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A method for routine analysis of recombinant immunoglobulins (rIgGs) by capillary isoelectric focusing (cIEF)

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Abstract

A capillary isoelectric focusing (cIEF) method was developed for routine analysis of recombinant immunoglobulins (rIgGs). The cIEF method used a dimethyl siloxane-coated capillary and a separation matrix of 2% ampholytes in 0.4% methylcellulose (MC). The rIgGs, and internal *pI* marker protein standards, were mixed with carrier ampholyte in MC, focused using high voltage, and then the protein bands were mobilized past a UV detector by simultaneous application of low pressure and voltage. Qualitatively and quantitatively equivalent rIgG focusing profiles were obtained via cIEF and gel-based IEF, with individual isoform peak area percentages and calculated peak *pI* values being comparable for the same samples. Linear relationships were obtained for peak area response versus sample concentration, and for the *pI* gradient developed between the internal *pI* marker standards. The relative standard deviation (RSD) in rIgG peak areas was less than 2% intra-day and less than 8% inter-day (72 h). The RSD for the mobilization times of rIgG peaks was less than 1% intra-day and less than 3% inter-day (72 h). There was no observed decrease in the performance of the capillary over 150 analyses. cIEF offers several important advantages over gel IEF, e.g. direct, quantitative detection of proteins by intrinsic UV absorbance at 280 nm, rapid analyses (≤ 30 min), capability of automation, and one-step, electronic data analysis and archival. These data demonstrate the superiority of the cIEF method for routine analysis of rIgGs.

Keywords: Recombinant IgG; Isoelectric focusing; Capillary electrophoresis

1. Introduction

Isoelectric focusing (IEF) is perhaps the best single technique for assessing charge heterogeneity

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in protein molecules. The predominant analytical IEF method employs a cross linked gel matrix containing ampholytes for separating the protein isoforms, which are subsequently detected by staining with either Coomassie Blue or silver salts. The individual sample isoforms can then be quantified by scanning the stained gels with a densitometer. More recently, capillary isoelectric focusing (cIEF) of proteins, especially recombinant immunoglobulins (rIgGs), has been employed to obtain similar results [1]. High resolution separations can be achieved within a capillary filled with viscous solution containing ampholytes, followed by on-line detection of the focused protein bands by UV [2]. A variety of cIEF approaches for protein analysis have been described [3–7] and reviewed [8].

Analysis of proteins by gel IEF requires several time consuming steps, e.g. electrofocusing, protein fixing, staining, destaining, scanning and band area integration. On the other hand, cIEF offers advantages over the gel IEF method with regard to all of these points, including: (a) rapid analysis times (typically ≤ 30 min); (b) direct, quantitative detection of proteins by intrinsic UV absorbance at 280 nm; (c) capability for automation, resulting in reduced time and labor requirements; and (d) capability of electronic data analysis and archival, of particular value for validation and cGMP compliance. However, the advantages of cIEF particularly in the analysis of therapeutic antibodies have not been explored.

In this report, a two-step cIEF method for routine analysis of rIgGs is described. First, the protein is focused in a capillary which has been internally derivatized (coated) in order to reduce electro-osmotic flow (EOF); then low pressure is applied to mobilize the protein bands past the detector window. This two-step method has been found to be suitable for rIgGs over a wide *pI* range. Factors effecting the separations, reproducibility, and results obtained when using the two-step cIEF method, as well as the comparative merits of this cIEF method vis-a-vis gel IEF are discussed in this report.

2. Experimental

2.1. Sample preparation

For concentrated solution of rIgGs, each preparation was diluted separately in water to 5 mg protein ml^{-1} and 25 μl of each was then diluted with 500 μl of 2% ampholyte (Pharmalyte, pH 3–10 for typical IgGs) (Pharmacia Biotech, Piscataway, NJ) in 0.4% methylcellulose (MC) solution. Myoglobin (horse heart, *pI* 6.8, 7.2), lysozyme (chicken egg, *pI* 9.6) and lectin (Lens culinaris, *pI* 8.2, 8.6, 8.8) (Sigma, St. Louis, MO) were used as standard markers for determining the *pI* of the rIgGs. Other ampholytes tested for their utility in the two-step cIEF of rIgGs included Servalytes 3–10 (Crescent, NJ) and Sigma ampholyte 3–10.

2.2. Preparation of 0.4% methylcellulose

Methylcellulose (1500 cp, Sigma, 0.4 g) was added to 100 ml of water in a 200 ml flat bottom boiling flask and stirred at room temperature for 10 min. The flask was placed in a boiling water bath until a homogeneous colloid was formed, and then the solution was stirred overnight at room temperature. The solution was filtered using a 0.45 μm filter (PTFE Acrodisc, Gelman) before use.

2.3. Capillary IEF with pressure mobilization

A Beckman P/ACE 2200 capillary electrophoresis unit, supplied with a data acquisition/analysis system consisting of an IBM PS/2 computer and Beckman System Gold software (Beckman Instruments, Palo Alto, CA), was used in these studies. However, other capillary electrophoresis systems capable of pressure mobilization can be used. The apparatus was operated in the normal polarity mode, using a Beckman capillary cartridge with a 50×200 μm aperture. Proteins were focused using a CE μSIL DB-1 capillary (50 μm i.d., dimethyl siloxane coated, 0.05 μm film thickness (47 cm total length (40 cm effective length), J&W Scientific, Folsom, CA). Catholyte consisted of 20 mM

NaOH in 0.4% MC, anolyte consisted of 120 mM phosphoric acid in 0.4% MC, and a separation matrix comprised of 2% Pharmalyte, pH 3–10, in 0.4% MC. For cIEF of rIgGs having very basic *pI*s, the Pharmalyte in the separation matrix was changed to a 30:70 mixture of Pharmalytes 3–10 and 8–10.5, respectively. For all of the cIEF experiments, a constant voltage setting of 30 kV was used and the capillary cartridge temperature was maintained at 30°C. After focusing (8–20 min), the protein bands were mobilized by applying a low pressure (0.5 psi) while maintaining the voltage typically at 30 kV. The migrating protein bands were detected by UV absorbance at 280 nm.

DB-1 coated capillaries (new or old) were preconditioned once by flushing with methanol, followed by water, and then 0.4% MC, for 10 min $step^{-1}$ when installed into the instrument. The capillary was rinsed with 2% Pharmalytes in 0.4% MC for 4 min. The rIgG samples were injected onto the column using high pressure (20 psi) for 21 s. Next, the *pI* marker proteins, lysozyme and myoglobin, were injected using low pressure (0.5 psi) for 50 s. Although the focusing time was protein dependent, typically 8–20 min was sufficient for most of the rIgGs. The current initially increased sharply, to a maximum of approximately 5 μA, and then steadily decreased to a plateau at 1 μA within 5–10 min. A low pressure of 0.5 psi was applied at 8–20 min into the run for about 20 min in order to mobilize the focused protein bands past the detector window monitored at 280 nm, while maintaining a constant voltage of 30 kV. After the cIEF run was completed, the capillary was rinsed consecutively with water for 2 min, phosphoric acid (0.1 M) for 1 min, and again with water for 3 min. This method is also refered to as a two step method.

2.4. Capillary IEF without pressure mobilization

The cIEF was performed using a 37 cm long eCAP neutral capillary (50 μm, 5.5 cm effective length, Beckman) as per manufacturer's instructions. Protein bands were monitored at 280 nm. Anolyte consisted of 10 mM phosphoric acid and catholyte consisted of 20 mM NaOH. rIgG sample (10 μl of approximately 5 mg ml^{-1}) was mixed with 3 ml of carrier ampholyte solution consisting of 4% Pharmalyte 3–10, 1.5% TEMED (Sigma) in 0.4% hydroxypropyl MC (Sigma). The column was preconditioned with 10 mM phosphoric acid for 1 min, and then filled with sample/carrier ampholyte solution. Focusing was carried out at 23°C using 15 kV with reversed polarity. After each run, the column was rinsed sequentially with 0.1 M HCl and water. This method is also refered to as a one-step method.

2.5. Gel IEF

Standard gel IEF analysis was performed with commercial pre-cast gels with pH range of 3–10 (IsoGel, FMC). Coomassie stained gels were analyzed using Image Quant Personal Densitometer system (Molecular Dynamics).

3. Results

3.1. IEF profiles

Results from the cIEF and gel IEF of three different rIgG preparations are shown in Figs. 1–3. In general, the overall cIEF profile for each rIgG preparation compared well with the corresponding densitometric scan profile from gel IEF. A comparison of the data for IgG-2 (Fig. 5) from gel IEF and cIEF is described in Table 1. Quantitative differences were observed between the two methods and these differences are likely due to differential dye binding and optical range of the instruments for scanning gels.

3.2. pI Determination

The cIEF method yielded a linear pH gradient over a wide *pI* range in the capillary. A typical calibration plot of migration times versus *pI* values of the marker protein standards is shown in Fig. 4. A linear calibration curve ($r^2 = 0.991$) was obtained for *pI* standards ranging from *pI* 9.6 (lysozyme) to *pI* 6.8 (myoglobin). The *pI*s of the rIgG isoforms were estimated from the calibration curve according to their migration times. The *pI*

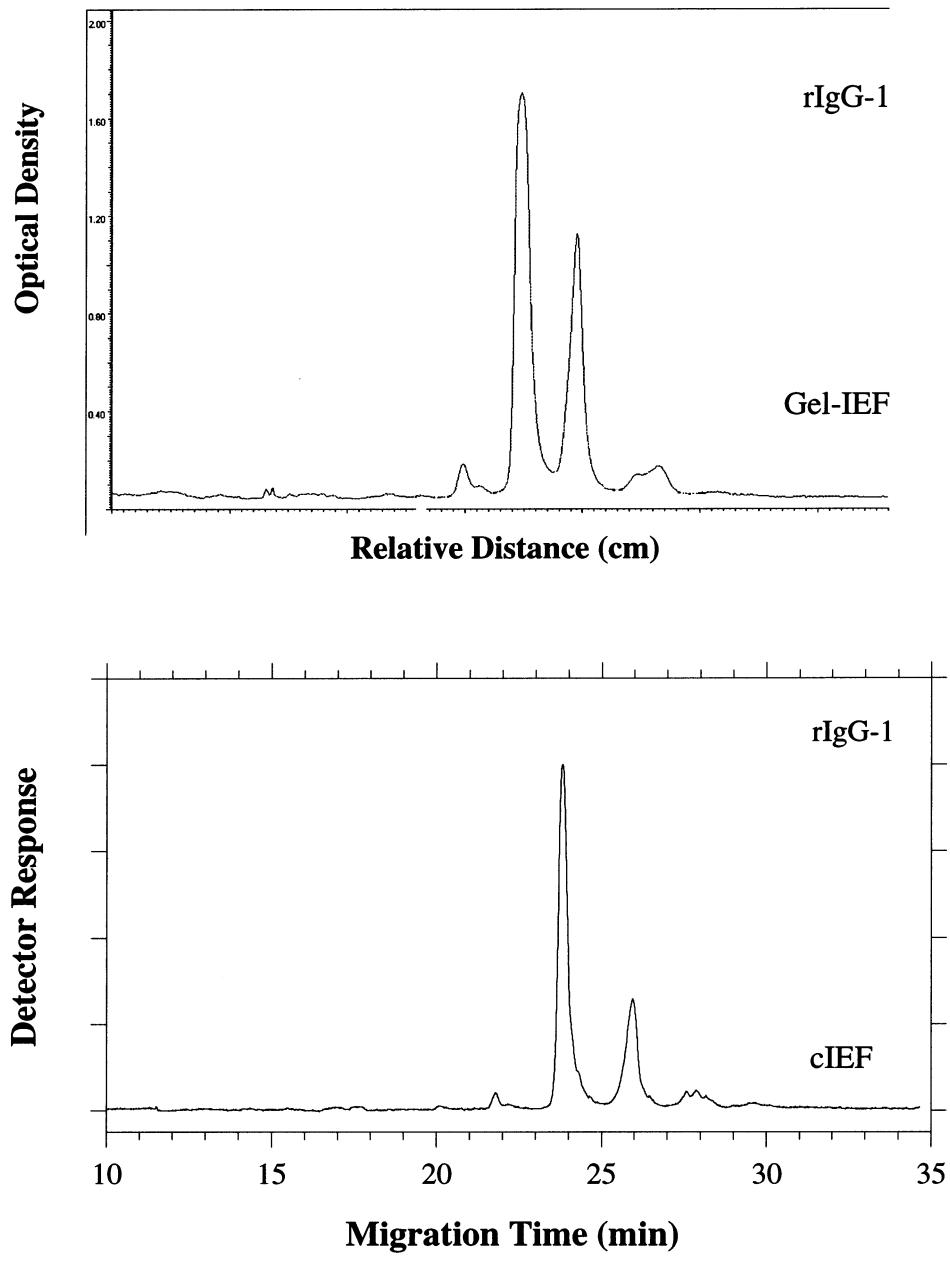

Fig. 1. Comparison of gel-IEF and cIEF methods for rIgG-1.

of unknown rIgGs were generally determined by linear extrapolation between two internal marker proteins whose *pI*s bracketed the expected *pI* of the rIgG (Fig. 5).

3.3. Linearity

A typical linear plot obtained with increasing concentrations of one of the rIgGs is shown in

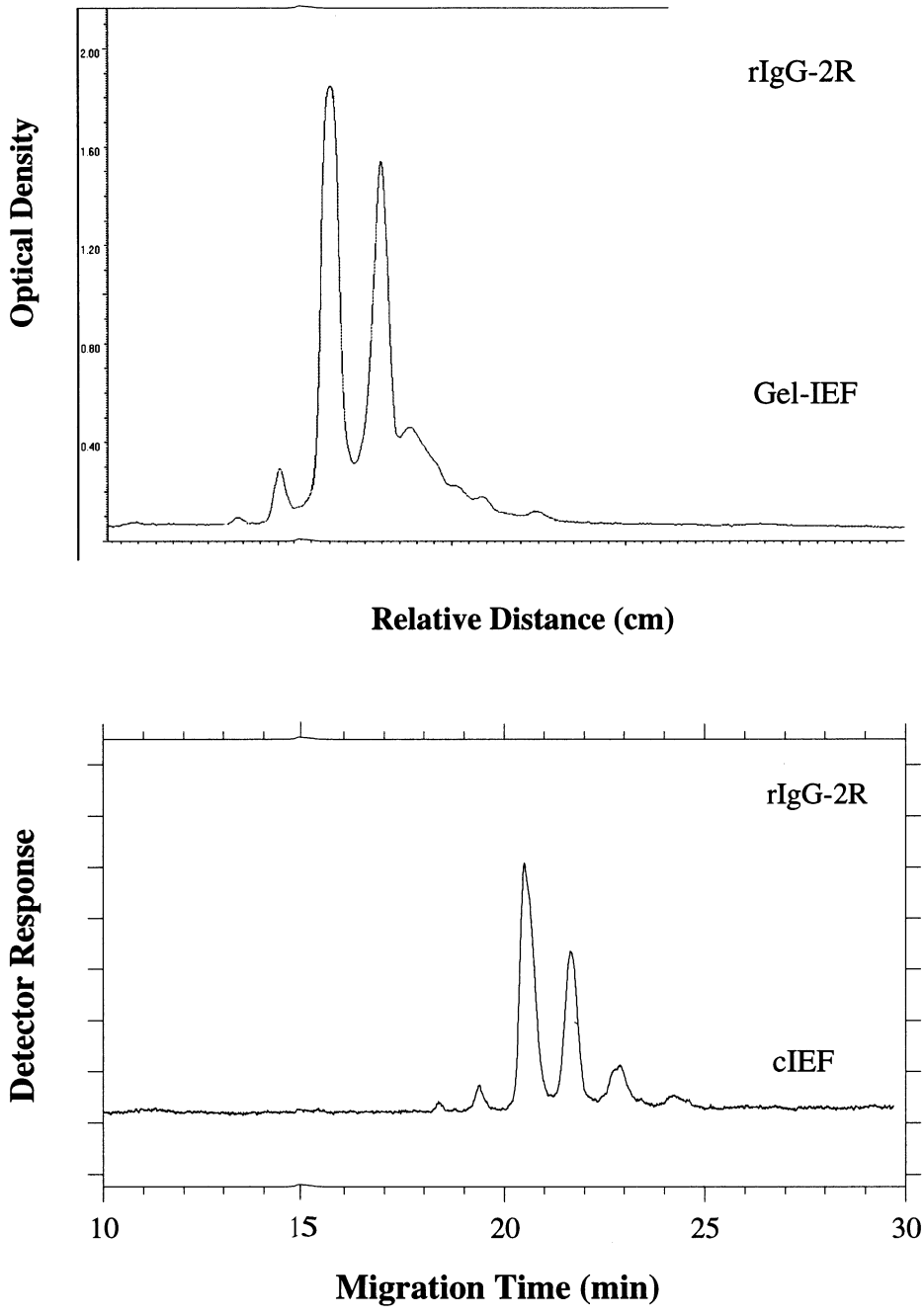

Fig. 2. Comparison of gel-IEF and cIEF methods for rIgG-2R.

Fig. 6. Six different protein concentrations, ranging from 0.625–20 mg ml^{-1} (IgG-2) were separately injected, and the data for the two major peaks obtained (Fig. 5) was analyzed. A linear relationship for peak area versus sample concentration was observed for both peaks ($r^2 \geq 0.998$

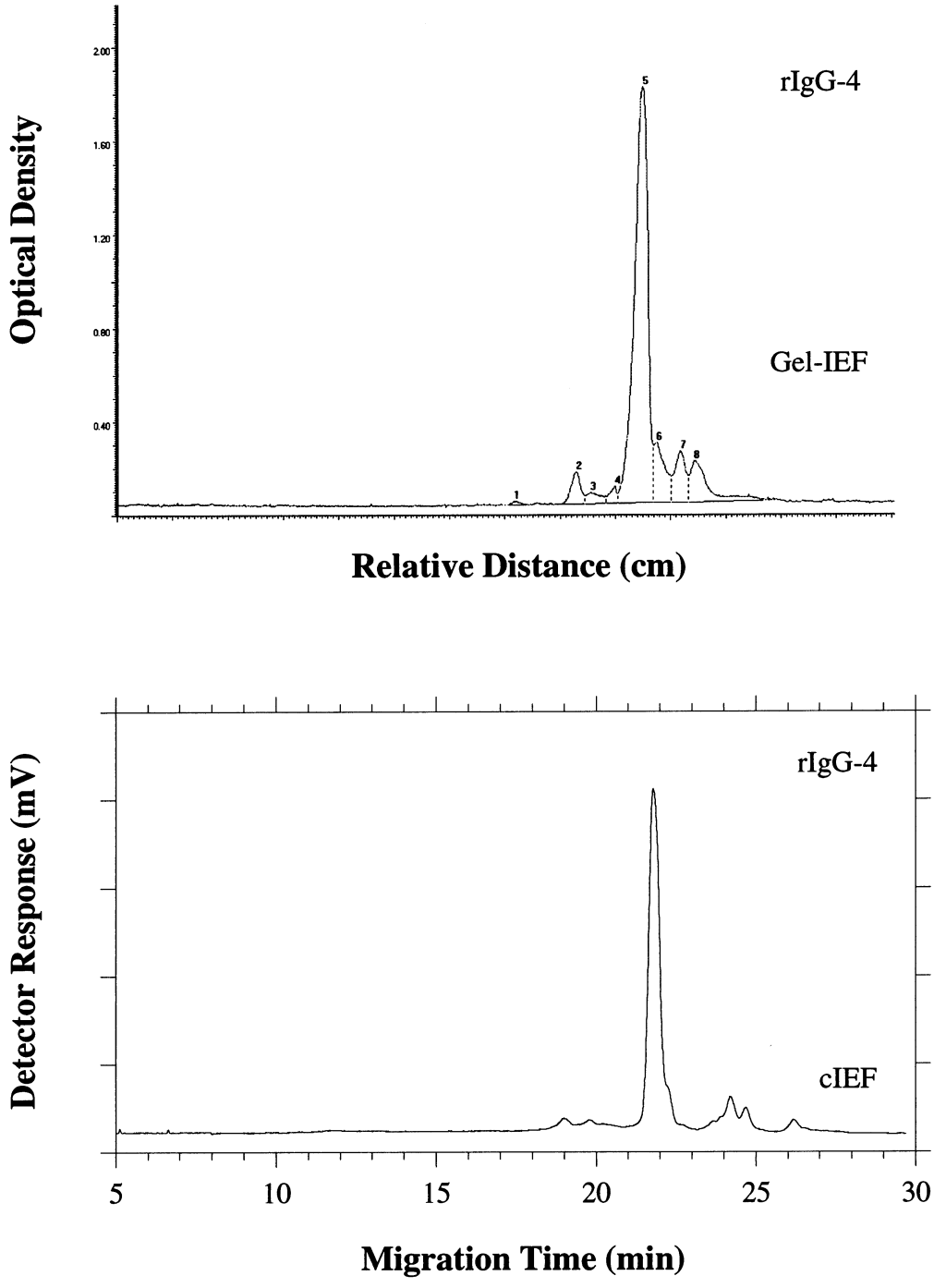

Fig. 3. Comparison of gel-IEF and cIEF methods for rIgG-4.

Table 1
Comparison of peak areas from gel IEF and cIEF

Peak	Gel IEF[a] relative %	cIEF[a] relative%
Peak 2	49.2 (7.8)[b]	57.1 (1.65)[c]
Peak 3	29.8 (12)	19.9 (1.73)
Peak 4	13.0 (15)	7.8 (1.33)
Peak 5	6.3 (22)	2.8 (0.72)

[a] Peak areas were determined for the IgG-2 shown in Fig. 5.
[b] %RSDs for a set of five analyses.
[c] % RSDs for a set of six analyses.

for both peaks) over the concentration range studied, demonstrating the quantitative capability of cIEF. of cIEF.

3.4. Reproducibility

Results from a reproducibility study (intra-day and inter-day) are shown in Table 2. The percent relative standard deviations (%RSDs) were determined for rIgG-2 on 3 separate days with six replicate analyses each day. The %RSDs for intra-day determinations were less than 2% for the major peak areas and less than 1% for the migration of the peaks. The %RSDs for inter-day determinations (using intra-day averages) over the course of 3 days, were less than 8% for peak area and less than 2.8% for the migration of the peaks.

3.5. Capillary stability

The capillary used in this method was a DB-1 column coated internally with GC-immobilized dimethyl siloxane (J&W Scientific). The stability of the capillary was tested by consecutive injections of rIgG-2. Fig. 7 shows the separations of the rIgG-2 after the first, 50th and 150th injection using the same DB-1 capillary. Reproducible migration times and good resolution without peak shape deterioration were observed after 150 injections. These results demonstrated that the dimethyl siloxane-derivatized (coated internally) capillary was stable under the conditions described for the two-step cIEF.

3.6. Isoform peak resolution

Under the experimental conditions described here, rIgGs having a wide range of *pI* values can be analyzed. For analysis of rIgGs with pIs < 9.0, the Pharmalyte with a pI range of 3–10 was found to offer acceptable isoform peak resolution (Figs. 1–3). However, this ampholyte preparation alone was unsuitable for analysis of highly basic rIgGs with $pI > 9.0$. For the highly basic rIgGs, peak resolution was greatly improved by using a 30:70 mixture of Pharmalytes 3–10 and Pharmalytes 8–10.5 (Fig. 8).

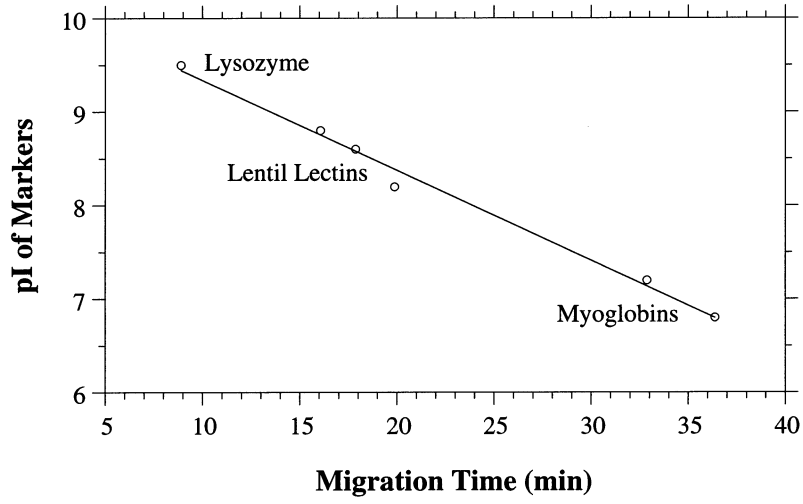

Fig. 4. Calibration curve for *pI* estimation.

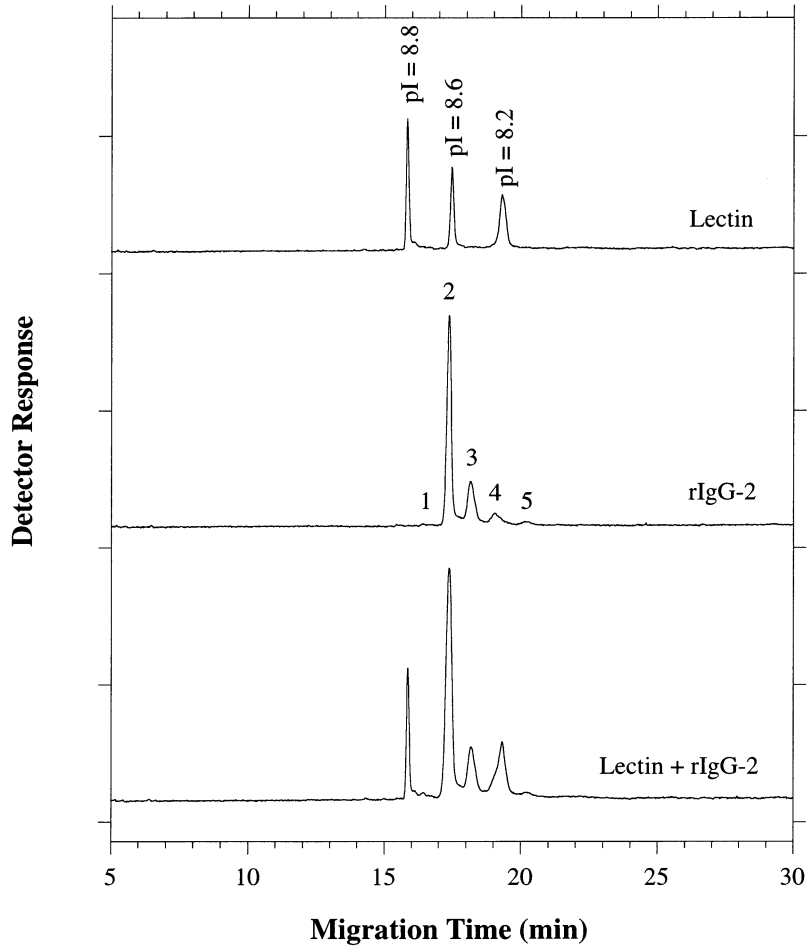

Fig. 5. Determination of *pI* values using internal *pI* markers.

Resolution of individual sample protein isoforms was highly dependent upon the capability of the ampholytes to form a sufficient pH gradient within the capillary. The cIEF profiles obtained by using different ampholyte preparations from different suppliers varied considerably. A working criterion for ampholyte acceptability was that the cIEF profile should at least match, or exceed, that of gel IEF with regard to the number of isoforms detected. It was found that, for the rIgGs studied, Servalytes did not give acceptable cIEF results; the number of isoform peaks obtained and the resolution between them did not match the results obtained using Pharmalytes and Sigma ampholytes, each of which met the criterion for acceptability stated above (Fig. 9).

4. Discussion

4.1. One-step cIEF versus two-step cIEF

Although the rIgGs can be separated by using the one-step cIEF (Fig. 10), the resolution and reproducibility of the rIgG isoforms are usually poorer than that obtained with the two step cIEF. In one-step cIEF, the speed of the protein mobilization depends upon electro-osmotic flow (EOF) which is strongly influenced by slight changes in the operating conditions, such as pH, capillary temperature, total salt concentrations, etc. If one-step cIEF is used for analysis of a basic rIgG, then EOF must be strictly controlled. Since the

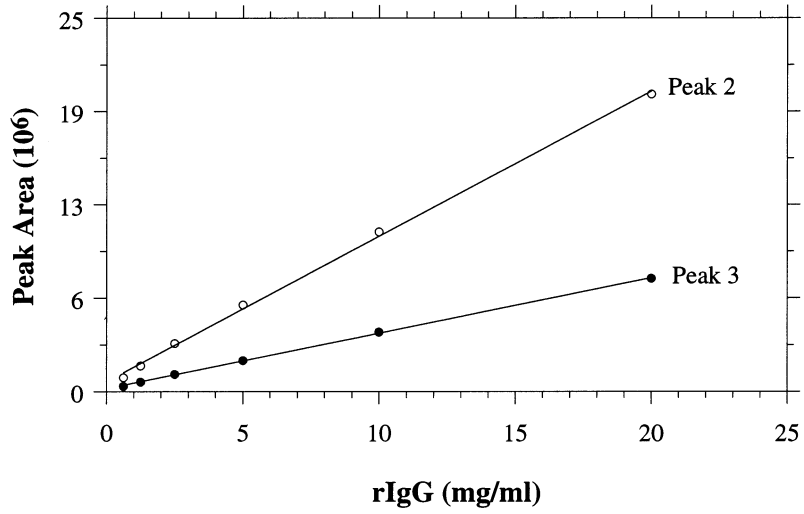

Fig. 6. Linearity response for isoform peaks in IgG-2 (Fig. 5).

basic rIgG bands focus near the end of the capillary, incompletely focused bands may be prematurely mobilized past the detector window by EOF. In contrast, the effect of EOF is minimized in the two-step cIEF method, and therefore, protein isoforms are completely focused prior to mobilization for acceptable resolution and reproducibility of rIgG analyses, including highly basic samples.

4.2. Capillary

In the two-step cIEF of rIgGs, coated capillaries (DB-1) are required in order to minimize the EOF and, therefore, uncoated capillaries can not be used. Therefore, stability and reliability of the internal column coating chemistry may be of some concern.

The DB-1 column was found to be robust, capable of providing equivalent and highly reproducible results over the course of repetitive analyses (>150) (Fig. 7), without suffering any obvious deleterious effects, when properly maintained. Other commercially available, coated capillaries can be tested for use with this method, however, the applied voltage and the time for focusing prior to mobilization must be determined and optimized for each column type.

4.3. Ampholytes

A number of different carrier ampholytes are commercially available for IEF. Since assignment of isoform peak *pI* values by cIEF is dependent upon the migration times of each peak relative to those of the internal *pI* marker protein standards included with each analysis, not all analysis times must necessarily be equivalent for the different ampholyte preparations. When optimizing the analysis for a specific protein, the ampholytes that will be used must be evaluated according to a variety of criteria, including the following: (a) viscosity (has an effect on migration time in two-step method); (b) conductivity (has an effect on resolution); (c) buffering capacity at the sample pI (resolution); and (d) transparency at 280 nm (artifact peaks). These parameters were evaluated by trial and error for their suitability in cIEF and their properties vary significantly among ampholytes produced by different manufacturers. A comparison of cIEF profiles using three commercial ampholytes is shown in Fig. 9. Differences such as baseline noise, peak response, migration time and, especially, isoform resolution, are apparent in the electropherograms of the same rIgG sample, which differed only in the ampholytes used for the analyses. It is our experience that the same carrier ampholytes used in gel IEF often

Table 2
Reproducibility of cIEF for rIgG-2

Experiment	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
	Area $\times 10^5$				
Day 1	0. 722[a]	57.11	19.89	7.792	2.789
	(1.65)[b]	(0.75)	(1.73)	(1.33)	(0.72)
Day 2	0.716	62.29	22.34	8.056	3.150
	(1.94)	(0.25)	(1.21)	(2.60)	(5.97)
Day 3	0.702	64.46	23.16	8.428	3.233
	(1.20)	(0.42)	(1.15)	(2.89)	(0.87)
Avg.	0.713	61.29	21.80	8.092	3.057
%RSD	1.43[c]	6.16	7.82	3.95	7.71
	Migration time (min)				
Day 1	16. 58[d]	17.59	18.47	19.46	20.66
	(0.81)[b]	(0.87)	(0.84)	(0.87)	(0.83)
Day 2	17.19	18.30	19.24	20.19	21.57
	(0.15)	(0.13)	(0.13)	(0.14)	(0.11)
Day 3	17.36	18.47	19.41	20.37	21.76
	(0.22)	(0.19)	(0.13)	(0.19)	(0.21)
Avg.	17.04	18.12	19.04	20.01	21.33
%RSD	2.39[e]	2.58	2.63	2.42	2.76

[a] Average peak area ($n = 6$).
[b] %RSDs for a set of six analyses.
[c] Day-to-day variation in the peak area.
[d] Average migration time ($n = 6$).
[e] Day-to-day variation in the migration time.

may not be used in cIEF. Most of the available ampholytes are not optimized and tested for cIEF. In our practice, only the ampholytes from Sigma and Pharmacia were suitable for the analysis of rIgGs. Some of the problems common to cIEF were addressed recently [10].

4.4. Precipitation

Problems of precipitation of proteins and methylcellulose matrix are well-known phenomena that may occur during cIEF [2]. Proteins easily precipitate at their *pI*s, and methylcellulose may precipitate due to excessive heat generated in the capillary. Protein precipitation results in a clogged capillary, which is indicated by no current during the analysis. Precipitation can be minimized by reducing the protein concentration, the capillary temperature, the voltage and the time for focusing and mobilization.

4.5. Internal pI marker standards

In cIEF, the peak shapes and migration times of the protein isoforms can vary significantly with the total ionic strength of the sample, including concentrations of the sample protein, salts, internal standards, etc. Preparing standard and sample solutions in an identical manner (protein and total ion concentrations, etc.) often may not be practical or even possible. Consequently, the use of an extrinsic *pI* calibration curve for the determination of sample protein *pI*s is typically unsuitable in cIEF. This difficulty is obviated by the inclusion of internal pI marker standards with the samples in each analysis.

4.6. Two-step cIEF method versus gel IEF

Although gel IEF is a widely, routinely used technique for protein charge heterogeneity analysis [9], cIEF methods have not yet become as well

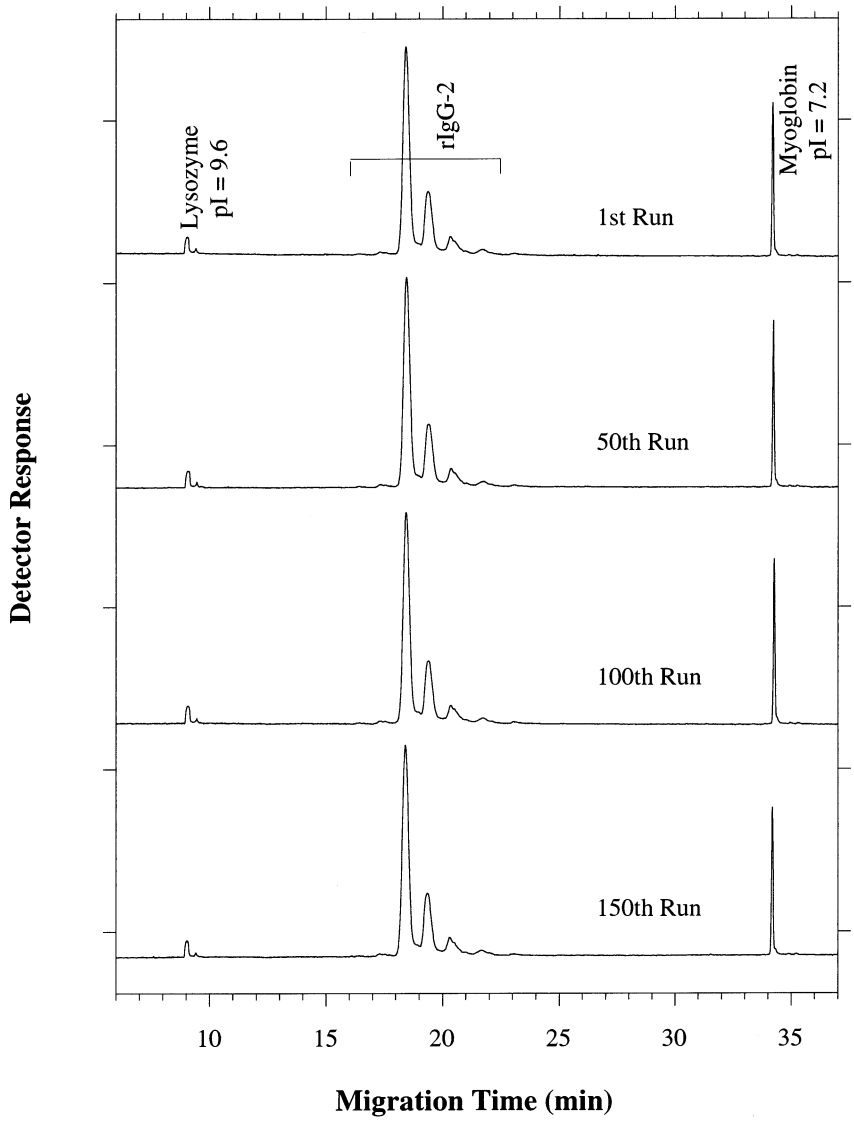

Fig. 7. Demonstration of DB-1 capillary stability with repeated injections.

established for this purpose. Perhaps this may be due to a variety of notions based on early work in this area [2,8], for example: (a) cIEF may not be an equivalent alternative to gel IEF because different IEF profiles are obtained by these methods; (b) cIEF results may be less accurate and/or reproducible in terms of both individual isoform *pI* estimates and the relative percentages of the total sample that they comprise; and (c) cIEF analyses are restricted to protein samples whose *pI* values fall only within a narrow pH range (i.e. the resolving power of cIEF is limited at extremes of pH) [2]. However, it is clear from the analytical results obtained using the two-step cIEF method described here that these concerns can be overcome. It should be also pointed out that in general CE methods are not as reliable as the HPLC methods due to higher rate of failures caused by instrument malfunction (pressure, voltage leaks, and clogging of the capillaries etc.)

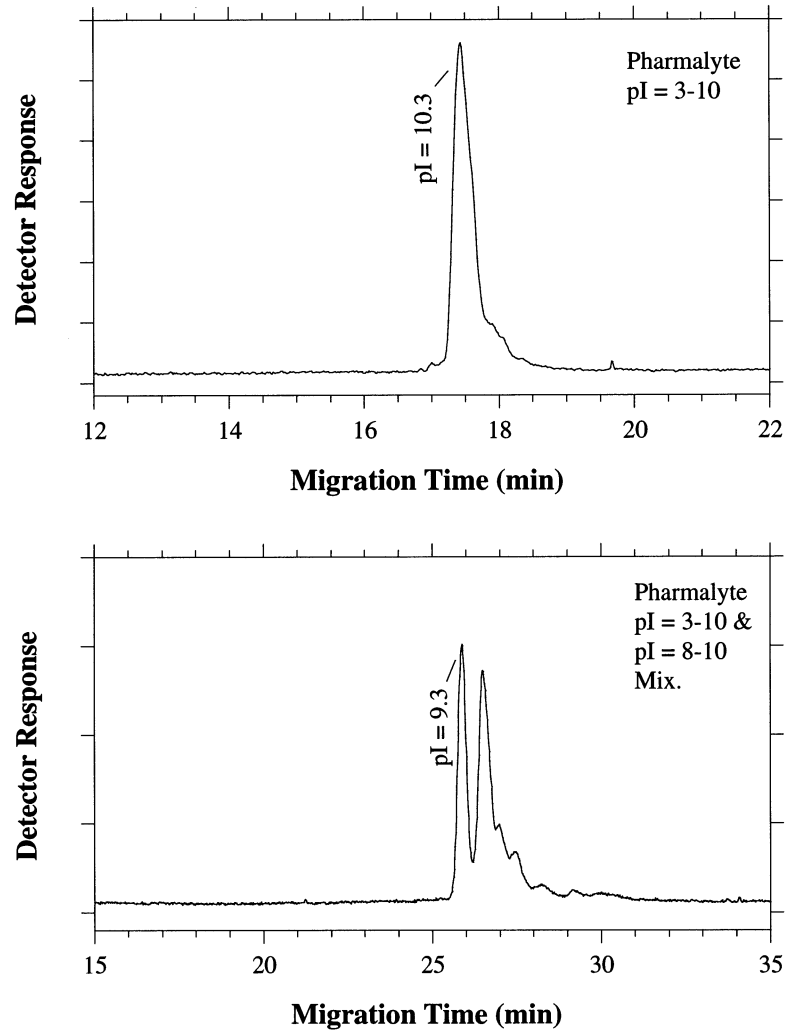

Fig. 8. cIEF separation of very basic rIgG with and without a mixture of ampholytes. Gel-IEF methods could not be used with this rIgG.

[10] and lack of high precision due to variability in the injection volumes.

The cIEF electropherogram is a plot of UV absorbance versus protein migration time, while the gel scan is a plot of light absorbance versus the stained isoform band's position along one dimension in the IEF gel. Both types of plot have the general appearance of a chromatogram, with peaks whose areas can be integrated and quantified. The overall graphic profiles obtained by gel IEF and cIEF were remarkably similar in appearance for the same samples, allowing for quick visual comparisons (Figs. 1–3). In addition, individual isoform peak areas determined from both plots were quite comparable, as were the *pI* values calculated for individual isoforms. These results were found to be highly consistent and reproducible.

Because cIEF utilizes a solution support medium within the capillary, movement of sample isoforms is not limited by gel matrix sieving effects (e.g. pore size). The graphic profile comparisons show that improved separations between peaks are typically obtained using cIEF as op-

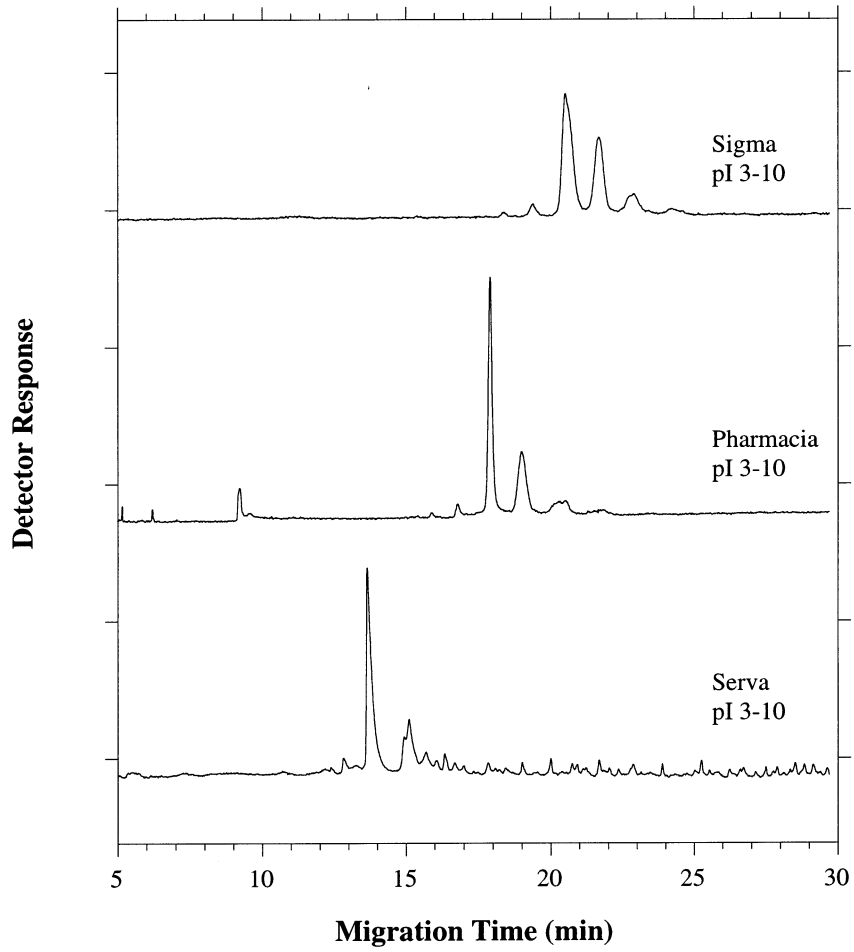

Fig. 9. Comparison of commercial ampholytes.

posed to those achieved via gel IEF (Figs. 1 and 2). The important difference between the two approaches is that cIEF incorporates on-line electronic data acquisition, analysis and archival, thus minimizing the time and effort required for generating the same information. The ease with which the cIEF solution support medium can be varied and tested, combined with the speed of cIEF analysis, also allows for rapid method development and optimization. For example, custom blending of different Pharmalyte solutions enabled separation of the highly basic rIgG isoforms, which were otherwise not separable using either a single Pharmalyte employed in our two-step cIEF approach, or with commercially available IEF gels (Fig. 8). Additionally, testing applicability of different ampholyte preparations is much more facile via cIEF than by pouring IEF gels.

5. Conclusions

A rapid, simple and highly reproducible cIEF method for routine analysis of therapeutic rIgGs has been developed. This method incorporates a two-step process in which the sample protein isoforms are focused first, and then mobilized past a UV detector window. For analysis of rIgGs, the two-step cIEF yields much higher resolution and

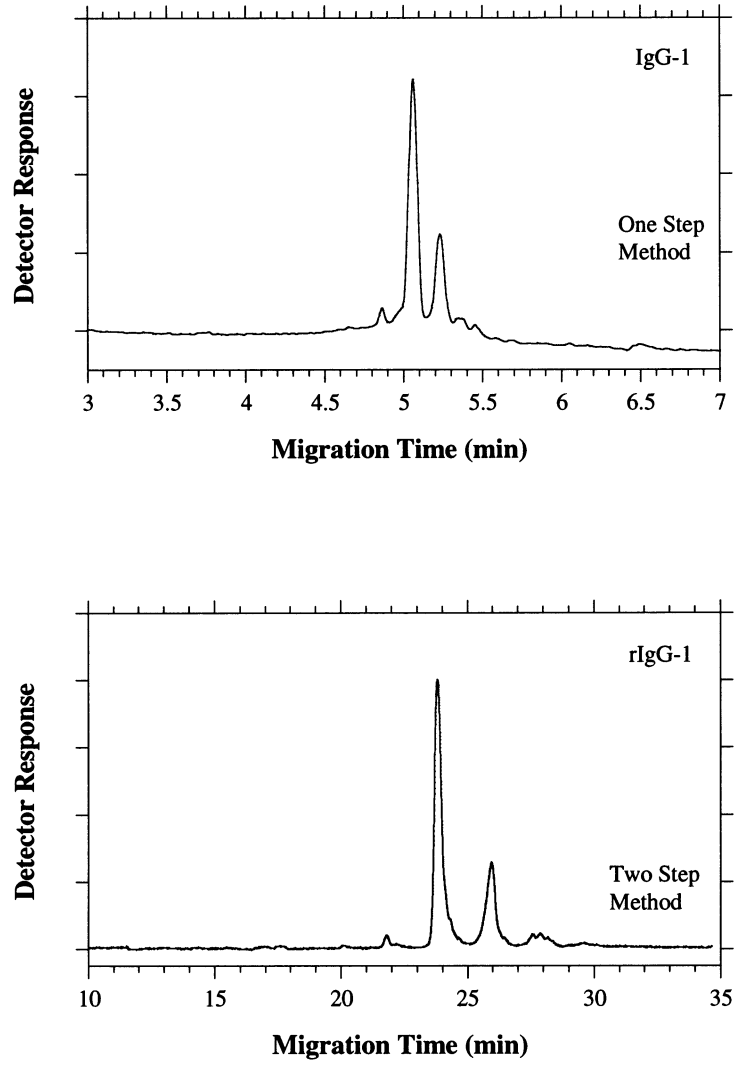

Fig. 10. Comparison of one-step and two-step cIEF methods using rIgG-1.

reproducibility than the one-step cIEF method, particularly for sample proteins having high *pI*s (Fig. 10). Since the EOF component is virtually eliminated, the two-step method is also much more precisely controlled and the resulting analyses are less subject to the variation in the sample (protein and buffer concentrations etc.).

The cIEF profiles of rIgGs are comparable to the densitometric scans obtained by gel IEF for the same samples, an indicator of the accuracy of the method. Moreover, the pI assignments and the relative proportions of individual rIgG isoforms in a given sample are comparable when determined by either the two-step cIEF method or gel IEF. An important advantage of the cIEF method is that development of defined mixtures of ampholytes optimized for the analysis of specific rIgGs can be easily accomplished within a short time. Major advantage, however, is the degree to which the two-step cIEF method saves time and effort compared to the gel IEF method due to its capacity to analyze samples in a manner similar to

HPLC analysis. The cIEF method for routine analysis of recombinant IgGs reported here offers following advantages over the other methods: (a) cIEF profiles are comparable to that of the slab gel IEF scans; (b) it has good reproducibility for the peak areas and the migration times; (c) it reduces time and effort required for the gel IEF; and (d) it can be used easily for the analysis of rIgGs with various *pI*s.

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