonal antibodies. The CZE running buffer system was optimized to be 20 mM acetate–acetic acid (pH 6.0) together with the co-addition of 0.3% polyethylene oxide (PEO) and 2 mM triethylenetetramine (TETA), which was further tested with advantages on the peak resolution improvements. The conditioning period was scheduled to 1 min for both 0.1 M HCl and CZE running buffer to reduce total separation time. Additionally, the applied voltage and effective separation length were optimized at 30 kV and 20 cm separately. Compared with the method reported by Yan [1], this newly developed method showed a higher resolution in separating the two unknown basic peaks by testing monoclonal antibody sample (mAb1). The further validation results showed that for all five of charge isoform peaks of test mAb1, repeatability, intraday and interday precision had a RSD less than 0.58% for migration time and less than 3.18% for corrected area percent. The correlation coefficients of more than 0.98 for all peaks also demonstrated the good linearity for the method. In addition to the application of distinguishing intact antibody from C-terminal Lys variants, the method also has advantage in separating the Fab, Fc and intact antibody-relevant substances quickly, which facilitated the rough evaluation of papain induced digestion.

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1. Introduction

Monoclonal antibodies (mAbs) have emerged as one of the most promising classes of therapeutics in the biopharmaceutical industry because of their advantages of being highly specific and having limited side effects. As these complex compounds often undergo post-translation like modification, deamidation, oxidation, glycosylation, mismatched disulfide bond, and aggregation [2–8], it can be very challenging to completely characterize the heterogeneity of mAbs.

Charge variants, as one kind of product heterogeneity, have been reported to correlate with biological activity and stability. The N-linked glycosylation pattern has received significant attention as it greatly influenced the affinity for Fcγ receptors (FcγR) which mediated antibody dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytoxicity (CDC) functions [9,10]. Methionine oxidation is another chemical modification known to change the stability and conformation of the CH_2 domain [11–13].

Therefore, many sophisticated techniques and methods have been required to detect the charge heterogeneity of mAbs. One of the traditional charge based methods has been slab gel isoelectric focusing (IEF), which provides high-resolution separation but can be time-consuming, labor-intensive and difficulty of quantitation. As an alternative, capillary isoelectric focusing (cIEF) was later developed and applied for the separation and analysis of charge variants of compounds in the areas of biochemical and biological research. The method allows for easy of quantitation, however, can take more than 45 min to accomplish the focusing and mobilization step for each separation. Many examples of application of the traditional cIEF method to monitor charge heterogeneity have been reported [14,15]. Imaging cIEF with short capillary and whole-column detection which could collect online focusing information and data directly was developed and avoided traditional mobilization problems. iCE280, the equipment manufactured by ProteinSimple (Santa Clara, CA, USA), was just based on this Imaging cIEF mechanism. Several applications of this method for charge

Abbreviations: ADCC, antibody dependent cell-mediated cytotoxicity; CDC, complement dependent cytoxicity; CE, capillary electrophoresis; cIEF, capillary isoelectric focusing; CPB, carboxypeptidase B; CZE, capillary zone electrophoresis; EACA, 6-aminocaproic acid; EOF, electroosmotic flow; Fab, fragment antigen-binding; Fc, fragment crystalline; HPMC, (hydroxypropyl)methyl cellulose; IEC, ion exchange chromatography; IEF, isoelectric focusing; mAb, monoclonal antibody; PEO, polyethylene oxide; TETA, triethylenetetramine.

* Corresponding author at: 899 Ha-lei Road, Shanghai 201203, China.
E-mail address: linmuyu0708@gmail.com (J. Lin).

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http://dx.doi.org/10.1016/j.jchromb.2012.08.022

heterogeneity analysis of mAb products using iCE280 have been described [16,17].

Capillary zone electrophoresis (CZE) separations are based on differences in electrophoretic mobility of charge isoforms, which are greatly influenced by both the molecular charge and the hydrodynamic radius of the protein. Nowadays, this method has been increasingly accepted as attractive alternatives to slab gel isoelectric focusing (IEF) and ion exchange chromatography (IEC) to assess charge heterogeneity of proteins. Dai [18] used the capillary zone electrophoresis (CZE) method to separate and quantitate the monoclonal antibodies in cell growth medium. Later Ma and Nashabeh [19] developed capillary zone electrophoresis (CZE) as a tool for the analysis of charge heterogeneity of therapeutic mAbs in a permanently coated capillary to minimize protein adsorption to the inner wall of the capillary. Yan [1,20] developed and optimized the CZE method in dynamically coated fused silica capillary to reduce separation time and cost.

In this work, the composition of the CZE running buffer and several parameters including effective separation length and applied voltage were developed and optimized to accomplish the high resolution separations in less than 10 min. Especially when used in analyzing the test sample mAb1, this newly developed method exhibited higher resolution for separation of the two unknown basic peaks compared with the similar method reported [1]. This promising platform method can be applied in many areas including charge variants analysis, high-throughput clone screening, product characterization, etc.

2. Experimental

2.1. Materials

All chemicals used were of analytical reagent grade. Bare fused silica capillaries were from Beckman-Coulter (Brea, CA, USA). Polyethylene oxide (PEO, Code No. 182028, average MW 600,000), triethylenetetramine (TETA, Code No. 90460), (hydroxypropyl)methyl cellulose (HPMC, Code No. H7509), 6-aminocaproic acid (EACA, Code No. A2504), papain (Code No. 76218, 12 U/mg protein) and carboxypeptidase B (CPB, Code No. C9584, ≥125 U/mg protein) were from Sigma–Aldrich (St. Louis, MO, USA). Commercial mAb1 (IgG1, CD20 targeted, 10 mg/ml, p*I* 9.2), mAb2 (IgG1, Her-2 targeted, 21 mg/ml, p*I* 9.0), mAb3 (IgG1, TNF-α targeted, 50 mg/ml, p*I* 8.9), and mAb4 (IgG1, TNF-α targeted, 10 mg/ml, p*I* 7.3) were from Roche (Basel, Switzerland), Roche (Basel, Switzerland), Abbot (North Chicago, IL, USA) and Johnson & Johnson (New Brunswick, NJ, USA). Other regents such as Tris/HCl, NaCl, EDTA, and L-cysteine, were commercially available.

2.2. CZE running buffer preparation

A mixture running buffer system was composed of 20 mM NaAc, 0.3% polyethylene oxide (PEO) and 2 mM triethylenetetramine (TETA), and its pH was adjusted to 6.0 by addition of hydrochloric acid.

2.3. Sample preparation

All the samples were diluted to 1 mg/ml with deionized distilled water.

2.4. CZE analysis in PA 800 plus platform

CZE analysis was carried out using PA 800 plus system (Beckman-Coulter) with a bare fused-silica capillary (Beckman-Coulter: 50 μm *i.d.*, 30.2 cm total length, 20.0 cm effective length) and a 214 nm UV detector. Data were collected and analyzed using 32 Karat software (Beckman-Coulter, version 10.0). Before the experiment, the capillary was first preconditioned by flushing deionized distilled water, 0.1 M HCl, and CZE running buffer for 5 min at a pressure of 50 psi separately and then applying a normal polarity voltage of 30 kV for 10 min. Conditioning steps before each injection was performed by flushing the capillary with 0.1 M HCl and running buffer for 1 min at a pressure of 70 psi, respectively. After sample was injected for 10 s at a pressure of 0.5 psi, separation was performed at 30 kV in normal polarity for 10 min. All steps were conducted at 20 °C and the data acquisition frequency was 8 Hz.

2.5. CPB enzymatic reaction

200 μg of mAb4 was mixed with 200 μl of CPB buffer (25 mM Tris/HCl buffer, pH 7.5, containing 0.1 M NaCl) and 4 μl of CPB (8.1 U/ml, 1 mg CPB dissolved in 10 ml CPB buffer). The resulting solution was incubated at 37 °C for 2 h. As a control sample, water was added instead of the enzyme. After incubation, the buffer system was changed into water using ultrafiltration membrane (Vivaspin 500, 10 kDa cutoff, recovery of 95–98%, Sartorius) with the final protein concentration of 1 mg/ml.

2.6. Papain enzymatic reaction

5 μl of papain (1 U) and 200 μg of mAb2 were added to the digestion buffer (100 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM L-cysteine) and then incubated at 37 °C for 24 h. As a control sample, water was added instead of the enzyme. Following incubation, the buffer system was changed into water using ultrafiltration membrane (Vivaspin 500, 10 kDa cutoff, recovery of 95–98%, Sartorius) with the final protein concentration of 1 mg/ml.

3. Results and discussion

3.1. Optimization of CZE running buffer composition

3.1.1. Background electrolyte composition

As the basis for assay optimization, a reasonable background electrolyte capable of providing steady low current and good separation was definitely important in our work. Common acidic running buffers involving acetate–acetic acid, citrate–citric acid, iminodiacetic acid and glycine–HCl at fixed concentration of 20 mM, pH 3.0–5.0 (pH adjusted by addition of glacial acetic acid) were first investigated to determine whether they met the above requirements. The separation current was unstable at the applied high voltage of 30 kV when utilizing iminodiacetic acid and citrate–citric acid buffers. In contrast, 20 mM acetate–acetic acid and glycine–HCl buffers at the different tested conditions (pH 3.0, 4.0, and 5.0) all provided steady current, among which 20 mM acetate–acetic acid, pH 5.0 provided lower current than any of the other buffers tested at the voltage of 30 kV. Furthermore, the separation profile acquired by utilizing this buffer was better than the others tested, though the separation still required further improvement (data not shown). Therefore, 20 mM acetate–acetic acid, pH 5.0 was chosen as the background electrolyte.

3.1.2. Addition of linear polymers and polyamines

Linear polymers are thought to form a dynamic protecting layer in the inner surface of the capillary and polyamines competed with the protein to interact with the surface Si—OH group. Use of both substances as additives could therefore help enhance CE-based protein separations. However, if the inner Si—OH of a new capillary was highly ionized during alkali preconditioning, adding polymers and polyamines would have little effect on the efficiency of suppressing EOF and inhibiting proteins from adsorption to the capillary wall.

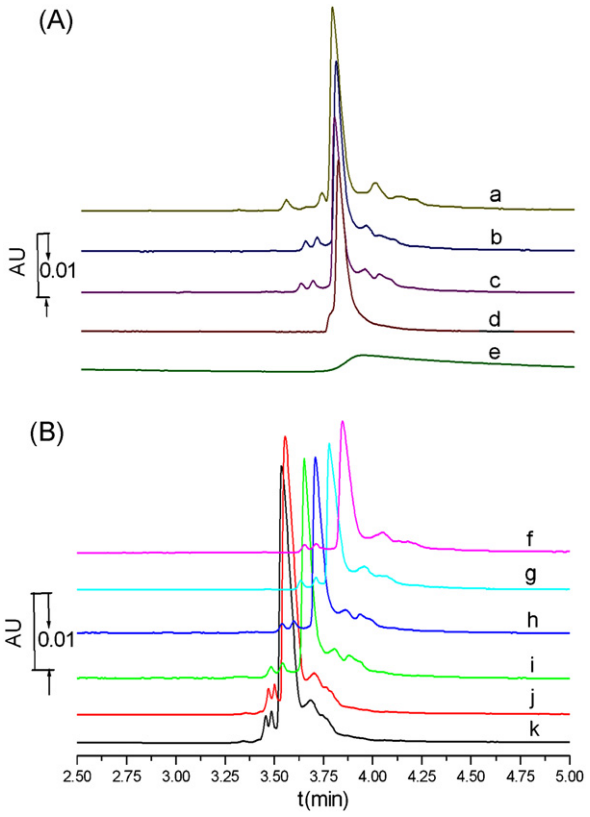


Fig. 1. Effect of TETA and PEO concentrations on the resolution of the separation: (A) TETA effect, (B) PEO effect, (a) 3 mM TETA, 0.5% PEO, (b) 2 mM TETA, 0.5% PEO, (c) 1 mM TETA, 0.5% PEO, (d) 0 mM TETA, 0.5% PEO, (e) 0 mM TETA, 0.5% PEO, capillary preconditioned by alkali, (f) 2 mM TETA, 0.6% PEO, (g) 2 mM TETA, 0.5% PEO, (h) 2 mM TETA, 0.4% PEO, (i) 2 mM TETA, 0.3% PEO, (j) 2 mM TETA, 0.2% PEO, and (k) 2 mM TETA, 0.1% PEO. Background electrolyte: 20 mM acetic–acetate, pH 5.0; capillary effective length: 20 cm; voltage: +30 kV; injection: 0.5 psi for 10 s; test sample: mAb1. All the capillaries were preconditioned using acidic buffer with the exception of trace E.

In our experiments, combinations of a linear polymer (0.1–0.6% PEO) and one polyamine (1–3 mM TETA) were added into the background electrolyte to improve the separation and the resulting electropherogram were compared. As shown in Fig. 1, alkali preconditioned capillaries failed to separate the charge variants, possibly due to the interaction of proteins with highly negatively charged surface of these capillaries. On the contrary, the separations were much better for acid preconditioned capillaries. In addition to preconditioning, adding TETA also had an obvious influence on the separation resolution of the charge variants peaks. When no TETA was added, both basic and acidic peaks which migrated before and after main peak respectively, failed to separate from the main peak. The resolution greatly improved after increasing the TETA concentration to 1 mM and 2 mM. When 2 mM TETA was added, the 5 peaks including 2 basic peaks, 2 acidic peaks, and 1 main peak were well separated from each other. However, when adding 3 mM TETA, some changes of charge variants profiles were observed unexpectedly. This may have been induced by the interaction of the excess amines from TETA with carboxyl groups of the protein samples, possibly modifying the charge heterogeneity of the test samples. Therefore, the optimal concentration of TETA was found to be 2 mM. Similarly, the impact of PEO concentrations on the separation performance including resolution and peak areas was investigated. Fig. 1 shows that the conditions of adding 0.1% and 0.2% PEO both failed to acquire acceptable resolution for the 2 basic peaks. While the separation performed much better upon the addition of 0.3% PEO. When the PEO concentrations increased from 0.3% to 0.5%, the separation resolution improved, however, the migration time increased and the peak areas decreased. This may be explained by an increase in buffer viscosity due to addition of more PEO, thus possibly decreasing the actual amount injected. Increasing the injection time or injection pressure resulted in larger peak areas but also wider peaks as the samples tended to broaden when the injection time was too long (data not shown). Meanwhile, the condition of adding 0.6% PEO was left out of consideration as it had no improvement on resolution and more separation time. To balance the resolution and migration time and peak areas, 0.3% PEO was chosen as the composition of the CZE running buffer.

3.1.3. pH of CZE running buffer

Protein isoform charge varied with buffer pH conditions which would affect the charge/mass based capillary electrophoresis separation and lead to varied migration times and peak resolutions. Most of the commercially available mAb samples whose isoelectric point (p*I*) were over 7 were positively charged in acidic buffer solutions. The charge of a protein, depending on their pH environment, significantly influenced migration time and separation resolution. When the buffer pH was farther from that of the protein p*I*, its charge increased and its migration time decreased, however, separation resolution was poor. Balance was therefore needed to ensure a rapid separation while not sacrificing the resolution of the samples. CZE running buffers were adjusted to pH 4.5, 5.0, 6.0, 7.0 and 8.0 separately by addition of hydrochloric acid, among which the condition of pH 6.0 assured both the fast separation (less than 5 min) and good peak resolution. Therefore in the following experiments, the pH 6.0 CZE separation buffers were used. The electropherograms acquired by testing the various pH conditions were shown in Fig. 2.

3.2. Optimization of CZE separation conditions

Applied voltage and effective separation distance influenced the speed and resolution of separations adversely. In this case, a high speed separation while not sacrificing resolution was achieved by choosing reasonable applied voltage and effective separation distance. As seen in Fig. 2, samples migrated earliest when applying short end injection and performing separation with an effective capillary length of 10 cm and reverse voltage of 30 kV, however, the resolution of charge variants peaks was unacceptable. In contrast, the results of using a 20 cm effective capillary length were improved as all the charge variants peaks separated well. With the fixed effective length of 20 cm, the separation was faster when applying high voltage of 30 kV compared to that of 20 kV and 25 kV. Thus 20 cm effective length with applied voltage of 30 kV was finally selected as our routine analysis conditions. This newly developed and optimized method was compared with a similar reported method [1] by testing the same sample mAb1. The results suggested that our method had advantages over the reported method in the separation resolution for the charge variants. Notably, our newly developed method facilitated the further characterization of the two basic peaks, which were relatively difficult to separate utilizing the referred method. The electropherograms of the two methods were shown in Fig. 3.

3.3. Qualification of the method

3.3.1. Specificity

The purpose of the specificity test is to discriminate between the molecule of interest and other molecules that have potentially similar profiles. In this study, 5 samples were tested, including mAb1 (Lot.2), mAb2, mAb1 (Lot.1), mAb3, and mAb4. As shown in Fig. 4, the 5 samples had significantly different CZE profiles. Interestingly, for the 2 lots of mAb1, though with same migration time of the main peak, they had quite great observable differences in CZE profiles.

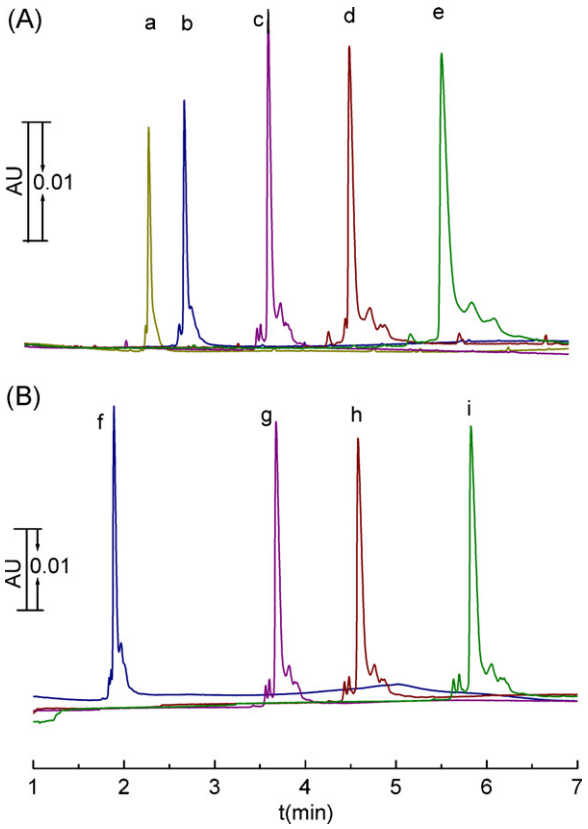


Fig. 2. Effect of pH, capillary effective length, and voltage on the migration time and resolution of the separation, (A) pH effect, (B) capillary effective length and voltage effect, (a) pH 4.5, effective length of 20 cm, +30 kV, (b) pH 5.0, effective length of 20 cm, +30 kV, (c) pH 6.0, effective length of 20 cm, +30 kV, (d) pH 7.0, effective length of 20 cm, +30 kV, (e) pH 8.0, effective length of 20 cm, +30 kV, (f) pH 6.0, effective length of 10 cm, −30 kV, (g) pH 6.0, effective length of 20 cm, +30 kV, (h) pH 6.0, effective length of 20 cm, +25 kV,and (i) pH 6.0, effective length of 20 cm, +20 kV. All other experimental conditions were as in Fig. 1.

These differences, mainly induced by basic peaks, could be further characterized by CPB treatment or other experiments, however, at least demonstrated the lot-lot inconsistence for this product. This method can therefore be highly sensitive to justify the specific molecule quality and to monitor in-process changes.

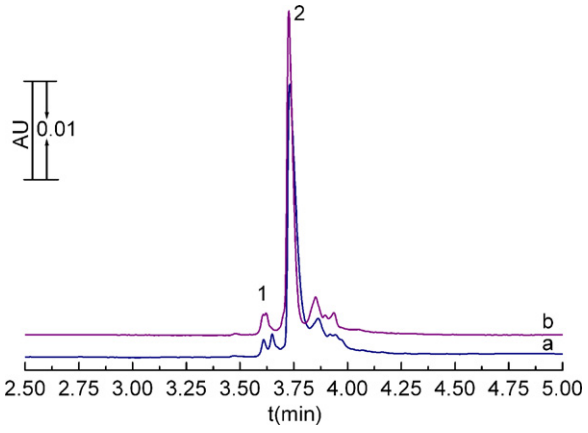


Fig. 3. Charge variants separations conducted by using two methods, (a) our newly developed method, CZE running buffer: 20 mM acetic–acetate, pH 6.0, 2 mM TETA, 0.3% PEO, (b) reference reported method, CZE running buffer: 400 mM EACA, pH 5.7, 2 mM TETA, 0.05% HPMC, (1) basic peaks, and (2) main peak. Capillary effective length: 20 cm; voltage: +30 kV; injection: 0.5 psi for 10 s; test sample: mAb1.

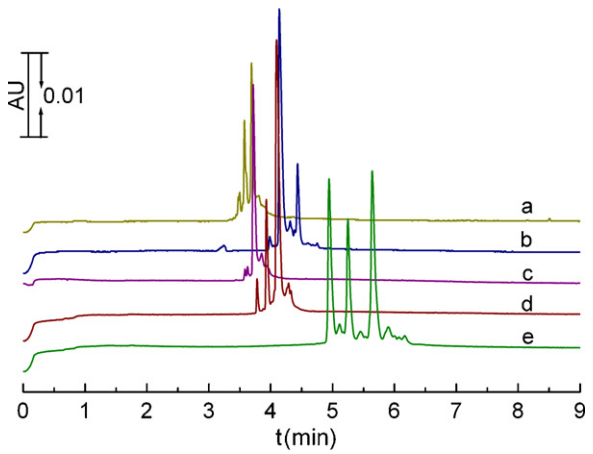


Fig. 4. Specificity test of the CZE method. (a) mAb1(Lot.2), (b) mAb2, (c) mAb1(Lot.1), (d) mAb3, (e) mAb4. CZE running buffer: 20 mM acetic–acetate, pH 6.0, 2 mM TETA, 0.3% PEO. Capillary effective length: 20 cm; voltage: +30 kV; injection: 0.5 psi for 10 s.

Table 1
Migration time precision tests of the CZE method for mAb1.

	Basic peak1	Basic peak2	Main peak	Acidic peak1	Acidic peak2
Intraday precision[a]					
Mean (min)	3.57	3.61	3.68	3.82	3.89
RSD (%)	0.19	0.19	0.13	0.22	0.24
Interday precision[b]					
Mean (min)	3.57	3.61	3.69	3.83	3.89
RSD (%)	0.50	0.52	0.54	0.58	0.57
Repeatability[c]					
Mean (min)	3.57	3.61	3.69	3.83	3.90
RSD (%)	0.25	0.26	0.28	0.30	0.30

[a] One sample preparation and six replicate injections in one day.
[b] One sample preparation, three injections per day for three consecutive days.
[c] Six sample preparation and one injection each in one day.

3.3.2. Precision

Intraday precision was evaluated by performing a single sample preparation and six replicate injections in the same day. Interday precision was evaluated by performing one sample preparation and three injections per day for three consecutive days. Repeatability was assessed by six sample preparation and one injection each in one day. The test sample for all the above experiments was mAb1. As shown in Tables 1 and 2, small migration time and corrected area (peak area divided by migration time) deviations assured good intraday, interday precisions and repeatability (RSD < 3.18%) of the method.

Table 2
% Corrected area precision tests of the CZE method for mAb1.

	Basic peak1	Basic peak2	Main peak	Acidic peak1	Acidic peak2
Intraday precision[a]					
Mean (%)	2.35	3.51	74.96	11.76	7.42
RSD (%)	1.84	0.46	0.28	1.81	0.61
Interday precision[b]					
Mean (%)	2.32	3.66	75.23	11.49	7.30
RSD (%)	1.50	3.15	0.50	3.18	1.86
Repeatability[c]					
Mean (%)	2.33	3.76	75.30	11.34	7.28
RSD (%)	1.44	0.76	0.17	1.00	0.49

[a] One sample preparation and six replicate injections in one day.
[b] One sample preparation, three injections per day for three consecutive days.
[c] Six sample preparation and one injection each in one day.

3.3.3. Linearity

Linearity was assessed by varying the mAb1 concentration from 2 mg/ml down to 0.3 mg/ml. The concentration levels were: 0.3 mg/ml, 0.5 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 1.5 mg/ml, and 2.0 mg/ml, for which 3 preparations and the following 1 injection each were performed. The corrected areas of acidic peaks, main peak, and basic peaks were plotted against the protein concentration, respectively. Our method showed a good linearity for detected peaks within the concentration range of 0.3–2.0 mg/ml, including acidic peaks (R^2 = 0.9941), main peak (R^2 = 0.9905) and basic peaks (R^2 = 0.9892). The linearity criteria range is a little narrow as the high concentration should not be more than 2.0 mg/ml. Larger amounts of samples injected would probably compete with TETA to interact with surface ionized silanol and thus adsorptions of proteins to the capillaries increase. Furthermore, corrected area percentages for acidic peaks, main peak, and basic peaks plotted against variant concentrations resulted in almost 3 horizontal lines, which indicated that peaks percentage measurement maintained constant with these protein concentrations.

3.3.4. LOD/LOQ

The LOD/LOQ of the method were calculated when S/N of main peak of the test sample mAb1were 3 and 10 separately. Noise on the baseline was calculated for sample buffer at 3.5–4.0 min when the main peak should appear. S/N of HC was calculated to be 167.5 while the test sample concentration was 0.3 mg/ml. When extrapolated to S/N ratio of 3:1 and 10:1, the concentration of LOD and LOQ was 5.4 μg/ml and 17.9 μg/ml separately. However, sensitivity for detection of charge impurities is critical in a purity determining method. In this case, the basic peak1, which had the smallest peak area among the charge variants, influenced the results and potentially overestimated the determined purity value if it was undetectable. It was therefore important to find the lowest total protein concentration when S/N of the basic peak1 was greater than 10 to ensure it was detectable and the result was quantitative. This concentration was just found to be 0.3 mg/ml, which became the lowest concentration for our routine charge heterogeneity testing by this CZE method.

3.4. Applications

3.4.1. Characterization of C-terminal Lys variant of mAb4

Lys and Arg residues at the C-terminal end of proteins and peptides can selectively be cleaved by CPB. In this study, when mAb4 was treated with CPB, the 2 predominant basic peaks disappeared, which significantly illustrated that the 2 basic peaks observed in the CZE analysis was originated from incomplete posttranslational cleavage of C-terminal Lys. Data illustrating CPB treatment analysis is shown in Fig. 5.

3.4.2. Characterization of papain induced degradation of mAb2

Papain can selectively cut mAbs into two parts: Fragment antigen-binding (Fab) and Fragment crystalline (Fc). The extent of this enzymatic reaction was evaluated by using our newly developed CZE method. After treating mAb2 with papain, the initial main peak disappeared and two new separate peaks which represented Fab and Fc respectively appeared in the electropherogram. It took less than 5 min to accomplish the whole separation which was much faster than other methods including RP-HPLC, SDS-PAGE and cIEF. Thus it was concluded that this newly developed CZE method was very efficient and capable of quickly monitoring the extent of papain-digestion of the mAb samples. An electropherogram illustrating papain-digestion analysis is shown in Fig. 6.

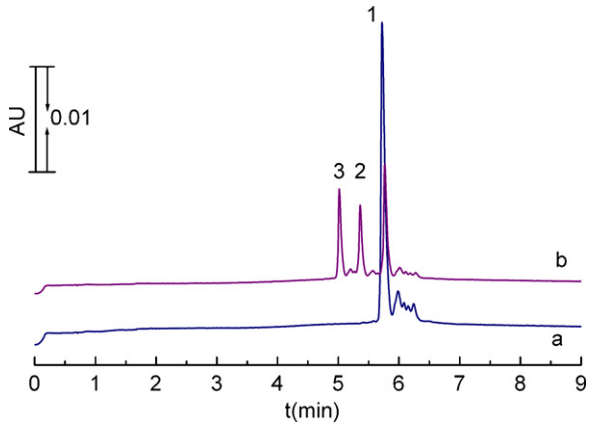


Fig. 5. CZE profiles of mAb4 with and without CPB treatment. (a) With CPB treatment, (b) without CPB treatment, (1) main peak, (2) one C-terminal Lys variant, and (3) two C-terminal Lys variant. Experimental conditions were the same as in Fig. 4.

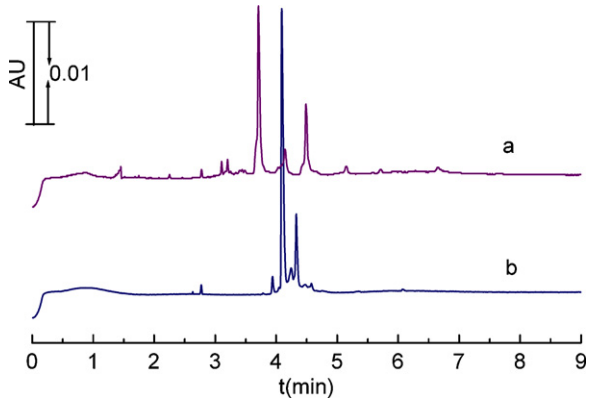


Fig. 6. CZE profiles of mAb2 with and without papain treatment. (a) With papain treatment and (b) without papain treatment. Experimental conditions were the same as in Fig. 4.

4. Conclusions

A fast and reproducible CZE method was newly developed and validated for routine analysis of therapeutic mAbs. Composition of the running buffer, effective separation length and applied voltage were optimized respectively on consideration of both the separation speed and resolution. The optimal running buffer was composited with 20 mM NaAc, 0.3% polyethylene oxide (PEO) and 2 mM triethylenetetramine (TETA), and its pH was adjusted to 6.0 by addition of hydrochloric acid. Additionally, use of applied voltage of +30 kV and a 20 cm effective separation length resulted in best separations when utilizing the above running buffer. The later validation data further demonstrated the specificity, robustness and reproducibility of this method. Compared to other traditional charge based methods including CEX-HPLC and cIEF, this developed method provided exactly similar information faster and easier. C-terminal Lys variants of one commercial mAb product were characterized quickly and easily by utilizing this CZE method. Information regarding papain induced degradation of the test mAb was also generated using this method. All these results demonstrated the potential application of this method in biopharmaceutical manufacturing processes.

Acknowledgements

The authors wish to acknowledge the National Natural Science Foundation of China (No. 31100264) for funding the research. The

authors also thank Mark Lies and Peng Zhang from Beckman Coulter and Guang Yang from DongHua University for reviewing this manuscript.

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