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Rapid high-performance isoelectric focusing of monoclonal antibodies in uncoated fused-silica capillaries

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Abstract

Rapid (<5 min) high-performance isoelectric focusing was performed in uncoated fused-silica capillaries to resolve isoforms of monoclonal antibodies and to determine their isoelectric points (p*I*). The methodology involved the use of a 32 cm (effective length 9 cm)×50 μm I.D. uncoated capillary. (Hydroxypropyl)methyl cellulose was used as an additive to suppress analyte–wall interaction and to precisely control electroosmotic flow so that focusing and mobilization of focused zones past detector occur simultaneously. Urea was included in the separation medium to solubilize the antibodies that precipitated at their point of focusing. The methods with and without urea used ampholytes pH 5–8 to generate a demonstrable linear gradient between pH 5.4 and pH 7.2, based on the separation of various protein standards. Reproducibility [<2% (R.S.D.)] of the migration times (corresponding to the detectable isoforms of the antibodies) was obtained by using two sets of reagents and capillaries on three consecutive days. p*I* values determined from day-to-day with a reference standard were shown to vary by only 0.01 pH unit. The described capillary isoelectric focusing methods provided a rapid, simple and reproducible way of monitoring micro-heterogeneity and p*I* of the murine monoclonal antibodies investigated. © 1997 Elsevier Science B.V.

Keywords: Isoelectric focusing; Monoclonal antibodies

1. Introduction

Isoelectric focusing (IEF) has traditionally been performed on anti-convective media such as slab gels to resolve proteins according to their isoelectric points (p*I*). The formation of pH gradient generated by ampholytes in an electric field is responsible for the separation. When analyzed by IEF, a murine monoclonal antibody often exhibits micro-heterogeneity predominantly due to post-translational glycosylation [1]. Variation in carbohydrate content may significantly affect a glycoprotein's biological activity, clearance, solubility and stability [2,3]. Mutations in hybridoma cell line may also alter p*I* of a monoclonal antibody, ultimately affecting its specificity [4]. In immunodiagnostics industry, monoclonal antibody is used in almost every assay. Monitoring the consistency of production and purification of monoclonal antibodies from lot-to-lot is one of the fundamental requirements for ensuring consistent reagent performance. IEF can be used to detect subtle changes in charge characteristics (p*I*) and in the banding pattern (isoforms or glycoforms) of monoclonal antibodies [5]. Unfortunately, IEF on the gel format requires intensive labor and is time consuming. Tedious gel staining procedure also generates hazardous waste. The stained gels often have to be photographed or dried in order to be kept

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as permanent records. Alternatively, IEF in a capillary format (cIEF) offers the advantages of high resolution, fast separation time (<5 min), on-column detection, better quantitation, automation, ease of data handling with electropherogram printout and low reagent consumption.

IEF in narrow bore glass capillaries (200 μm I.D.) was introduced by Hjertén and Zhu [6] in 1985. Heat generated by the high voltage and buffer resistance could dissipate efficiently with minimal convection in small diameter silica capillaries. However, ionizable silanols ($pK_a \approx 3.0$) on the capillary wall created two obstacles: namely adsorption of proteins to the wall and electroosmotic flow (EOF) due to electrical double layer. The former would cause peak broadening and/or irreversible protein binding, and the later would prevent the attainment of a stable pH gradient. One way to circumvent those obstacles was to chemically modify the inner capillary wall by neutral hydrophilic molecules such as methylcellulose [6] or non-cross-linked polyacrylamide [7]. The use of such coated capillaries, however, required a separate mobilization step after interruption of focusing voltage to move the focused proteins past the detector. While reapplying and maintaining voltage to prevent diffusion, Hjertén and Zhu [6,7] carried out elution by hydrodynamic pumping and, based on electroneutrality, performed chemical mobilization in the direction of cathode by either changing the catholyte with an anolyte or adding salt to the catholyte. The reverse would be true for anodic elution chemically. Other researchers used pressure [5] or vacuum [8] and voltage simultaneously to mobilize the focused proteins past the detector.

In general, the two-step cIEF methods have the following disadvantages: the need to purchase costly capillaries, capillary coating instability, longer analysis time than the one-step cIEF (see below), and complication associated with a separate mobilization step. Siloxane (Si–O–Si–C) linkage, commonly employed for attaching a neutral modifier onto the inner capillary wall, is susceptible to hydrolysis under alkaline conditions. This hydrolysis is amplified when using reagent such as 0.1–1 *M* NaOH solution to rinse capillary between separations. Nevertheless, two-step cIEF kits are commercially available (for a review see Ref. [9]).

Two-step cIEF was used to separate isoforms of monoclonal antibodies [10,11]; however, the separations were not shown to be reproducible. Huang et al. [5] also showed separation of isoforms of a monoclonal antibody by a two-step cIEF method with pressure-driven mobilization. The separation time was in the range of 11–12 min, and reproducibility for p*I* measurement [S.D.=0.03 (pH unit); $n=5$] was reported.

In 1991, Mazzeo and Krull [12] showed that two-step cIEF may be condensed into an one-step process by using polymer methylcellulose (MC) as a dynamic modifier and by maintaining sufficient EOF as a means of mobilization. The adhesion of MC to the inner capillary wall decreases the density of the electrical double layer so that electroosmosis is significantly retarded, allowing sufficient time for focusing to occur. At the same time, MC also prevents close contact between proteins and the wall, thus minimizing their interaction. Increased viscosity also serves to minimize diffusion during electrophoresis. This one-step approach was demonstrated for the separation of model proteins in uncoated fused-silica capillaries and in C_8-coated capillaries by Krull and co-workers [12–15]. Thormann and co-workers [16–18] also reported similar findings using (hydroxypropyl)methyl cellulose (HPMC) in uncoated capillaries. However, instead of filling the capillary completely with the ampholyte–sample mixture, the later group used only partial filling.

One-step cIEF was also used to separate isoforms of monoclonal antibodies in an octyl-bonded capillary under native conditions [19] and in an uncoated capillary under denatured conditions [20]. In the former case, HPMC was used to further control EOF and minimize analyte–wall interaction. The authors [19] used two standards (above and below p*I* of a monoclonal antibody) within a separation to directly determine p*I* based on a fitted non-linear curve analysis. p*I* measurement by the method was shown to exhibit a wide range of variation [0.00–0.12 (S.D.)]. In the later study, Kubach and Grimm [20] demonstrated the separation of isoforms of one monoclonal antibody in a separation time of 30–35 min in an uncoated fused-silica capillary under denatured condition with urea. Reproducibility of the separation was not reported.

In this study, simple and reproducible one-step cIEF methods with and without urea were developed

to carry out rapid (<5 min) separation of isoforms of murine monoclonal antibodies using only uncoated capillaries. Separations of six murine monoclonal antibodies were shown. Reproducibility of migration times was demonstrated on three consecutive days with two sets of reagents and capillaries. Determination of p*I* from day-to-day was shown to be highly reproducible [0.1% (R.S.D.)]. The developed methods have been routinely used for monitoring consistency in charge characteristics (p*I*) and/or in banding pattern (isoforms or glycoforms) of many murine monoclonal antibodies.

2. Experimental

2.1. Reagent and materials

Pharmalytes pH 5–8 and urea were purchased from Sigma (St. Louis, MO, USA). (Hydroxypropyl)-methyl cellulose, orthophosphoric acid 85% and sodium hydroxide (1 *M*) were obtained from Fluka (Ronkonkoma, NY, USA). Sodium phosphate (Fisher Chemical, Fair Lawn, NJ, USA) was used to prepare the dialysis buffer (5 m*M*) at pH 7.0. Hydrochloric acid (1 *M*) and sodium hydroxide (1 *M*) were also obtained from Fisher Chemical for capillary conditioning. Fused-silica capillaries with 50 μm I.D.× 360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). IEF standards (bovine carbonic anhydrases, p*I* 5.4 and 5.9; human carbonic anhydrase, p*I* 6.6; and horse myoglobin, p*I* 7.2) were purchased from Sigma and used without further purification or treatment. Purified murine monoclonal antibodies ''a'', ''b'', ''c'', ''d'', ''e'', and ''f'' were obtained from product development and manufacturing groups in Chiron Diagnostics (East Walpole, MA, USA). A mixture of human hemoglobin variants (Hb C, S, F and A) was purchased from Isolab (Akron, OH, USA), and human HbA1c was obtained from Fitzgerald Industries International (Concord MA, USA).

2.2. Instrumentation

cIEF was performed on a Hewlett-Packard ^{3D}CE ChemStation (Waldbronn, Germany) outfitted with a HP Vectra 486 computer. Detection was at 280 nm using the electropherograph's built-in diode array detector. Temperature was regulated to 35°C by the instrument's air-circulating heating and cooling system. Operation of the instrument and data collection/analysis were controlled by HP ChemStation System Software Revision A.03.01.

2.3. Methods

2.3.1. Capillary preparation

Fused-silica capillaries 32 cm (effective length from anodic end to the detector 9 cm)×50 μm I.D. were used. Prior to use, columns were preconditioned daily with the following sequence of solvents by applying the electropherograph's high pressure ''flush'' (920–940 mbar) from the inlet vial for each solvent: deionized water (8 min), 1 *M* NaOH (20 min), deionized water (8 min), 1 *M* HCl (8 min) and 0.4% HPMC (8 min). Previously unused capillaries were filled with deionized water overnight and followed by the daily preconditioning described above.

2.3.2. Reagents

Pharmalytes pH 5–8 solution was mixed (6:94, w/w) with 0.5% HPMC to prepare R1, and was mixed (1:9, w/w) with 0.8% HPMC to prepare R2. HPMC solutions (0.5% and 0.8%) were prepared from 1% HPMC. The urea-containing reagent (R3) was prepared by mixing (1:9, w/w) Pharmalytes pH 5–8 with the solution [urea (7 *M*) and HPMC (0.3%)]. Catholyte (20 m*M* NaOH) and anolyte (25 m*M* phosphoric acid) were prepared from stock solutions of 1 *M* NaOH and 1 *M* phosphoric acid, respectively. All reagents were filtered through 0.2 μm membrane and stored in refrigeration at 4°C until needed.

2.3.3. Sample preparation

All purified antibodies (0.3–10 mg/ml) were dialyzed against 5 m*M* Na_2HPO_4, pH 7. Samples were prepared by mixing with R1 [1:4 (v/v), sample: R1], with R2 [1:1 (v/v)], or with R3 [1:1 (v/v)]. For p*I* determination, antibody samples were spiked with a small volume of a protein standard [e.g., antibody–standard (17:3, v/v), (p*I* 5.9)].

2.3.4. cIEF

Prior to each separation, the capillary was ''flushed'' by 1 *M* or 0.1 *M* NaOH solution for 1.5 min, by 0.4% HPMC solution for 1 min and by appropriate sample for 2 min. Each flushing completely filled up the capillary with appropriate solvent or sample. Because voltage polarity was reversed, anodic end of the capillary was located closer to the detector. A constant voltage of −13 kV was applied for 5 min, unless indicated otherwise. Separation time for p*I* determination was longer due to the later migration of carbonic anhydrase (p*I* 5.9). Separation of myoglobin (horse heart) or Isolab mixture (human Hbs C, S, F and A) as test standard was performed prior to separating samples of interest.

3. Results and discussion

Fig. 1 shows the electropherograms of five different monoclonal antibodies ''a–e'' whose isoforms were separated in less than 5 min. The p*I* ''fingerprint'' or the unique band pattern observed for each antibody is predominantly due to variation in glycosylation of its polypeptide during post-translational modification [21,22]. Carbohydrate moieties can affect a glycoprotein's hydrophobicity, charge, mass, shape and conformation [3] and may therefore also affect its biological activity, specificity, solubility, clearance and stability [2,3]. Because IEF separates proteins on the basis of their net charges (p*I*), this methodology is used for detecting subtle changes in charge heterogeneity of a monoclonal antibody after repeated freeze–thaw cycles, after long-term storage of hybridoma cell line, and/or from a hybridoma over long periods in culture [23]. Mutations in the cell line may also alter detectable p*I* and specificity of a monoclonal antibody [4]. Other forms of post-translational modification such as deamidation of the glutamine and/or asparagine residues may also contribute to the micro-heterogeneity of a monoclonal antibody [1,24].

Separations were initially attempted in the longer portion of the capillary under normal (i.e., positive) polarity configuration with or without tetramethylethylenediamine (TEMED) as a basic extender. However, this normal capillary configuration gave excessive peak broadening due to long migration before detection. TEMED, when present, added complication to the already complex system of cIEF and made the separation more difficult to be reproduced and method(s) validated. It was soon realized that the reversed polarity configuration without TEMED would circumvent those difficulties. Based on this later configuration with ampholytes pH 5–8, only 0.84 pH unit was expected to span across the 9 cm section (28%) of the 32 cm long capillary, detecting only proteins with p*I* values between 5 and 5.84. However, antibodies and proteins with p*I* values $\gg$ 5.84 were still detected.

In order to rationalize this electrophoretic behavior, the following mechanism is proposed. During the cIEF, protons from anolyte enter the capillary to titrate not only the ampholytes but also the negatively charged silanols on the uncoated wall, whereas hydroxyls in catholyte when entered are repelled by the wall and participate solely in titrating the ampholytes. As the results, there are more free hydroxyls than free protons available for titrating the ampholytes to form the pH gradient in the capillary. The excessive free hydroxyls probably then function as a basic extender, compressing the pH gradient to the anodic end so that the 9 cm segment of the capillary contains most of the available gradient (see also Table 3).

Many antibodies were found precipitated at their point of focusing under native cIEF conditions (e.g., Fig. 2, top). Reagent R3 that contained urea was then used to produce a well-behaved separation (e.g., Fig. 2, bottom). The use of this urea-containing reagent to circumvent the precipitation problems has been successful in numerous cases (data not shown).

Collectively, the electropherograms (Figs. 1 and 2, bottom) suggest that slight driftings (cathodic and anodic) of the pH gradient (i.e., leading and tailing portions of first and last peaks, respectively) exist. Such drifts, however, appear to be approximately symmetrical under the conditions used and are reproducible (see below). The relationship between anionic electrophoretic migration and cathodic EOF has been thoroughly reviewed and described [9,25].

Tables 1 and 2 show that the migration times of the resolved isoforms of the antibodies were highly reproducible under native and non-native conditions, respectively. In both cases, the separations were

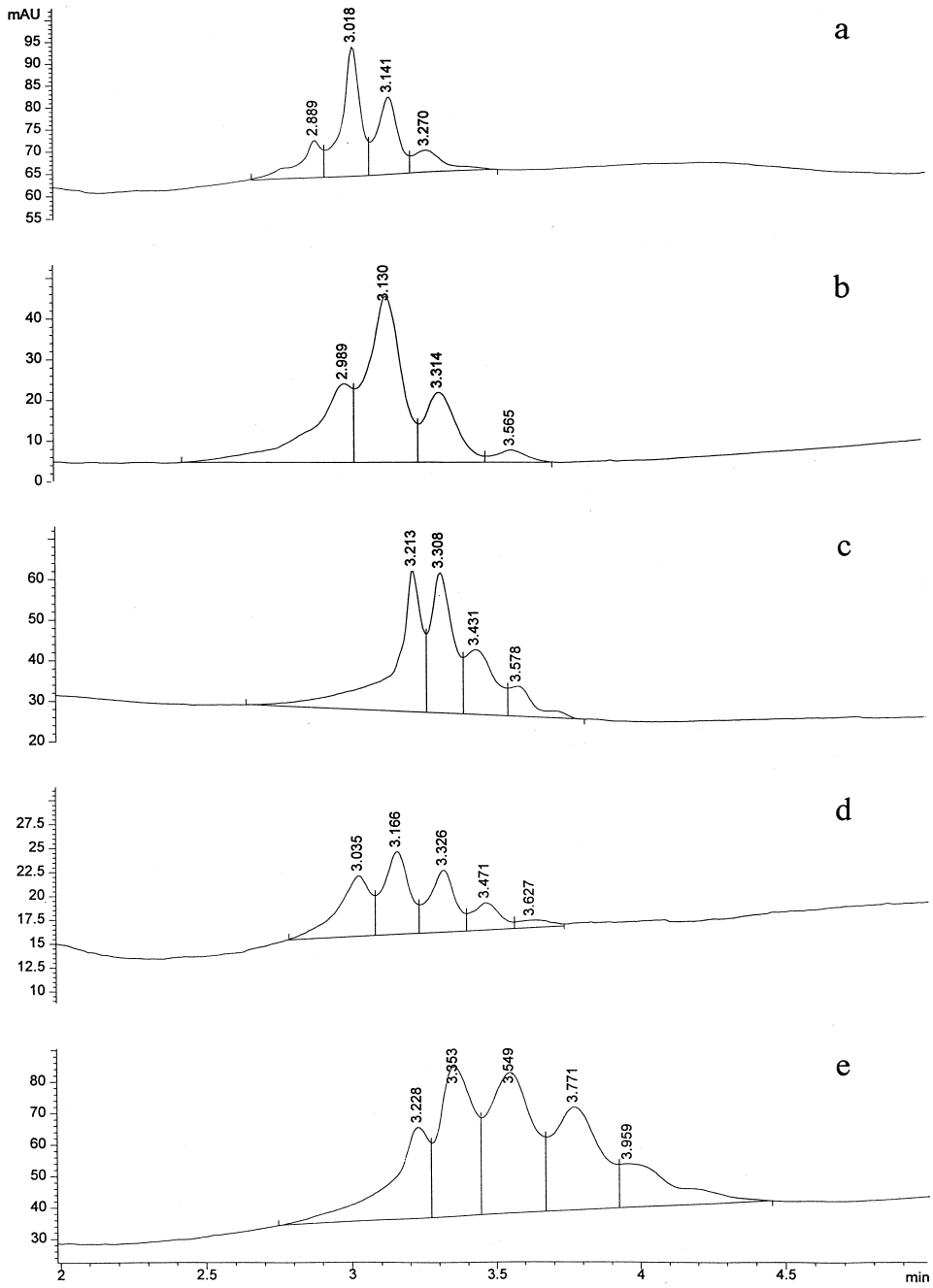

Fig. 1. Electropherograms of antibodies ‘‘a–e’’. Figures a–e represent the separations of antibodies ‘‘a–e’’, respectively. Migration time is indicated on top of every electrophoretic peak. R2 was used in Figure a, and R1 in Figures b–e. A constant voltage of −13 kV was used in Figures a and c, whereas −15 kV was used in Figure d. In Figures b and e, a linear voltage gradient of −17 kV to 0 kV in 8 min was created for the 5 min window monitored.

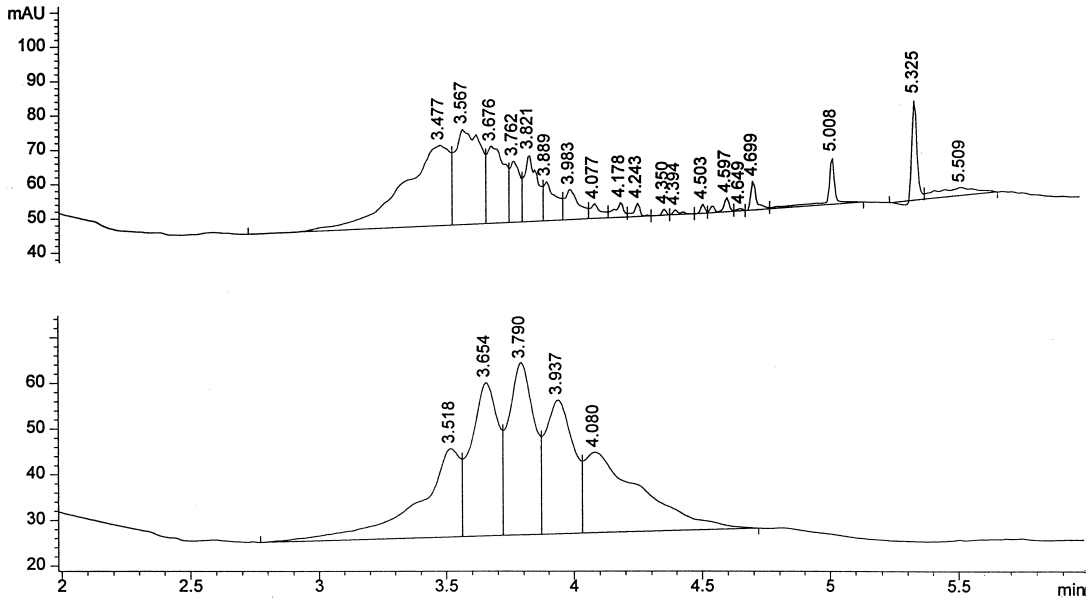

Fig. 2. Electropherograms of antibody ''f'' without (top) and with (bottom) urea. R2 was used in the top figure, and R3 in the bottom figure. In both cases, a constant voltage of −13 kV was applied.

Table 1
Reproducibility of migration time for antibody ''e''

Peak	Migration time (min)					R.S.D.[d] (%)
	Day 1[a]	Day 2[a]	Day 3[b]	Average	S.D.[c]	
1	4.020, 3.942	4.031, 3.980	4.032, 3.964	3.995	0.038	1.0
2	4.151, 4.073	4.161, 4.105	4.168, 4.101	4.127	0.039	0.9
3	4.314, 4.239	4.328, 4.263	4.329, 4.280	4.292	0.037	0.9
4	4.500, 4.426	4.502, 4.460	4.519, 4.455	4.477	0.035	0.8

[a] Experiments were performed using R1 lot A and capillary I, whereas [b] used R1 lot B and capillary II. In both cases, data were obtained in duplicate daily.
[c] S.D. = Standrad deviation ($n = 6$).
[d] R.S.D. = Relative standard deviation.

Table 2
Reproducibility of migration time for antibody ''d'' in the presence of urea

Peak	Migration time (min)					R.S.D. (%)
	Day 1[a]	Day 2[b]	Day 3[a]	Average	S.D.	
1	4.272, 4.206	4.332, 4.136	4.331, 4.274	4.259	0.076	1.8
2	4.500, 4.432	4.564, 4.361	4.560, 4.500	4.486	0.078	1.7
3	4.750, 4.680	4.815, 4.612	4.799, 4.749	4.734	0.076	1.6
4	4.968, 4.915	5.044, 4.851	5.003, 4.957	4.956	0.068	1.4

[a] Experiments were performed using R3 lot A and capillary I, whereas [b] used R3 lot B and capillary II. In both cases, data were obtained in duplicate daily.

Table 3
Linear equations and R^2 values from the plots of migration time (y-axis) vs. pI (x-axis) of standards

Separation	Equation, $y=mx+b$, and R^2 value	
	Native	With urea
1	$y=-2.714x+22.615$, 0.999	$y=-3.131x+26.294$, 0.991
2	$y=-2.730x+22.611$, 0.999	$y=-3.053x+25.739$, 0.991
3	$y=-2.693x+22.173$, 0.999	$y=-3.024x+25.471$, 0.991

carried out on three consecutive days using two different sets of reagents and capillaries. Data were acquired in duplicate daily. As shown in Table 1, R.S.D.s ($n=6$) are 1% or less for the four detectable peaks of antibody ''e''. R.S.D.s ($n=6$) are $<2\%$ for the four peaks of antibody ''f'' separated in the presence of urea (Table 2). Both tables also show that the second value of the duplicate consistently exhibited faster migration by 1–2% (except the 4% in day 2 of Table 2). One possible explanation is that mixing the same sample (40 or 50 μl) from repetitive usage with the carry-over solution on the outer surface of the capillary might affect the sample's viscosity/pH such that the EOF was slightly increased in the second separation. Further (but unnecessary) improvement in the reproducibility when two same sample vials were used to obtain the duplicate also supports this explanation.

Four pI standards (bovine carbonic anhydrases, pI 5.4 and 5.9; human carbonic anhydrase, pI 6.6; and horse myoglobin, pI 7.2) were used to investigate the linearity of the pH gradient for the proposed cIEF systems. This pH range (5.4–7.2) covered the pI values of all monoclonal antibodies investigated here and in the results obtained by Kundu and Fenters [19]. Table 3 shows the line equations obtained from the plots of pI (x-axis) vs. migration time (y-axis) for three consecutive separations of the above standard mixture. The differences in slopes and in y-intercepts were expected because of migration time variation, as discussed above. Despite the observed differences among the triplicate, pH gradient for each separation turned out to be highly linear based on the linear regression trendline analysis ($R^2=0.999$). However, the same analyses revealed an R^2 value 0.991 for the separations in the presence of urea. The latter could be explained by the deviation of pI values of the protein standards under denatured or partially denatured conditions.

Fig. 3 shows an electropherogram that was obtained for the purpose of determining pI for antibody ''a''. A standard (carbonic anhydrase, pI 5.9) was included in the separation. To calculate pI for each of the four isoforms detected, experimental migration times were first corrected by the factor [(migration time of the standard in selective equation of Table 3)/(migration time of the standard in the separation)]. The corrected migration times were then converted into pI values using the appropriate equations in Table 3. The results are shown in Table 4 for the isoforms of antibody ''a'' and antibody ''f''. R.S.D.s (0.2%) are consistently higher for the

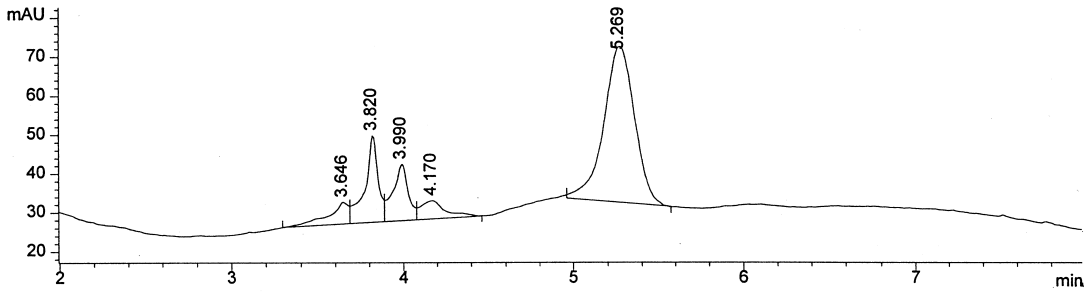

Fig. 3. Electropherogram of antibody ''a'' and carbonic anhydrase (pI 5.9). Carbonic anhydrase (migration time = 5.269 min) was included as the pI standard. R2 was used for the separation, and voltage = −12 kV.

Table 4
Reproducibility of p*I* determined by the equations of Table 3

Condition	Antibody	Peak	p*I*					R.S.D. (%)
			Eq. 1	Eq. 2	Eq. 3	Average	S.D.	
Non-urea	"a"	1	6.656	6.642	6.624	6.641	0.016	0.2
		2	6.577	6.564	6.548	6.563	0.015	0.2
		3	6.499	6.488	6.473	6.487	0.013	0.2
		4	6.416	6.407	6.394	6.406	0.011	0.2
Urea	"f"	1	6.935	6.945	6.942	6.941	0.005	0.1
		2	6.878	6.887	6.884	6.883	0.005	0.1
		3	6.820	6.828	6.825	6.824	0.004	0.1
		4	6.756	6.763	6.761	6.760	0.004	0.1
		5	6.698	6.704	6.702	6.701	0.003	<0.1

p*I* values determined under native condition vs. those (R.S.D. = 0.1%) obtained in the presence of urea. Such data indicate that p*I* measurement in the former case is more susceptible to slope, in good agreement with the fact that the slopes generated without urea in Table 3 are less steep than those with urea. Only one chosen equation, however, is needed for routine p*I* determination. Table 5 shows that variation in p*I* measurement becomes consistently low (R.S.D. = 0.1%) between days under both native and non-native conditions when p*I* values were determined with a fixed equation from each condition. This low R.S.D. value translates to the measurement of p*I* values with day-to-day variation of only 0.01 pH unit.

Finally, the method using R3 was applied to the separation of HbA1c. In Fig. 4, the peak at the migration time of 3.139 min (arrowed) was absent without including HbA1c in the mixture of human Hb standards (C, S, F and A), demonstrating the ability of the method to also rapidly resolve HbA1c from HbA in a separation time of less than 3.5 min.

4. Conclusions

Simple, rapid, automated and reproducible cIEF methods with and without urea in uncoated fused-silica capillaries have been successfully demonstrated for routine separation of murine monoclonal antibodies. Migration times were highly reproducible [<2% (R.S.D.)] when examined on three consecutive days with two different sets of reagents and capillaries. A narrow but sufficient pH gradient to cover the murine monoclonal antibodies investigated was shown to be linear (Table 3) between pH 5.4

Table 5
Reproducibility of p*I* determined with and without urea

Condition	Peak	p*I*				R.S.D. (%)
		Day 1[a]	Day 2[a]	Average	S.D.	
Native (antibody "a")	1	6.656, 6.661	6.648, 6.647	6.653	0.007	0.1
	2	6.577, 6.581	6.567, 6.569	6.574	0.007	0.1
	3	6.499, 6.503	6.490, 6.496	6.497	0.005	0.1
	4	6.416, 6.420	6.412, 6.419	6.417	0.004	0.1
With urea (antibody "f")	1	6.935, 6.951	6.946, 6.946	6.945	0.007	0.1
	2	6.878, 6.893	6.886, 6.886	6.886	0.006	0.1
	3	6.820, 6.835	6.827, 6.827	6.827	0.006	0.1
	4	6.756, 6.772	6.762, 6.762	6.763	0.007	0.1
	5	6.698, 6.712	6.699, 6.700	6.702	0.007	0.1

[a] Data were obtained in duplicate daily.

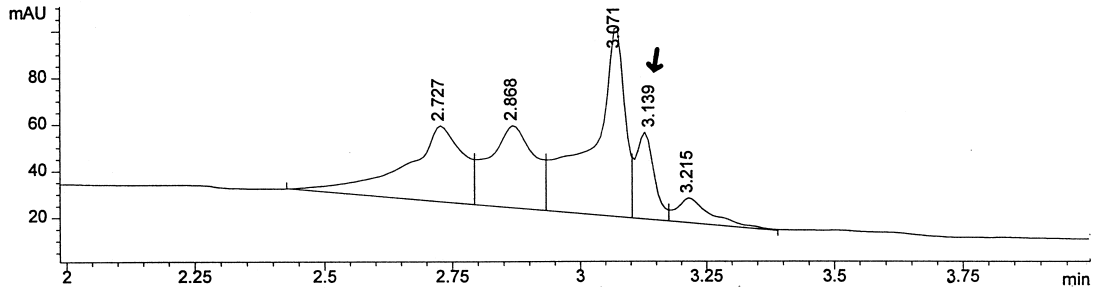

Fig. 4. Electropherogram of human Hb mixture (C, S, F and A) and HbA1c. The peak indicated by arrow (migration time = 3.139) was absent without including HbA1c in the separation. R3 was used for the separation.

and pH 7.2. p*I* values determined from day-to-day were shown to vary by only 0.01 pH unit, allowing for the detection of slight changes (>0.01 unit in p*I*) in charge characteristics of the monoclonal antibodies due to potential cell line mutation. The micro-heterogeneous fingerprints obtained in form of electropherogram for different lots of the same antibody can be conveniently stored and used for detecting changes in banding patterns. Significant variation of fingerprint may indicate for instability of cell line or poor sample handling. The proposed one-step cIEF methods have simplified (i.e., one-step vs. two-step) and made economical (i.e., inexpensive uncoated fused-silica capillaries vs. expensive coated capillaries) the process of determining p*I* and of monitoring micro-heterogeneity for the monoclonal antibodies.

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