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Charge heterogeneity of a therapeutic monoclonal antibody conjugated with a cytotoxic antitumor antibiotic, calicheamicin

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ABSTRACT

A robust and highly reproducible capillary isoelectric focusing (cIEF) method for the evaluation of charge heterogeneity of monoclonal antibody (mAb) pharmaceutical which contains covalently bound antitumor compounds was developed using a combination of commercially available dimethylpolysiloxane-coated capillary and carrier ampholyte. In order to optimize major analytical parameters for robust mobilization, experimental responses from three pI markers were selected. The optimized method gave excellent repeatability and intermediate precision in estimated pI values of charge variants with relative standard deviations (RSDs) of not more than 0.06% and 0.95%, respectively, when using IgG_4 as a model. Furthermore, RSDs of charge variant compositions were less than 5.0%. These results suggest that the proposed method can be a powerful tool for reproducible evaluation of charge variants of both naked mAbs and their conjugates with high resolution, and it is applicable to quality testing and detailed characterization in the pharmaceutical industry. In addition, it should be noticed that the method provided non-linear pH gradient within the tested ranges, from pI 9.50 to 3.78, and the pH gradient caused the inconsistency of estimated pI ranges between cIEF and gel IEF. This result indicates that selecting appropriate pI markers based on the target pI ranges of charge variants for each mAb related pharmaceutical is highly recommended for the precise determination of pI values.

1. Introduction

Recently, a number of monoclonal antibody (mAb) pharmaceuticals have been developed and used worldwide for treatment of cancer, rheumatoid arthritis and other diseases [1–3]. The mAb pharmaceuticals possess various heterogeneities in their molecular size, charge and carbohydrate composition. These heterogeneities are mainly due to post-translational modifications such as glycosylation, aggregation, oxidation, deamidation, mismatched disulfide bonds, and are often observed when manufacturing process are changed [4–10].

A charge profile represents the specific "fingerprint" due to the charge heterogeneity of mAb products as well as other protein pharmaceuticals. During development stages of these biopharmaceuticals, evaluation of charge heterogeneity is necessary for assurance of product quality and stability [11,12]. In the previous reports, some charge variants derived from several post-translational modifications or degradation showed different biological activity and stability compared to their original variants. For example, peptides of which methionine residues are oxidized, show significantly reduced activity in the antigen binding potency assay [13]. Removal of 20% of the total sialic acid can result in a 50% loss in the circulating proteins [14].

The charge heterogeneity of proteins has been usually analyzed by isoelectric focusing (IEF) method based on gel format. The method allows separation of proteins into their charge variants based on their charge differences in a pH gradient under electric field. However, gel IEF method usually requires long analysis time for both separation and staining steps, and provides semi-quantitative results with poor resolution. To overcome these disadvantages of gel based electrophoresis, capillary electrophoresis has emerged as an alternative which allows rapid and quantitative analysis with high resolution, and has been applied to the analysis of several biomolecules such as nucleic acids [15,16], proteins [17–19], carbohydrates [20–23] and cells [24].

Capillary isoelectric focusing (cIEF) is one of the separation modes in capillary electrophoresis, and the first report using carrier ampholyte was made by Hjertén and Zhu in 1985 [25]. Silverman et al. reported the separation of a recombinant mAb by cIEF into five major and one minor band in a pH 5–8 gradient, and a direct comparison was made with other separation techniques [26]. In comparison with slab gel IEF and ion-exchange chromatography

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(IEC), cIEF was found to give the same resolution as gel IEF, but better than IEC. cIEF was much faster than gel IEF, and the reproducibility of peak areas was much better than gel IEF. However, the major limitations of cIEF were found to be the poor reproducibility of migration time. The mobilization often causes distortion of the pH gradient and loss of resolution due to non-uniform mobilization speed and buffer diffusion, especially in two-step method. Several improvements in reproducibility were made during last two decades [27–30], and some excellent reviews of cIEF that cover recent developments and advances of the technique have also been published [31,32]. Two-step methods of cIEF are frequently adopted with single-point detector: charge variants are focused into their pI values, and then mobilized to the detector by hydrodynamic or chemical mobilization [28,33–37]. One-step methods with single-point detector which enable us to perform focusing and mobilization simultaneously using moderate electroosmotic flow (EOF) toward the cathode were also reported [28,31]. Furthermore, imaging cIEF with short capillary and whole-column detection which can eliminate the need for mobilization was developed, and several applications for charge heterogeneity analysis of mAb products using a commercial imaging cIEF instrument have been described [38,39].

Newly developed carbohydrate-containing mAb pharmaceuticals to which antitumor compounds are linked, i.e. mAb-antitumor conjugates, are the newly emerged biopharmaceuticals [11,40,41]. They have highly complex heterogeneities in their structures because of additional variations based on conjugations between native mAb and the antitumor compound of low molecular weight. For the analyses of mAb conjugates, considering the numbers of attached antitumor compounds at various binding sites is extremely important for assessing constant quality of them to ensure a clinical efficiency and minimize unwanted toxicity. In addition, evaluation of heterogeneity due to attached carbohydrate chains is another important target, and several reports have been reported previously [29,30,42,43]. From these reasons, further improvement and optimization have been needed to achieve highly reproducible cIEF method with high resolution for mAb pharmaceuticals especially for mAb conjugates like gemtuzumab ozogamicin.

The present study describes a two-step cIEF method for not only a native mAb pharmaceutical but also a new type mAb pharmaceutical, gemtuzumab ozogamicin, which is a recombinant humanized IgG_4 monoclonal antibody against the CD33 antigen that is modified with a cytotoxic antitumor antibiotic, calicheamicin, obtained from *Micromonospora echinospora* ssp. *Calichensis* [44]. In order to establish a robust mobilization conditions, carrier ampholyte, hydroxypropyl methylcellulose (HPMC), urea, arginine and iminodiacetic acid concentrations are selected as major analytical parameters and optimized using experimental responses from three pI markers of pIs 9.50, 5.50 and 4.10. To investigate the reproducibility of the optimized method, repeatability and intermediate precision studies are conducted using IgG_4 as a model. Furthermore, to prove the usefulness of the optimized method for the analysis of mAb conjugates, both intact and deglycosylated gemtuzumab ozogamicin, are analyzed. In addition, based on the results obtained by both cIEF and gel IEF, recommendation in selecting pI markers for precise determination of pI values is proposed.

2. Materials and methods

2.1. Materials

A commercial mAb pharmaceutical, gemtuzumab ozogamicin, was kindly donated from Kinki University Nara Hospital. The solution of gemtuzumab ozogamicin (approximately 1.0 mg/mL) was kept at 5 °C. A purified IgG_4, kappa, from human myeloma plasma was purchased from Sigma–Aldrich (St. Louis, MO, USA). A pH 3–10 Pharmalyte was purchased from GE Healthcare (Buckinghamshire, UK). Another carrier ampholyte, Ampholyte buffer (pH 3–10), and cIEF gel buffer were obtained from Beckman (Fullerton, CA, USA). Three pI markers of pIs 9.50, 5.50 and 4.10 were also obtained from Beckman, and other 11 synthetic pI markers used for the evaluation of a detailed pH gradient were prepared according to the method reported previously [36]. An agarose IEF gel and an IEF accessory kit were from Lonza (Basel, Switzerland), and a pI marker solution (pH 3–10) for gel IEF was from Serva Electrophoresis (Heidelberg, Germany). HPMC, urea, acetic acid, iminodiacetic acid, 2-mercaptoethanol and trichloroacetic acid were from Sigma–Aldrich. Sodium hydroxide and phosphoric acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Peptide-N^4-(acetyl-β-D-glucosaminyl) asparagine amidase (PNGase F, EC 3.5.1.52, recombinant) was from Roche Diagnostics (Mannheim, Germany).

2.2. Deglycosylation of N-linked oligosaccharides from gemtuzumab ozogamicin

Deglycosylation of gemtuzumab ozogamicin was conducted as follows. Briefly, 10 units (10 μL) of PNGase F were added to 50 μL of 1.0 mg/mL mAb solution, and the solution was incubated at 37 °C for 24 h. As a reference sample, water was added instead of the enzyme in order to compare charge profiles. After incubation, sample solutions were desalted as described below, and used for cIEF and gel IEF.

2.3. Sample preparation

After centrifugation of the mAb solutions using a Microcon™-100 (Millipore: Billerica, MA, USA), the remaining solution on the cup was diluted with water to 5.0 mg/mL and 1.0 mg/mL for cIEF and gel IEF, respectively. A portion (20 μL) of 5.0 mg/mL mAb solution was added to 120 μL of 0.2% or 0.4% (w/v) HPMC solutions containing 2 mol/L or 4 mol/L urea, and mixed with 3 μL of pI marker mixture of pIs 9.50, 5.50 and 4.10 (1 μL each) or 5.5 μL of pI marker mixture of 11 synthetic pI markers (0.5 μL each). Then, the solution was diluted by dilution solution (a mixture of 500 mmol/L arginine solution, 200 mmol/L iminodiacetic acid solution, carrier ampholyte and water) to adjust the concentration for each condition (Table 1). The final volume was adjusted to 175 μL. All the sample solutions were used immediately after preparation.

2.4. cIEF analysis

cIEF was carried out using a PA800 system (Beckman) with a Neutral capillary (Beckman: 50 μm i.d., 30 cm, 20 cm effective length) or a DB-1 capillary (Agilent Technologies: Palo Alto, CA, 50 μm i.d., 30 cm, 20 cm effective length). Data were collected and analyzed using a 32 Karat software (Beckman, version 8.0). New capillaries were rinsed with water for 2 min, 350 mmol/L acetic acid for 2 min and a 0.2% or 0.4% (w/v) HPMC solution containing 2 mol/L or 4 mol/L urea for 5 min before analysis. After each analysis, capillary was rinsed with 6 mol/L urea solution for 3 min and water for 2 min. Focusing was performed at 25 kV in normal polarity for 15 min. 200 mmol/L phosphoric acid and 300 mmol/L sodium chloride were used as inlet and outlet solutions, respectively. After focusing, the outlet solution was replaced with 350 mmol/L acetic acid, and the charge variants were mobilized to outlet at 25 kV in normal polarity. All the steps were conducted at 20 °C, and both focusing and mobilization were monitored at 280 nm.

Table 1
Experimental designs for Ampholyte buffer and Pharmalyte.

Experiments	Parameter				
	Carrier ampholyte (%)[a]	HPMC (%)	Urea (mol/L)	Arginine (mmol/L)	Iminodiacetic acid (mmol/L)[a]
1	2.0 (2.0)	0.2	4	20	2.0 (2.0)
2	2.0 (2.0)	0.4	4	20	5.0 (4.0)
3	2.0 (2.0)	0.4	4	50	2.0 (2.0)
4	2.0 (2.0)	0.2	4	50	5.0 (4.0)
5	2.0 (2.0)	0.4	2	50	5.0 (4.0)
6	2.0 (2.0)	0.2	2	20	5.0 (4.0)
7	5.0 (4.0)	0.4	2	50	2.0 (2.0)
8	5.0 (4.0)	0.2	2	20	2.0 (2.0)
9	5.0 (4.0)	0.4	4	50	5.0 (4.0)
10	5.0 (4.0)	0.4	2	20	5.0 (4.0)
11	5.0 (4.0)	0.2	2	50	5.0 (4.0)
12	2.0 (2.0)	0.4	2	20	2.0 (2.0)
13	5.0 (4.0)	0.2	4	50	2.0 (2.0)
14	5.0 (4.0)	0.4	4	20	2.0 (2.0)
15	3.5 (3.0)	0.3	3	35	3.5 (3.0)
16	2.0 (2.0)	0.2	2	50	2.0 (2.0)
17	5.0 (4.0)	0.2	4	20	5.0 (4.0)

[a] Numbers listed in bracket were applied for Pharmalyte.

2.5. Gel IEF analysis

Gel IEF was conducted using a horizontal IEF instrument consisted of a Multiphor II, a Multitemp III and an EPS3501 XL (GE Healthcare). Approximately 5 μg of mAbs were added onto the agarose gel (Lonza), gone into the gel in pre-focusing at 1 V (constant) for 75 volt hours (Vhr), and then focused at 1500 V (constant) for 1500 Vhr. After focusing, the gels were immersed in 20% trichloroacetic acid solution for 30 min for fixing, and dried completely with a hair dryer prior to staining of the gel. Fifty milliliter of Gelcode® blue (Thermo Scientific, Rockford, IL, USA) was used for staining for 30 min, and the gels were dried again after destaining by water for ca. 5 min. Gels were scanned using a GS-800 apparatus and analyzed by a Quantity One software (Bio-Rad Laboratories: Hercules, CA, USA).

2.6. Optimization of cIEF parameters

Major analytical cIEF parameters and interactions between two parameters were examined using a design of experiments (DOE) software, JMP® (SAS Institute: Cary, NC, USA). Analysis of experimental responses obtained from a study generated using JMP® highlights the effect of both the parameters and interactions on each response. This type of effect is not observed when each parameter is studied independently. In this study, four experimental responses (relative migration times (RMT) between two pI markers of pIs 5.50 and 9.50, peak area of pI 9.50 marker, migration time of pI 4.10 marker and signal to noise (*S*/*N*) ratio of pI 5.50 marker) were selected for evaluation of cIEF parameters in terms of resolution, detection sensitivity, high-throughput analysis and *S*/*N* ratio, respectively. Then, the parameters (carrier ampholyte, HPMC, urea, arginine and iminodiacetic acid concentrations) which are supposed to affect the responses were identified. Experimental plans generated using JMP® were composed of a half-factorial design (2^{5-1}) with middle point as shown in Table 1.

After 17 runs using the parameters indicated in Table 1, data were analyzed in JMP® using analysis of variance (ANOVA) method by least-square fit in order to identify which parameters and interactions between two parameters had a significant effect on the experimental responses. Calculated probabilities (*p*-values) from ANOVA were used in order to classify the observed effect of the parameters and interactions when both carrier ampholytes, Pharmalyte and Ampholyte buffer, were used. A low *p*-value indicates that the results are statistically significant. The values of significance probabilities are normally set as 0.05 or less. However, in this experiment, the values between 0.05 and 0.10 were additionally included as "negligible impact" in order to emphasize which system is proper for cIEF. Therefore, the values greater than 0.10 indicate the results are not significant. In addition, JMP® also provided optimum conditions based on the experimental data. The statistically optimized conditions for both carrier ampholytes were used for further analysis in order to confirm their reproducibility.

3. Results and discussion

3.1. Selection of capillaries for cIEF

In the present study, we examined two chemically modified capillaries, a Neutral capillary and a DB-1 capillary, as candidates for cIEF. In order to achieve robust mobilization, the EOF has to be decreased because it often causes flow variety. Several successful separations for glycoproteins using DB-1 capillary, of which the inner wall is modified with dimethylpolysiloxane, has been reported previously [29,30]. Even if DB-1 capillary shows a small EOF, the flow could be reduced when a small amount of a neutral polymer like HPMC was added in the running buffer. In addition, by adding a small amount of neutral polymer in the running buffer, the polymers cover the inner wall of the capillary and prevent the irreversible adsorption between glycoprotein and the capillary wall.

For selection of capillaries, focusing and mobilization were conducted at 25 kV for 15 min and 45 min, respectively. All other analytical conditions are the same in Section 2.4. The sample solution was prepared by mixing pI markers (1 μL each), 0.1 mg of mAb solution and a 3 mol/L urea containing cIEF gel buffer (Beckman). The sample solution also contained 50 mmol/L arginine, 5 mmol/L iminodiacetic acid and 4.0% Ampholyte buffer. From the experimental results, both capillaries tested in this study gave similar profiles of gemtuzumab ozogamicin (data not shown). Therefore, for this study, we selected a DB-1 capillary for further experiments because it was easily available from commercial source for capillary gas chromatography.

3.2. Optimization of cIEF parameters

The effects of the five analytical parameters described in Section 2.6 and their interactions were studied for both Ampholyte

buffer and Pharmalyte using three different concentrations for all the parameters, respectively (Table 1). Each sample solution was prepared according to the concentrations described in Table 1, and all the separations were conducted by using the conditions described in Section 2.4. The tested concentration ranges of each parameter were set according to the previous reports [36,38,39]. In this study, slightly different parameter ranges were set between the two carrier ampholytes because the combination of high concentrations of carrier ampholyte and iminodiacetic acid caused disappearance of pI markers from electropherogram when Pharmalyte was used. Thus, for Pharmalyte, maximum concentrations of carrier ampholyte and iminodiacetic acid were set at 4% and 4.0 mmol/L, respectively.

The effects of each parameter and interaction between two parameters were categorized as summarized in Table 2. For example, HPMC concentration showed moderate impact (p-value, <0.05) on S/N ratio and negligible impact (<0.10) on migration time of pI 4.10 marker when using Ampholyte buffer. The quality of fit to the model between expected and actual values of each response was expressed by coefficient of determination (R^2). For both carrier ampholytes, all the responses tested in this study showed good fitting with R^2 values of higher than 0.92. According to Table 2, cIEF method using Ampholyte buffer was obviously affected by all the parameters and interactions with p-values of less than 0.10. These results suggested that the method using Ampholyte buffer is not robust within the tested ranges. In contrast, in the method using Pharmalyte, concentration of carrier ampholyte and arginine showed moderate and negligible impact on RMT, respectively. Higher concentration of carrier ampholyte forms wider range of pH gradient in the capillary, and higher arginine concentration provides straitened RMT by the presence of arginine because it is focused into the most basic region in the capillary due to its pI value. Considering these points, the concentrations of both carrier ampholyte and arginine affect the RMT. Other parameters and interactions had no effect on the method using Pharmalyte, and Pharmalyte seems better than Ampholyte buffer to achieve the robust results.

JMP® provides statistically optimized cIEF conditions based on the testing results. In case of cIEF using Pharmalyte, the parameters for carrier ampholyte, HPMC, urea, arginine and iminodiacetic acid were 4.0%, 0.2%, 4.0 mol/L, 20 mmol/L and 2.0 mmol/L, respectively. In case of Ampholyte buffer, the concentrations of carrier ampholyte, urea, and iminodiacetic acid were changed to 4.1%, 2.0 mol/L and 5.0 mmol/L, respectively.

3.3. Repeatability

To investigate the repeatability of the optimized cIEF conditions, 11 continuous analyses were performed. Sample solutions of IgG_4 with three pI markers (pIs 9.50, 5.50 and 4.10) were prepared using both carrier ampholytes and separated by cIEF, respectively (Fig. 1). The obtained electropherograms were analyzed to evaluate migration times of each pI marker and estimated pI values of charge variants. For pI estimation, pI 9.50 and 5.50 markers were used for assuming a linear relationship between pI values versus migration times. Statistical analysis was performed on both the migration times and estimated pI values to determine the average and relative standard deviations (RSDs), and the results were summarized in Table 3. The RSDs of pIs 9.50, 5.50 and 4.10 markers for the Pharmalyte showed excellent values of 0.16%, 0.17% and 0.22%, respectively, and were much smaller than those obtained for Ampholyte buffer (2.76%, 0.60% and 0.64%, respectively). In addition, the RSDs of estimated pI values of peaks a–d in Fig. 1(A) for Pharmalyte were no more than 0.06%, and were much smaller than those observed for peaks e–h in Fig. 1(B) using Ampholyte buffer (≤0.45%). Although Pharmalyte gave slightly poor resolution than

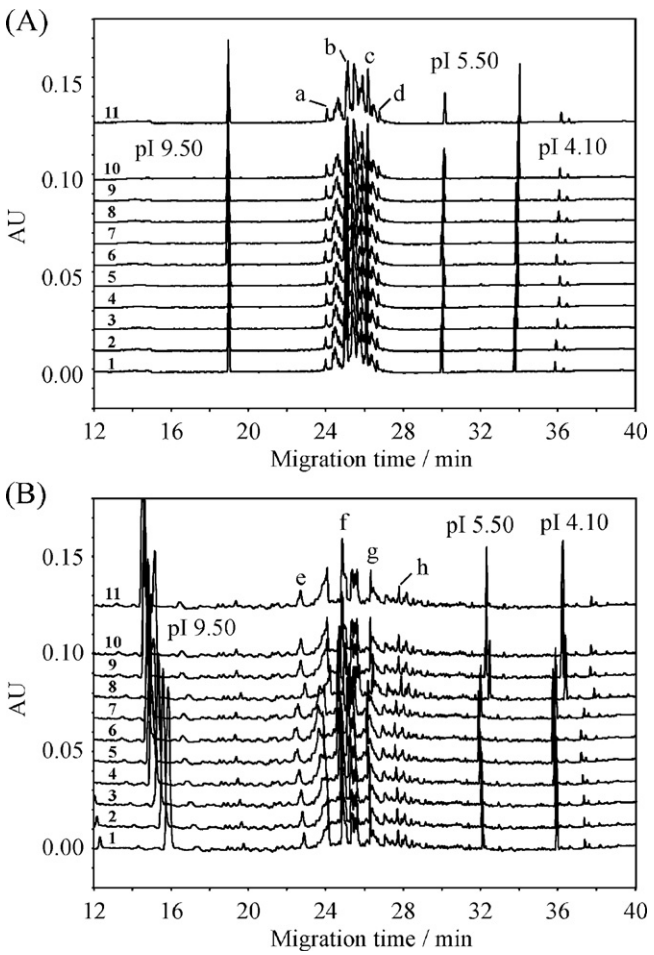

Fig. 1. Repetitive analysis of a mixture of IgG_4 and pI markers using Pharmalyte (A) and Ampholyte buffer (B). The numbers in both panels indicate the order of the measurements. (A) Sample solution using Pharmalyte was prepared by mixing pI markers (1 μL each), 0.1 mg of mAb solution and 0.2% hydroxypropyl methylcellulose solution containing 4 mol/L urea. The solution also contained 4.0% Pharmalyte, 20 mmol/L arginine and 2 mmol/L iminodiacetic acid. (B) Sample solution using Ampholyte buffer was prepared by mixing pI markers (1 μL each), 0.1 mg of protein and 0.2% hydroxypropyl methylcellulose solution containing 2 mol/L urea. The solution also contained 4.1% Ampholyte buffer, 20 mmol/L arginine and 5 mmol/L iminodiacetic acid. Analytical conditions: capillary, DB-1 capillary (Agilent Technologies, 50 μm i.d., 30 cm, 20 cm effective length); injection, 99 s at 25 psi; focusing, 25 kV for 15 min; mobilization, 25 kV for 25 min; temperature, 20 °C; detection, UV at 280 nm.

Ampholyte buffer, it provided approximately 10-fold higher S/N ratios than Ampholyte buffer. These results strongly show that the optimized conditions using Pharmalyte were robust and highly repeatable, and were consistent with the results obtained from JMP®.

3.4. Intermediate precision of estimated pI values and percent composition

In order to investigate the reproducibility of the cIEF method optimized above, an intermediate precision study was performed using IgG_4 as a model. Sample solutions were prepared using two different lots of Pharmalyte (in duplicate) and analyzed by cIEF using two different lots of DB-1 capillary. Experiments were conducted on separate two days, and eight data sets were collected on each day to generate 16 electropherograms in total. The pI values of each peak were estimated in the same manner as mentioned in Section 3.3. The RSDs of estimated pI values were less than 1.0% for all charge variants, peaks a through h in

Table 2
Effects[a] of major analytical parameters and interactions between two parameters.

Parameter/interaction[b]	Ampholyte buffer				Pharmalyte			
	RMT[c]	Peak area	Migration time	*S*/*N* ratio	RMT[c]	Peak area	Migration time	*S*/*N* ratio
Parameter								
Carrier ampholyte (%)	B	B	A	A	B	–	–	–
HPMC (%)	–	–	C	B	–	–	–	–
Urea (mol/L)	–	–	–	B	–	–	–	–
Arginine (mmol/L)	C	C	C	B	C	–	–	–
Iminodiacetic acid (mmol/L)	–	C	B	C	–	–	–	–
Interaction between two parameters								
Carrier ampholyte–HPMC	–	–	C	B	–	–	–	–
Carrier ampholyte–urea	–	–	B	–	–	–	–	–
Carrier ampholyte–arginine	–	–	B	B	–	–	–	–
Carrier ampholyte–iminodiacetic acid	–	C	B	C	–	–	–	–
HPMC–urea	–	–	B	C	–	–	–	–
HPMC–arginine	–	–	B	B	–	–	–	–
HPMC–iminodiacetic acid	–	–	–	C	–	–	–	–
Urea–arginine	–	–	B	–	–	–	–	–
Urea–iminodiacetic acid	–	–	–	B	–	–	–	–
Arginine–iminodiacetic acid	–	–	–	B	–	–	–	–

[a] Effects are categorized using calculated *p*-values: (A) significant impact, <0.01; (B) moderate impact, <0.05; (C) negligible impact, <0.10; (–) no impact, ≥0.10.
[b] The tested ranges are shown in Table 1.
[c] RMT: relative migration time between two pI markers of pIs 5.50 and 9.50.

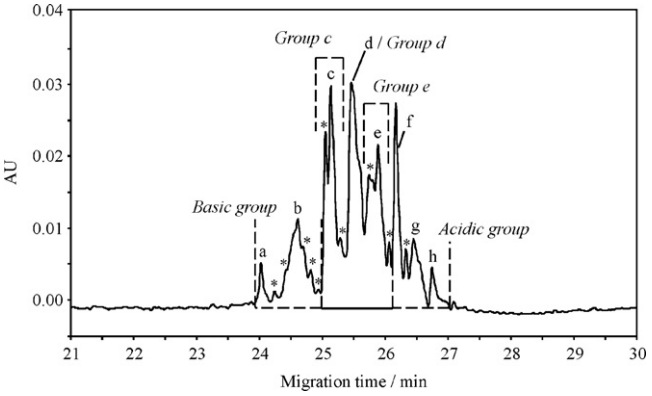

Fig. 2. Expanded view of cIEF analysis of IgG_4. The eight signature peaks and five groups of peaks observed in the analysis of IgG_4 were selected for the intermediate precision study. Actually, peaks (a through h) were used to calculate variation in the estimated pI values and percent compositions. Peaks denoted with an asterisk were only used to estimate variation of the percent compositions. Analytical conditions were the same as in Fig. 1.

Fig. 2. The detected charge variants in the basic (peaks a and b) or acidic regions (peaks f through h) on the electropherogram were assigned as basic or acidic groups, respectively. Furthermore, other variants were separated into three groups, group c, group d and group e. The RSDs of percent composition of each group were less than 5.0% as shown in Table 4. These values were comparable to those obtained by imaging cIEF (less than 6.3% in [38], and less than 5.7% in [45]) which are supposed to show excellent reproducibility because the method does not require mobilization. Although the cIEF method needs relatively longer analysis time than the imaging cIEF, it can provide higher resolution because of the use of a longer capillary. These results demonstrated that the present method can be used as a charge profiling method for mAb pharmaceuticals.

Table 4
Intermediate precisions on estimated pI value and percent composition of charge variants of IgG_4 under the optimized cIEF method.

Peak/group	Mean (*n* = 16)	Standard deviation	RSD (%)[a]
Estimated pI value			
Peak a	7.70	0.070	0.91
Peak b	7.50	0.071	0.95
Peak c	7.29	0.061	0.84
Peak d	7.15	0.045	0.63
Peak e	7.01	0.042	0.60
Peak f	6.90	0.023	0.34
Peak g	6.77	0.026	0.39
Peak h	6.66	0.024	0.36
Percent composition of charge variant			
Basic group	16.53	0.817	4.94
Group c	21.01	0.765	3.64
Group d	23.86	0.805	3.37
Group e	19.54	0.921	4.72
Acidic group	19.06	0.726	3.81

[a] Relative standard deviation, *n* = 16.

Table 3
Repeatability on migration time of pI markers and estimated pI value of IgG_4 under the optimized cIEF conditions.

Source	Ampholyte buffer		Pharmalyte	
	Mean (*n* = 11)	RSD (%)[a]	Mean (*n* = 11)	RSD (%)[a]
Migration time (min)				
pI 9.50	15.01	2.76	18.96	0.16
pI 5.50	32.11	0.60	30.06	0.17
pI 4.10	35.98	0.64	33.89	0.22
Estimated pI value[b]				
Peak a or e	7.70	0.45	7.68	0.00
Peak b or f	7.72	0.43	7.32	0.04
Peak c or g	6.87	0.39	6.92	0.00
Peak d or h	6.53	0.32	6.71	0.06

[a] Relative standard deviation, *n* = 11.
[b] Peaks a–d (in Fig. 1(A)) for Ampholyte buffer and peaks e–h (in Fig. 1(B)) for Pharmalyte.

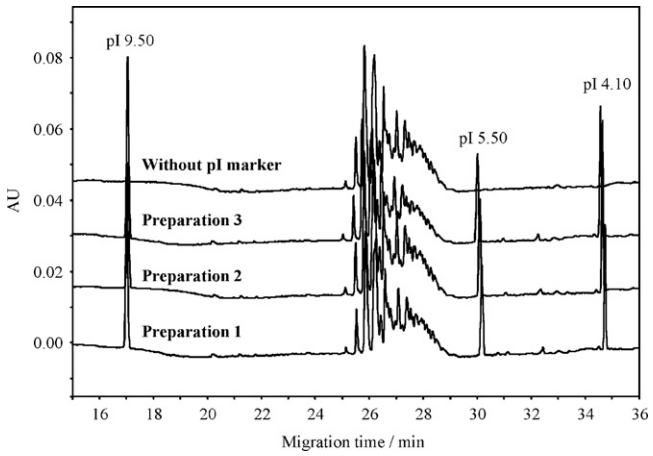

Fig. 3. cIEF analysis of gemtuzumab ozogamicin with and without pI markers under the optimized method. Analytical conditions were the same as in Fig. 1.

3.5. cIEF and gel IEF separations of intact and deglycosylated gemtuzumab ozogamicin

The optimized cIEF method was applied to the analysis of gemtuzumab ozogamicin. The sample solutions prepared in triplicates gave excellent reproducible electropherograms, and more than 20 peaks were detected in each analysis (Fig. 3). On the other hand, in gel IEF, only 13 charge variants were observed (Fig. 4(A)). These results demonstrate that there are some charge variants which are not separated in gel IEF, and the optimized method provides excellent separation with higher resolution than gel IEF.

The influence of deglycosylation on charge heterogeneity of gemtuzumab ozogamicin was examined using the optimized cIEF method. The sample solution of the deglycosylated preparation was prepared and separated by optimized cIEF and gel IEF. Complete deglycosylation of gemtuzumab ozogamicin was confirmed by CE-SDS analysis using SDS-Gel MW Analysis Kit from Beckman (data not shown) [23]. In gel IEF, although the charge variants of both deglycosylated and intact preparations were observed in a range of pI 6.0–7.8, the deglycosylated preparation showed lower and narrower pI range than the intact one (Fig. 4(A)). In addition, the number of detected bands was increased from 13 to 15 after PNGase F digestion. Similarly, in cIEF, several peaks which were observed ca. at pI 7 were completely disappeared or decreased, and shift of the peaks to lower pI values was observed (Fig. 4(B)). These data indicate that deglycosylation effects the number of charge variants, and the present method could distinguish two charge profiles between intact and deglycosylated preparations with higher resolution than gel IEF. Gemtuzumab ozogamicin has sialic acid containing *N*-glycans in its structure and their percent compositions in total *N*-glycans is approximately 9% (unpublished results). Therefore, deglycosylation causes the loss of the negatively charged variants and the deglycosylated variants should show higher pI values than the original one. However, it was surprising that the deglycosylated preparations showed lower pI values than those of the intact one by both cIEF and gel IEF methods. Chu mentioned that deglycosylation with PNGase F converts an asparagine residue to an aspartic acid in protein products [46]. Due to generating additional negative charges caused by deglycosylation, the deglycosylated variants showed lower pI values than the original one. These results indicate that the method can provide detailed information related to deglycosylation of mAb preparations even if they have highly complex structures like gemtuzumab ozogamicin.

3.6. pI values observed in cIEF and gel IEF

Estimated pI ranges for IgG_4 obtained by cIEF and gel IEF showed slightly different ranges; from 6.5 to 7.7 for cIEF and from 7.4 to 8.1 for gel IEF, respectively. Gemtuzumab ozogamicin showed similar results; pI range from 5.9 to 7.1 and from 6.1 to 7.7 for cIEF and gel IEF, respectively.

The optimized cIEF method developed in the present study contains urea as well as HPMC to prevent nonspecific adsorption of proteins to capillary walls. Cifuentes et al. reported that the presence of urea caused shift of the intrinsic pI values of protein samples [37]. However, our study showed that the maximum differences in pI values were approximately 0.1 pI unit in the presence of urea at 4 mol/L when we compared in the absence of urea. In gel IEF, a mixture of 13 pI markers between pI 10.7 and pI 3.5 was used for estimation of pI values. In contrast, only two pI markers having pI 9.50 and 5.50 were used in cIEF for estimation of pI values. In addition, carrier ampholyte sometimes forms non-linear pH gradient as reported previously [47,48] and this is one of the reasons why we observed different pI ranges in gel IEF and cIEF. In order to

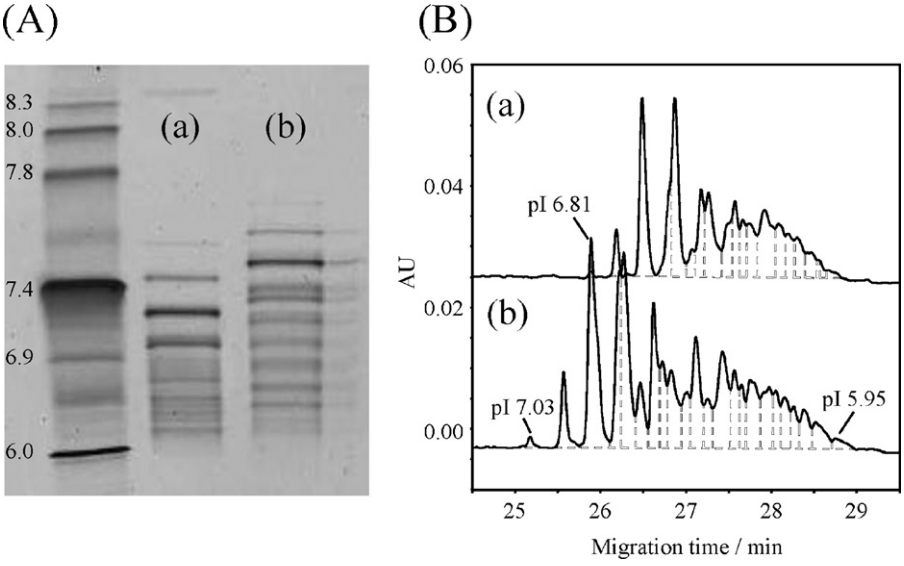

Fig. 4. Gel IEF image (A) and cIEF analysis (B) of gemtuzumab ozogamicin after treatment with PNGase F (a) and gemtuzumab ozogamicin after treatment with water instead of enzyme (b). Analytical conditions: pre-focusing, 75 Vhr at 1 V; focusing, 1500 Vhr at 1500 V. Analytical conditions for cIEF were the same as in Fig. 1.

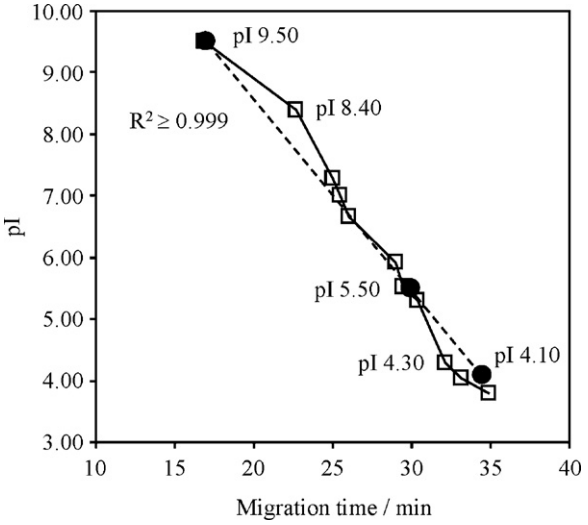

Fig. 5. The relationship between pI values of pI markers and their migration times under the optimized method. White squares and black circles represent 11 synthetic pI markers (marker number 31 and 33–42 in [36]) and three pI markers of pIs 9.50, 5.50 and 4.10, respectively. Each migration time is a mean of three determinations (standard deviations <0.3 for any pIs). The regression curve for the three pI markers was generated by least-square method, and the correlation coefficient of it gave an excellent linearity ($R^2 \geq 0.999$). Analytical conditions were the same as in Fig. 1.

investigate the linearity of pH gradient in optimized cIEF method, 11 synthetic pI markers [36] were used. Fig. 5 shows that the relationship between pI values of pI markers and their migration times. The observed pI values showed obvious differences approximately at pIs 8.40 and 4.30. In particular, the biggest difference between estimated pI value using the regression curve and intrinsic pI value within the tested ranges was 0.67 at pI 8.40.

In order to re-estimate the pI values for precise pI determination of charge variants of IgG_4, two appropriate pI markers which do not interfere with the peaks of the charge variants were selected. The pI 9.50 marker was also added to the sample mixture as reference. The obtained electropherograms of both IgG_4 and 11 synthetic pI markers are shown in Fig. 6. Re-estimated pI range of IgG_4 using pI 8.40 and 5.91 markers is from 7.2 to 8.1, and the range became much closer to that observed in gel IEF. The results demonstrate that Pharmalyte forms non-linear pH gradient during cIEF in the optimized method. From the results above, it is strongly suggested that appropriate commercially available pI markers should be selected based on the target pI ranges of charge variants when accurate pI values are required for detailed characterization of the products.

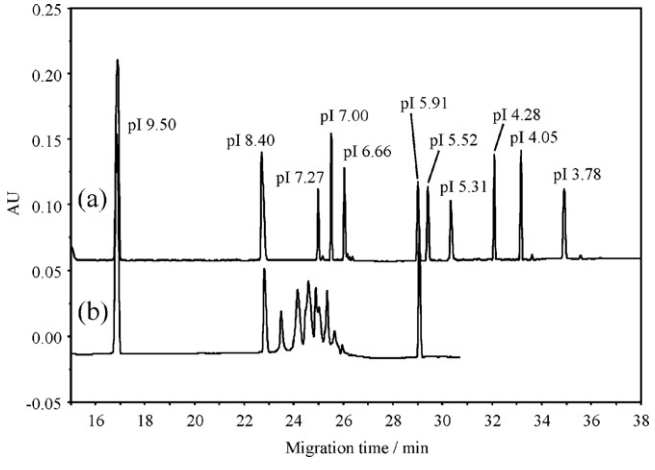

Fig. 6. cIEF analysis of IgG_4 for re-estimation of pI values. (a) Eleven synthetic pI markers (marker number 31 and 33–42 in [36]) and (b) IgG_4 with three pI markers of pIs 9.50, 8.40 and 5.91, respectively. Analytical conditions were the same as in Fig. 1.

4. Conclusions

The study for the effect of several major analytical parameters on the two-step cIEF separation of pI markers and the charge variants of model mAb has led to a reproducible cIEF methodology to analyze mAb conjugates having highly complex structure. The repeatability and intermediate precision of the present method have been demonstrated using a model mAb, IgG_4, and the feasibility of the method has been proved by applying it to a mAb conjugate. This method, which competes favorably with both classical gel IEF and newly developed imaging cIEF in terms of reproducibility with high resolution [26,38,45], is envisaged as a promising tool to analyze mAb conjugates in quality testing and detailed characterization in the pharmaceutical industry. However, we have to carefully consider whole procedures, i.e. from desalting to focusing step, for each biopharmaceutical, because some biopharmaceuticals may lead to form new impurities during analytical procedures. Since the proposed method can provide more detailed information on the charge heterogeneity even if the samples have highly complex structure, this method has a capability to deal with such concerns. In addition, the feasibility of utilization of DB-1 capillary which could provide excellent separation on both native mAb and mAb conjugates has to be evaluated individually when applying the method to other biopharmaceuticals. Furthermore, demonstrated non-linear pH gradient in a capillary indicates that selection of an appropriate set of pI markers based on the target pI ranges of charge variants for each mAb related product is highly recommended for the precise determination of pI values. We believe that the information described in the manuscript will be a useful guideline for evaluation of the newly developed chemically modified biopharmaceuticals.

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