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Research Article

Analysis of recombinant monoclonal antibodies by capillary zone electrophoresis

Analytical platforms that characterize charge heterogeneity in therapeutic proteins, such as mAbs, are important tools that can be used to define quality attributes. CZE separates protein moieties close to their native state and is a valuable physicochemical analytical method that can be used in parallel with other orthogonal methods for characterization and comparability. In this study, custom conditions for the analysis of charge heterogeneity of two mAbs were developed with regard to critical parameters in the BGE, running conditions, and sample treatment. The method application was tested for up to four mAbs and one mAb fragment. The electropherograms showed specific profiles and contrasting levels of basic and acidic isoforms with respect to the main isoform. Issues that surround this method, such as peak tailing and capillary lifetime, are summarized. Using this method, the identities of rituximab and trastuzumab were confirmed, based on the correspondence between the biosimilars and reference products, noninterference of the sample matrix, and the ability to separate spiked samples of related mAbs. The RSD of the isoform content and migration time for the method repeatability were less than 2 and 1%, respectively.

Keywords:
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1 Introduction

The development of analytical platforms is crucial to increase our understanding of the physicochemical properties of biopharmaceuticals, of which mAbs constitute an important segment of biotherapeutics that are being developed. Some mAbs have been approved and prescribed for several diseases [1,2]. During its biosynthesis, extraction, purification, formulation, and shelf life, mAbs can undergo a wide range of physicochemical modifications, including deamidation, glycosylation, oxidation, aggregation, proteolytic cleavage, and formation of disulfide bond isoforms [3–6].

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Abbreviations: API, active pharmaceutical ingredient; **CEX**, cation exchange chromatography; **EACA**, 6-aminocaproic acid; **HIC**, hydrophobic interaction chromatography; **HPMC**, hydroxypropylmethyl cellulose; **MT**, migration time

Powerful analytical methods are thus needed to analyze mAbs that are generated using recombinant DNA technology and ensure product quality, consistency, and stability at all stages of the product life cycle—from early research to the final drug.

Among other chromatographic and electrophoretic techniques such as cation exchange chromatography (CEX), hydrophobic interaction chromatography (HIC); and CIEF, respectively, the use of CZE has increased significantly in recent years, because it is valuable in analyzing charge heterogeneity [7–10]. For this purpose, a capillary is filled with a BGE, and in an applied electric field, subtle differences in molecular charge to size ratio and hydrodynamic volume of a protein are resolved in an electropherogram due to changes in electrophoretic mobility [11,12]. Unlike orthogonal analytical methods, such as chromatography, CZE does not require denaturants or solid-phase interfaces, permitting the inherent heterogeneity of mAbs to be analyzed close to their native state.

The first application of CZE as an analytical tool for therapeutic mAbs entailed the use of a permanently coated capillary [7]. A more recent study demonstrated the value of CZE in analyzing the identity, charge variants, and disulfide isoforms of IgG1 and IgG2 mAbs using uncoated capillaries [8]. The optimization of BGE composition and pH; the

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use of dynamic coatings; and modulation of the electric field strength to achieve rapid, selective, and repeatable separation have been reported [9]. The development of a microchip CZE method for high-throughput testing of charge heterogeneity in mAbs has also been described [10].

We report the development of an alternative CZE platform—to other chromatographic (HIC and CEX) and electrophoretic (CIEF) techniques—for the analysis of charge heterogeneity of two mAbs. By tailoring conditions and using a coated capillary with a hydrophilic surface, we resolved sharp peaks with null peak tailing. Further, our data suggest that no single method can encompass all mAb diversity—the ideal results are affected through customized conditions.

We describe the applicability of this method for analyzing four mAbs and one mAb fragment, using seven commercially available brands. The performance of the method is also discussed.

2 Materials and methods

2.1 Materials

6-Aminocaproic acid (EACA), histamine dihydrochloride, hydroxypropylmethyl cellulose (HPMC), and lithium acetate were obtained from Sigma-Aldrich (Saint Louis, MO). Acetic acid was obtained from J.T. Baker (Center Valley, PA). Acidic wash solution (0.1 N HCl), eCAP Tris buffer (50 mM, pH 8.0), and coated capillaries with a polyacrylamide-based hydrophilic surface (ref. 477441) of 50 μm id were obtained from Beckman Coulter (Fullerton, CA).

The monoclonal antibodies were Mabthera® (F. Hoffmann-La Roche, Basel, Switzerland) batches B60360, B60380, and B60420; Kikuzubam® (Probiomed S.A. de C.V., Mexico City, Mexico) batch RPPP12002; Reditux® (Dr. Reddy''s Laboratories, Hyderabad, India) batch RIAV0910; Herceptin® (F. Hoffmann-La Roche) batches B34130, B3417B010, B34310, and B3431B010; Trastuzumab's biosimilar active pharmaceutical ingredient (API) (Probiomed S.A. de C.V.) batch TZPP12001; Remicade® (Janssen Biotech, PA, USA) batch 1RMKA83006; Infliximab's biosimilar API (Probiomed S.A. de C.V.) batch INPD11005; Avastin® (F. Hoffmann-La Roche) batch B60030; and Lucentis® (Novartis International, Basel, Switzerland) batch S0055.

2.2 Instrumentation

A ProteomeLab PA800 CE instrument, equipped with an ultraviolet detector, 32 Karat software, and a Waters Empower chromatography data collection system, was used.

2.3 Procedures

The CZE separation was performed in a 50 μm id coated capillary with a hydrophilic surface (total length 40.2 cm, effective length 30.2 cm) at 20°C. The BGE for the CZE of rituximab, trastuzumab, and ranibizumab contained 200 mM EACA-acetic acid, 30 mM lithium acetate, and 0.05% w/v HPMC, pH 4.8, and the BGE for the CZE of infliximab and bevacizumab contained 150 mM EACA-acetic acid, 20 mM lithium acetate, and 0.05% m/v HPMC, pH 5.5.

The mAb samples were diluted to 1 mg/mL with 50 mM Tris buffer. The internal mobility marker solution of histamine was added to a final concentration of 1 mg/mL. Spiked samples were obtained by mixing two individually prepared mAb solutions at a 1:1 ratio.

Capillary conditioning included rinses with HCl 0.1 N for 1 min at 30 psi, water for 2 min at 30 psi, and BGE for 10 min at 30 psi, followed by the application of 15 kV for 10 min. A final BGE rinse was performed for 10 min at 40 psi. Prior to sample injection, the capillary was rinsed with HCl 0.1 N for 1 min at 30 psi, water for 2 min at 30 psi, and BGE for 4 min at 30 psi.

Samples were injected for 20 s at 0.5 psi and separated for 30 min at 15 kV, with detection at 214 nm. The data acquisition rate was set to 4 Hz. For short- and long-term storage, a 5 min high-pressure rinse with water was performed, and the two ends of the capillary were immersed in water and stored at 4°C.

HIC was performed using a butyl-NPR 4.6 × 35 mm (TOSOH Bioscience, Japan) column on an Alliance HPLC system (Waters, Milford, MA), with detection at 214 nm; the samples were separated with a linear gradient of 1 M ammonium sulphate/20 mM Tris-HCl, pH 7.0, to 20 mM Tris-HCl, pH 7.0, at a flow rate of 1 mL/min. CEX was performed using a Protein-Pak Hi Res CM 4.6 × 100 mm column on an Acquity UPLC system (Waters, Milford, MA), with detection at 280 nm; the separation was conducted on a linear gradient from 0 to 500 mM NaCl in a solution of sodium phosphate 10 mM, pH 6.5, at a flow rate of 0.8 mL/min. CIEF was performed on a ProteomeLab PA800 instrument using standard procedures recommended by the manufacturer (Beckman Coulter).

3 Results and discussion

3.1 Method development

The CZE method for analyzing mAb charge heterogeneity required us to select the appropriate conditions for the following parameters: EACA and HPMC concentration in the BGE, pH, sample injection time, and sample preparation. Ultraviolet detection was fixed at 214 nm to maximize the sensitivity for low-concentration variants [8–10].

A neutral coated capillary with a hydrophilic surface was used to prevent nonspecific and undesirable adsorption of proteins, which occurs frequently on the surface of conventional-fused silica, uncoated capillaries [13]. Further, a coated capillary diminishes the EOF [10] and improves resolution during the separation.

To evaluate the performance of the coated capillary during the development of our method, capillary absorption was examined using the mAb fragment ranibizumab (1 mg/mL) as a protein model. The procedure for monitoring capillary

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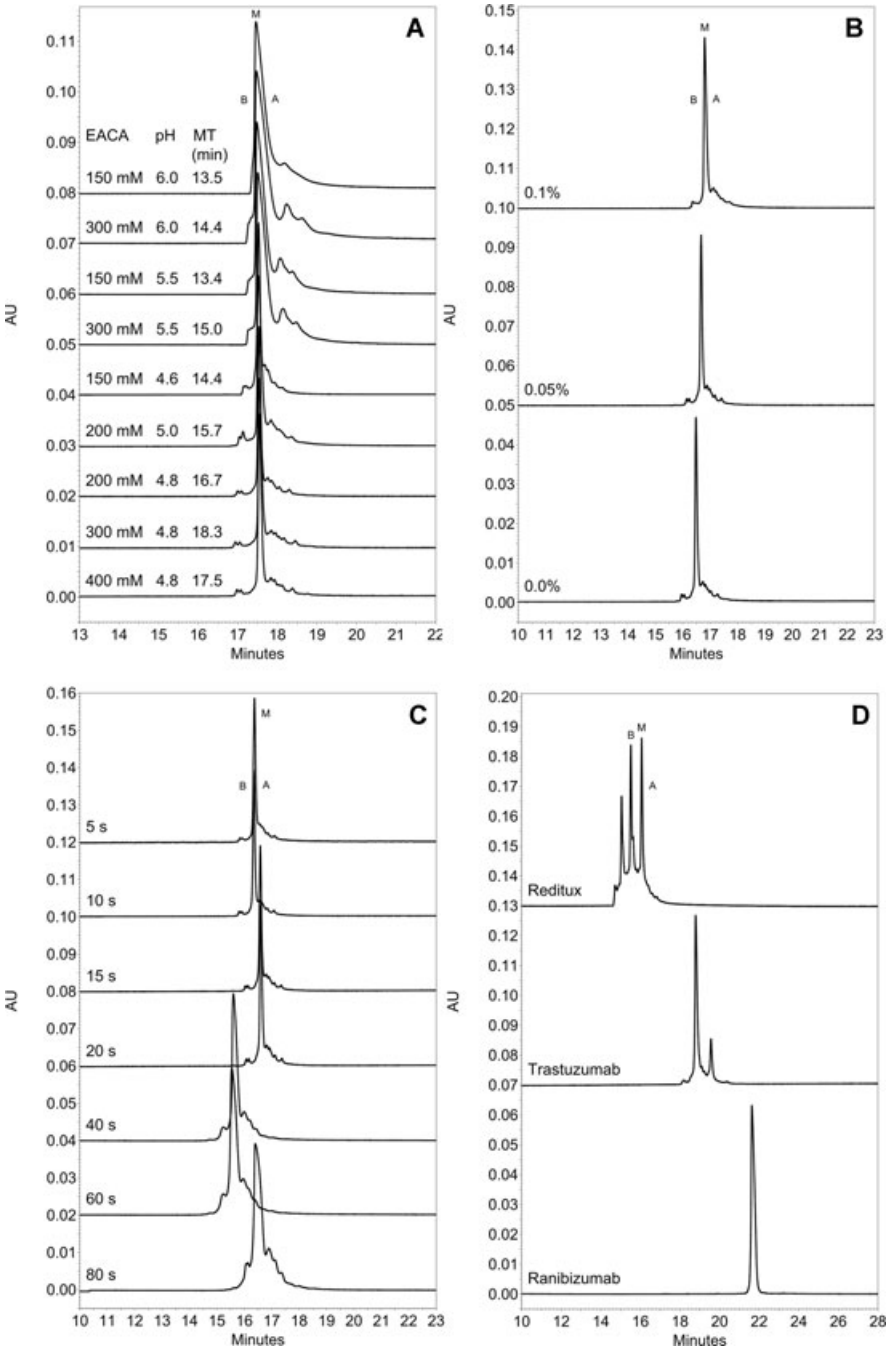


Figure 1. CZE method development for charge heterogeneity analysis of rituximab (Mabthera®): effect of (A) EACA concentration and pH, (B) HPMC concentration, and (C) sample injection time on resolution. Results of application of final conditions on (D) Reditux®, trastuzumab, and ranibizumab. Peak identity: B, basic; M, main; A, acidic. For experimental conditions, see Section 2.3.

absorption was adapted from a previous report [14] using two batches of coated capillaries with a hydrophilic surface. Reversible adsorption of proteins to the capillary wall retards their migration time (MT); thus, the RSDs of MTs were calculated for 18 successive injections.

Conversely, irreversible adsorption causes loss of proteins within the capillary; thus, the increase in area of the protein, expressed as area recovery percentage, was calculated for 18 successive injections.

For capillary batch M110312, no reversible adsorption occurred—the MT RSD was lower than 0.25%—and no irreversible absorption, because the peak area recovery from the second to 18th injection was 101.34 ± 0.47%. Moreover, for capillary batch M205020; no reversible adsorption occurred; the MT RSD was below 0.30%. Irreversible adsorption was higher than with batch M110312, as evidenced by the increase in peak area recovery from the second to ninth injection, after which stabilization was observed, with a peak area recovery of 105.55 ± 0.73% from the tenth to 18th injection. Based on these results, the coated capillaries were appropriate, because no reversible adsorption occurred and because irreversible adsorption occurred for a limited number of injections.

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3.1.1 Rituximab

The CZE method was first developed for rituximab; the p*I* of its main isoform is 9.3, as determined by CIEF.

A concentration of EACA of 45 mM has been shown to be appropriate for the separation of mAbs in a coated capillary [7]. Further, the use of 50 mM EACA in an uncoated capillary decreases the BGE capacity, causing peaks to broaden, and effecting poor resolution [8].

In our study, 50 mM EACA in a coated capillary with a hydrophilic surface did not improve the peak broadening or resolution. Proper separation of charge heterogeneity was obtained after several electrophoretic runs, in which EACA concentration and pH varied from 150 to 400 mM and from 4.6 to 6.0, respectively (Fig. 1A). At pH 6.0, loss of separation was observed, because it approximated the mAb's p*I*, thus enhancing the probability of adsorption of mAb to the capillary's inner surface.

Further increases in EACA concentration and decreases in pH led to a continuous improvement in resolution—similar resolutions were observed with 200, 300, and 400 mM EACA and at pH 4.8. A RSD of 1.4% was obtained on analysis of the percentage content of the main isoforms between the three conditions. However, in order to test the effect of a more concentrated BGE on resolution, EACA concentrations of 500, 600, and 700 mM were also tested (data not shown). At higher EACA concentrations, the complete charge heterogeneity profile was obtained in 26 min compared with 19 min at 200 mM. Thus, we selected 200 mM EACA and pH 4.8 as the best condition.

Acetic acid was used to lower the pH and increase BGE conductivity and ionic strength to approximately the pKa of the carboxylic acid of EACA (pH 4.43). Lithium acetate base was used to level the buffering capacity of BGE.

The separation was also affected by the pH of BGE [15]. Since the net protein charge in the vicinity of its p*I* become close to zero, the solubility of proteins may be limited [9]. The pH 4.8 was favorable, because the mAb acquired a net positive charge, nonspecific adsorption of protein to the capillary's inner surface was prevented, maintaining an appropriate distance from the p*I* of the mAb [16]. However, lowering pH even more in order to avoid adsorption could be detrimental for the protein integrity because protein hydrolysis.

At 0, 0.05, and 0.1% m/v HPMC, the viscosity of the BGE rose, but there was no improvement in resolution; the resolution actually decreased at 0.1% (Fig. 1B). A concentration of 0.05% was selected.

Between 5 and 20 s, increases in sample injection time and, ultimately, the sample volume, led to a continuous improvement in resolution (Fig. 1C). Injection time increased the concentration of protein along the capillary, and during separation, it increased concentrations in the various analyte zones, which correspond to each group of charged isoforms or variants of the mAb. The high concentration of sample creates different resisting zones along the capillary because of the disparate conductivity values between the sample and BGE [17]. This is supported by the slightly difference in the current profile observed when the sample injection time was 20 s. These results suggest that a sample focusing occurs, which, in turn, led to enhanced separation efficiency. Conversely, increases in sample injection time from 40 to 80 s led to a continuous decrease in resolution (Fig. 1C). This is consistent with the sample overloading effect description [17], which causes the peak broadening.

An appropriate balance between resolution and separation time was achieved with an effective capillary length of 30.2 cm.

Based on this evidence, the most important factors that have an impact in resolution of charge heterogeneity are conductivity, pH, and sample injection time.

After the primary running conditions were fixed, the sample preparations were examined. Samples should be diluted with water to adjust their concentration [18]; although this step is a common practice [8, 9], we found that it caused variability in the electrical current profile when the sample injection time was 20 s (Supporting Information Fig. 1). This effect can be explained by the disparate conductivity values between the sample and BGE. Thus, 50 mM Tris buffer was used as sample diluent to increase sample conductivity and level off the electrical current profile (Supporting Information Fig. 1). This is important for reducing variability of the injection between samples.

3.1.2 Infliximab

The favorable conditions of resolution for rituximab (200 mM EACA and pH 4.8) failed for infliximab (Fig. 2A), likely due to the dissimilarity in charge isoform heterogeneity between them (the p*I* of the main isoform of infliximab is 7.3, as determined by CIEF).

Various BGE concentrations and pH values can be used for mAbs with disparate p*I* values to achieve optimal separation [10]. Lowering ionic strength by reducing EACA concentrations from 200 to 150 mM and increasing pH from 4.8 to 5.5 improved the separation of infliximab (Fig. 2A). One possible explanation for this is that the relative charge differences among charge isoform heterogeneity could be increased when pH becomes closer with mAb's p*I* [9]. Also, infliximab requires a BGE with lower conductivity and ionic strength to reduce its electrophoretic velocity inside the capillary to improve resolution (Fig. 2). HPMC concentration in the BGE, sample injection time, and sample preparation were the same as with rituximab.

3.1.3 Other mAbs

To verify the suitability of our CZE platform for diverse mAbs, we used our method to analyze charge heterogeneity of the rituximab's biosimilar Reditux® (Fig. 1D); trastuzumab (Fig. 1D); and bevacizumab (Fig. 2B); and the mAb fragment ranibizumab (Fig. 1D). The p*I* of the main isoform of trastuzumab is 8.7, as determined by CIEF; those of the main

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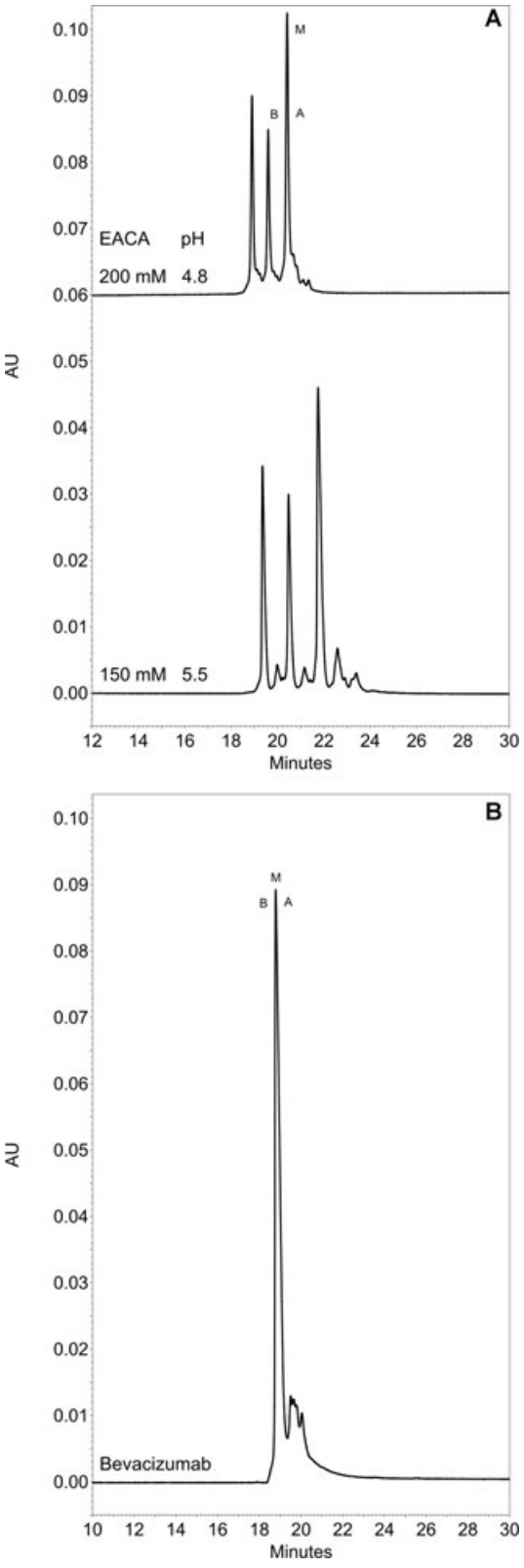


Figure 2. CZE method development for charge heterogeneity analysis of infliximab (Remicade®): effect of (A) EACA concentration and pH on resolution. Results of application of final conditions on (B) bevacizumab. Peak identity: B, basic; M, main; A, acidic. For experimental conditions, see Section 2.3.

isoforms of bevacizumab and ranibizumab are approximately 8.8 [19].

The ideal conditions for Reditux®, trastuzumab, and ranibizumab were 200 mM EACA and pH 4.8 versus 150 mM EACA and pH 5.5 for bevacizumab. Under the both conditions of BGE, the mAbs acquire a net positive charge, allowing the methods that were developed for rituximab and infliximab to be applied. Overall, CZE is a suitable platform for analyzing charge heterogeneity of mAbs, achieving separation within 25 min. In contrast with other CZE methods that have been previously reported [8, 9]—which use a single method for the analysis of several mAbs—the approach here described had considered the specific properties of mAbs in order to develop custom conditions to improve resolution and peak tailing. Also, this platform can be used as a quality control tool for batch release and product development.

3.1.4 Method issues

Several issues can arise with the use of CZE in routine operation that can alter its reliability, for which peak tailing and separation efficiency are key parameters in maintaining the performance of the CZE platform.

For example, peak tailing was observed when new-coated capillaries were used, which has been interpreted to result from protein adsorption to the capillary surface [20]. This phenomenon occurred in two of three newly installed capillaries, which might be an indication of the variability in capillary polyacrylamide-based surface coating between batches. The peak-tailing problem can be mitigated by increasing the number of injections; in our study, ten injections rectified the problem. This issue is consistent with the data in Section 3.1, which discussed the existence of irreversible adsorption of proteins, which halted at approximately the tenth injection for a specific capillary batch.

Although no MT variability was observed (RSD was below 0.30%), further analysis of this data showed presence of peak tailing. This phenomenon was stabilized after the tenth injection as well. This evidence also suggests that this issue was corrected primarily with the incorporation of protein that was present on the sample in the residual active sites on the capillary's inner surface. Once saturated, the irreversible incorporation of proteins could be avoided.

Other phenomena that can contribute, albeit less extensively, are the gradual adsorption of HPMC to a capillary surface that has been protonated by acidic rinses (HCl 0.1 N) and the interaction of EACA with the remaining groups that have not been covered by HPMC [11]. The idea of variability between capillaries from distinct batches is also supported by the data in Section 3.1, which demonstrate that specific batches of the same type of capillary perform differently with regard to adsorption.

On another example, the resolution decreased after multiple runs. Under the selected conditions, the lifetime of the coated capillary was 120–150 injections. This effect might be

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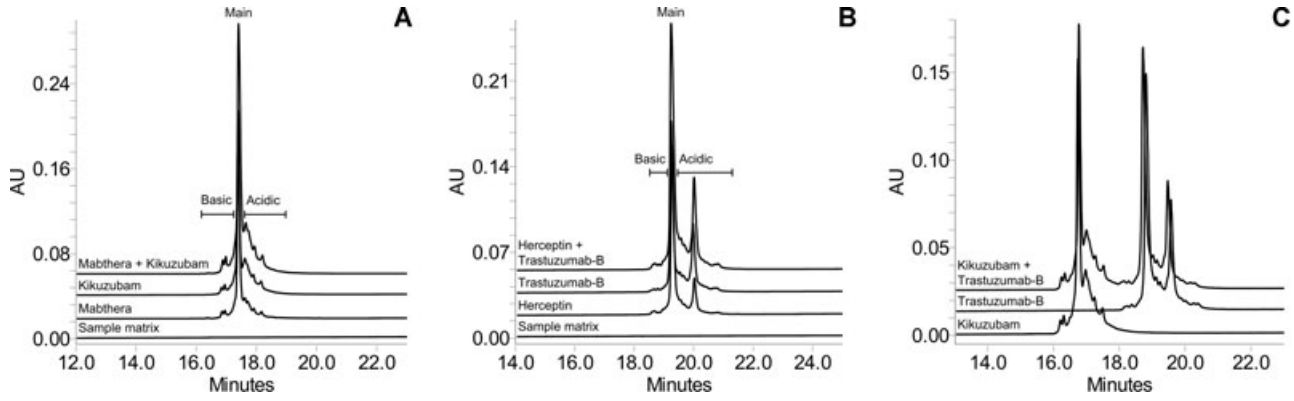


Figure 3. CZE separation of charge heterogeneity of (A) rituximab, (B) trastuzumab, and (C) rituximab- and trastuzumab-spiked sample. B: Biosimilar. For experimental conditions, see Section 2.3.

attributed to the progressive degradation of the inner coating of the capillary due to the continuous acidic rinses during conditioning and prior to sample injection.

3.2 Method performance

The performance of our CZE method was tested in identifying rituximab and trastuzumab. The system's suitability and specificity were assessed as validation characteristics according to ICH Q2(R1) guideline [21]. The system's suitability was verified measuring the MT of the internal mobility marker histamine and the electric current profile in all tests. The MT range of histamine was 3.7–4.1 min, and that of the electric current profile was 30–35 μA. The MTs of the basic, acidic, and main isoforms were relative to the MT of histamine.

3.2.1 Method selectivity

In the selectivity tests, the electrophoretic profiles of Kikuzubam® and Trastuzumab's biosimilar API corresponded to those of commercially available reference products: Mabthera® and Herceptin®, respectively, obtained through mixtures of both rituximab products and both trastuzumab products. Further, there was no interference from any component of the sample matrix (Fig. 3A and B), and we noted proper separation selectivity, as evidenced by the distinction between mAbs with similar p*I* values and structure and the same isotype (Fig. 3C).

3.2.2 Method repeatability

We examined six individual sample preparations of Kikuzubam®, Trastuzumab's biosimilar API, and Infliximab's biosimilar API in the method repeatability tests. Although the analysis of performance focused on the identity of rituximab and trastuzumab, the method repeatability were also evaluated for infliximab, as a model mAb, to determine the performance of each method that we developed.

Table 1. Performance of CZE method for charge heterogeneity analysis of mAbs

Characteristic	Parameter	Result		
Method repeatability	RSD% of area%	1.21%[a)]	0.89%[a)]	1.12%[a)]
		1.90%[b)]	0.25%[b)]	0.38%[b)]
		0.15%[c)]	0.12%[c)]	0.31%[c)]
	RSD% of MT	n.d.	0.27%[a)]	n.d.
		n.d.	0.21%[b)]	n.d.
		n.d.	0.91%[c)]	n.d.

a) Kikuzubam®.
b) Trastuzumab's biosimilar API.
c) Infliximab's biosimilar API.
n.d.: not determined.

These characteristics were evaluated using RSD values of the area percentage of the basic, acidic, and main isoforms and using the RSD values of the MTs of the main isoforms. In all cases, the content RSD values were less than 2%, and the MT RSD values were below 1% (Table 1). The specific and reproducible MTs for each mAb complemented the results of the identity tests.

3.2.3 Comparison with other techniques

A single method is not sufficient to resolve and characterize a protein [13]. HIC, CEX, and CIEF were used as orthogonal analytical techniques to separate charge heterogeneity in rituximab and trastuzumab and compare their resolution against that of CZE (Fig. 4). Overall, the electrophoretic techniques (CIEF and CZE) showed greater resolution than the chromatographic methods (HIC and CEX). One possible explanation is that the absence of solid interfaces and lower ionic strength solutions in electrophoretic techniques improve the sensitivity of subtle psychochemical changes in the mAbs in

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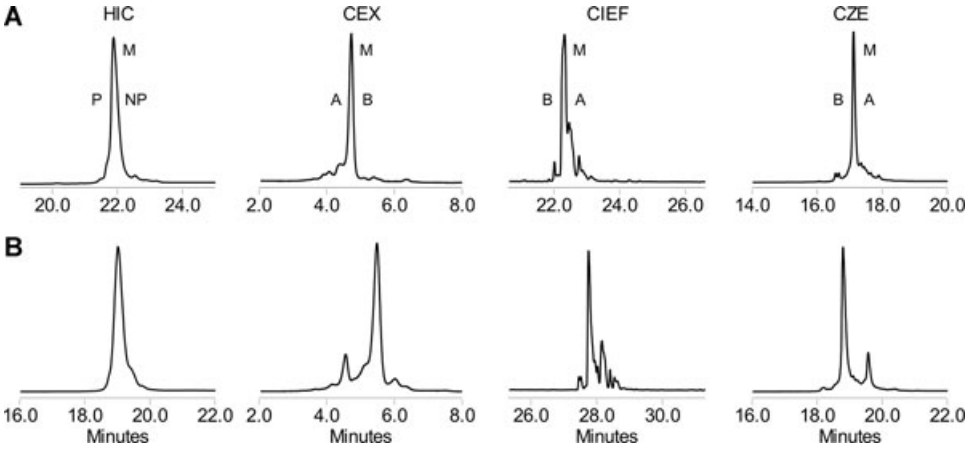


Figure 4. Comparison of separation of charge heterogeneity of (A) Mabthera® and (B) Herceptin® with CZE and other orthogonal methods. Peak identity: NP, nonpolar; P, polar; B, basic; M, main; A, acidic. For experimental conditions, see Section 2.3.

the separation agent (electric field strength). Further, CZE analysis more closely approximates the native conditions of the mAb.

3.3 Assessment of biosimilars

The rituximab and trastuzumab biosimilars were compared with regard to their content of basic, acidic, and main isoforms with those of the corresponding commercial reference products Mabthera® and Herceptin®, respectively. In both cases, the electrophoretic profiles had characteristic variants before and after their main isoform. We calculated a basic and acidic isoform content, respectively, of 11.1 and 29.4% for Mabthera®; 7.1 and 43.7% for Kikuzubam® (rituximab's biosimilar mAb); 62.7 and 15.1% for Reditux® (rituximab's biosimilar mAb); 4.1 and 33.1% for Herceptin®; and 3.8 and 44.7% for the trastuzumab's biosimilar API. The isoform contents found on all samples represent a physicochemical state of the mAbs. In order to define acceptable content ranges, it is important to evaluate the consequences of such variants on the bioactivity and safety of biopharmaceutical products [12] through biological methods.

4 Concluding remarks

Analytical platforms must be developed to increase our understanding of the physicochemical properties of biopharmaceuticals and define and monitor quality attributes at all stages of the development and production life cycles.

In this study, we developed two CZE methods that can analyze several mAbs closer to their native conditions. The performance of CZE was mAb-selective and obtained through an assessment of the critical parameters in the BGE (pH and ionic strength). During the development of the method, peak tailing, and capillary lifetime emerged as specific issues that had affected the quality of the analysis.

Once the performance of the method was tested, it became a suitable, selective, and precise analytical tool for identity testing. This method compared the composition of charge variants of rituximab and trastuzumab biosimilars with respect to their reference products and constituted an orthogonal technique of HIC, CEX, and CIEF. The use of different sample treatments like reduction or digestion, as well as other analytical techniques will be needed to fully characterize the charge heterogeneity of mAbs. Based on our data, using this method to analyze other mAbs and proteins requires adjusting their conditions properly to attain optimal performance.

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The authors have declared the following potential conflict of interest: All authors are employees of Probiomed S.A. de C.V., which is developing, manufacturing, and marketing biosimilar products, and are involved in the development of biosimilar products for Probiomed.

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