ELSEVIER

Journal of Chromatography A, 772 (1997) 145–160

JOURNAL OF CHROMATOGRAPHY A

Strategies to improve performance of capillary isoelectric focusing

R. Rodriguez-Diaz, M. Zhu, T. Wehr*

Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547, USA

Abstract

Acceptance of capillary isoelectric focusing (cIEF) as a routine analytical method has been limited by variable migration times, irreproducible patterns, and sample precipitation. Poor separation reproducibility can be traced to a number of factors. In this paper we explore the possibility of eliminating reagents without buffering capacity, and the use of internal markers. An often overlooked optimization stage of cIEF is mobilization. We demonstrate that hydraulic mobilization can be finely tuned by manipulating the pressure applied, and that ion addition mobilization can be greatly improved by properly selecting the type of ion used and its concentration. By limiting the pH range of the ampholytes to 8.5 or less, hundreds of runs on an individual capillary are possible, which is a necessity for routine analysis.

Keywords: Isoelectric focusing; Optimization; Proteins; Haemoglobin

1. Introduction

Capillary isoelectric focusing (cIEF) is an equilibrium method that relies on the formation of a pH gradient to achieve separation. One of the main characteristics of equilibrium techniques is the presence of an analysis end point (attainment of equilibrium), and no further resolution is achieved after this stage has been accomplished. In isoelectric focusing, resolution strongly depends on the slope of the pH gradient, and when resolution is not compromised by other factors of the process, gradient manipulation is the main parameter used to improve resolution.

Since not all molecules possess an isoelectric point (p*I*), IEF has been applied almost exclusively to the analysis of polypeptides. Until recently, IEF has been universally performed using gels as a matrix to eliminate convection and as a support for staining.

The development of capillary electrophoresis (CE) as a simplified form of electrophoresis diminished the problems inherent to electrophoretic analysis. The advantages of capillaries prompted researchers to adopt the different methodologies used in slab gels, and IEF was successfully transferred to this new approach [1].

Capillary IEF combines the high resolving power of conventional gel isoelectric focusing with the advantages of capillary electrophoresis instrumentation. Just as in gel electrophoresis, proteins are separated according to their isoelectric points in a pH gradient formed by carrier ampholytes when an electric potential is applied. The use of small diameter capillaries allows the efficient dissipation of Joule heat and permits the application of high voltages for a rapid focusing of the protein zones. The separations can be performed in free solution, without the need of gels, and this allows the content of the capillary to be replaced in-between runs, and therefore automation of the analysis. The use of UV-transparent fused-silica capillaries enables direct on-tube optical detection of focused protein zones, without the requirement of staining.

Protein analysis by cIEF has a higher resolving

*Corresponding author.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(97)00104-0

power than other protein analysis techniques, including other modes of capillary electrophoresis.

The transfer of IEF to the capillary format [1] alleviated the drawbacks of gel IEF. Capillary IEF not only provides high resolution and information essential to the characterization of unknown polypeptides (e.g., isoelectric point), but also provides quantitative information [2]. cIEF applications include isoelectric point determination, analysis of complex protein mixtures, microheterogeneity determination [3,4], clinical diagnostics [5,6], purity analysis and product degradation monitoring [3]. Capillary IEF also extended the use of IEF to molecules other than polypeptides, such as small peptides and dyes [8,9].

Despite the advantages of cIEF over its gel counterpart, cIEF has not reached a level of acceptance similar to that of other forms of CE, and it is seldom used as a routine analysis method. Besides the high number of parameters not completely understood, the main drawbacks of cIEF include variable migration times, irreproducible patterns, and sample precipitation. Poor separation reproducibility can be traced to a number of factors. In this paper we describe some recent advances in cIEF that increase reproducibility, capillary longevity, and show the potential use of cIEF as a routine analysis technique. We explore the possibility of eliminating reagents without buffering capacity (especially NaOH commonly used as catholyte and as base to prepare the mobilizer). We also show that internal markers [9] are essential to attain reproducibility, especially when analyzing samples that differ in the amount of salt present.

Since IEF is an equilibrium technique, and there are a limited number of parameters available for optimization in this type of method, any area where loss of resolution can occur must be taken into consideration. An often overlooked optimization stage of cIEF is mobilization. Besides the choice of hydraulic [1,11,13,14], electroosmotic [7] or ion addition mobilization [1], not much else has been explored in detail. In the following experiments, we demonstrate that hydraulic mobilization can be finely tuned by manipulating the pressure applied, and that ion addition mobilization can be greatly improved by properly selecting the type and concentration of ion used.

2. Material and methods

2.1. Capillary electrophoresis instrumentation

All analyses were performed on a BioFocus 2000 or BioFocus 3000 CE System (Bio-Rad Laboratories, Hercules, CA, USA) equipped with capillaries coated according to a modified version of the method first described by Hjertén [12], and thermostated by circulating water.

2.2. General reagents and samples

Human transferrin (iron free) was obtained from Sigma (St. Louis, MO, USA), human hemoglobins variants A, F, C and S were purchased from Isolab (Akron, OH, USA). Catholyte (40 m*M* NaOH), anolyte (20 m*M* phosphoric acid), zwitterionic mobilizer, ampholytes (Biolyte) and synthetic p*I* markers (BioMark) were obtained from Bio-Rad Laboratories.

Transferrin was diluted to a final concentration of 0.5 mg per ml with a 2% solution of various narrow range Biolytes mixed to provide a pH range from 3 to 8.5. Hemoglobin variants were diluted in a similar fashion to a concentration of 1 mg per ml. Where specified, a zwitterion (p*I* of 8.7), or N,N,N′,N′-tetramethylethylenediamine (TEMED) were used as spacers to block the blind segment of the capillary between the monitor point and the capillary outlet.

2.3. Analysis conditions

Typically, the capillary was filled with the sample+ampholyte solution for 10–30 s at 0.667 MPa. When internal standards were employed, they were introduced into the capillary post-sample injection at 0.033 MPa for 4–5 s. Detection was performed by UV absorption at 280 nm. Unless otherwise specified, focusing was carried out at 15 kV for 4 min. Mobilization was performed by the employment of a zwitterion [17], or by applying pressure or liquid height differential (referred as gravity) under an applied voltage of 15 kV. Variations to this basic protocol were made as specified for the different experiments. During the coating lifetime study all reagents and samples were replaced every 10 runs.

2.4. Effect of sample salt concentration

To show the effect of salt concentration, a 1 mg per ml solution of human hemoglobins A, F, S, and C was prepared in 2% (w/v) Biolyte 3-10, and variable volumes of 100 m*M* Tris phosphate buffer (pH 8.0) were added to the hemoglobin solution to achieve final concentrations of 10, 20, 30, and 40 m*M* buffer. The final concentration of the hemoglobins was adjusted to 0.5 mg/ml with water.

2.5. Effect of reagents with buffering capacity on the reproducibility of cIEF

To evaluate the effect of pH buffering, a taurine (Aldrich, Milwaukee, WI, USA) buffer was prepared to a final concentration of 500 m*M*, and the pH was adjusted to 8.8 by adding solid NaOH. A 40 m*M* NaOH solution was used as control. These two solutions were used directly as catholyte, or as base for the preparation of mobilizers. Bovine hemoglobin (Sigma) dissolved in 2% ampholytes (pH range approx. 3–8.5) to a concentration of 1 mg/ml was used as sample matrix.

2.6. Chemical mobilization

2.6.1. Effect of mobilizing salt

Samples consisted of human hemoglobin variants AFSC at a concentration of 0.5 mg/ml in 2% (w/v) solution of Biolyte 3–10 (Bio-Rad Labs.).

The capillary was filled with the sample+ampholyte mixture using pressure from a nitrogen source (100 p.s.i.; 1 p.s.i.=6894.76 Pa). A 20 m*M* phosphoric acid (Bio-Rad Laboratories) solution was used as anolyte, and 40 m*M* NaOH was used as catholyte.

Analyses were performed in a 24 cm×50 μm capillary, internally coated with linear polyacrylamide (LPA) (Bio-Rad Labs.). Focusing was carried out at 15 kV for 4 min. During mobilization the voltage was maintained at 15 kV. Detection was performed by UV absorption at 280 nm.

Mobilization solutions were prepared by addition of sodium salts of the following compounds in 40 m*M* NaOH to a concentration of 100 m*M*: dibasic phosphate, acetate (Sigma), and tetraborate (Aldrich). Mobilization using sodium chloride (Aldrich) at 100 m*M* was used as control. The pH was not adjusted after addition of the salt.

2.6.2. The effect of the concentration of the salt used for mobilization

Dibasic sodium phosphate was also used to determine the effect on resolution of the salt concentration in the mobilizer solution. The final concentrations of the added salt were 25, 50 and 100 m*M* diluted in a 40 m*M* NaOH. Phosphoric acid 20 m*M* was used as anolyte. The sample used was a standard mixture of human hemoglobin A, F, S and C. Analysis conditions were identical to those described above.

2.7. Hydraulic mobilization

2.7.1. Pressure mobilization

Pressure mobilization was performed using the procedure described by Huang et al. [13] The anolyte used was 20 m*M* phosphoric acid and the catholyte was 40 m*M* NaOH. Methyl cellulose (MC, 1500 cps at 2% concentration, Sigma) was added to the anolyte, catholyte and sample+ampholyte solution to a final concentration of 0.4% MC. Mobilization was performed by applying 0.007, 0.005, or 0.003 MPa of pressure (from a nitrogen cylinder). A 27 cm×50 μm, LPA coated capillary was used. The column was filled first with catholyte at 0.133 MPa of pressure, and the sample was then introduced for 24 s (also at 0.133 MPa). Focusing was performed for 2 min at 13.5 kV. The same field strength was maintained during mobilization. Sample zones were detected by UV absorption at 280 nm.

Preliminary results suggested that pressure mobilization produces better resolution at lower settings at a given MC concentration. To determine if higher viscosity could neutralize the negative effects of applying higher pressure, the concentration of the polymer was increased (anolyte, catholyte, and sample+ampholytes) to 0.6%. Mobilization was performed at 0.007 MPa pressure.

2.7.2. Gravity mobilization

For gravity mobilization, 20 m*M* phosphoric acid was used as anolyte, 40 m*M* NaOH was used as catholyte, and the ampholyte concentration was adjusted to 2%. Reagents contained no other addi-

tives. A 24 cm×75 μm coated capillary was used. The sample was introduced into the column by pressure for 10 s at 0.667 MPa. Focusing was conducted at 15 kV for 240 s. Mobilization was started by replacing the destination vial containing 40 m*M* NaOH for a vial containing a smaller volume of the same solution (thus creating a height difference). The total height difference used was approximately 2 cm.

3. Results and discussion

3.1. Protein pI distribution

Capillary IEF with focusing and mobilization as two distinct stages offers added control to the optimization process. This type of cIEF is normally performed in fused-silica capillaries whose internal wall has been chemically modified to eliminate electroosmosis and non specific adsorption of the sample to the capillary wall. Silica has been used as a chromatographic support for an extended period of time, and it is well known that the stability of bonded chemistries decreases with increasing pH. The most typical cIEF setup incorporates the use of a spacer in addition to the ampholyte range of pH 3–10. Under these conditions, approximately 30–40% of a 17–24 cm capillary is filled with a solution of pH 8.0 or higher. Righetti et al. [10] listed approximately 2380 p*I* values for different proteins. The p*I* distribution was as follows: 89.66% with a p*I* below 8.0; 4.12% with a p*I* between 8.0 and 8.5; and 6.22% with p*I* above 8.5. According to this distribution of protein p*I* values, a cIEF system that extends from a pH of 3 to 8.5 can be used to analyze approximately 94% of all proteins. This is of significance because at this pH range, hundreds of runs can be achieved using a single capillary [11].

3.2. Reproducibility and capillary lifetime

Preserving the integrity of the coating is beneficial to capillary lifetime, but it also improves overall performance of the cIEF process. Fig. 1 displays three electropherograms of human transferrin superimposed to show the reproducibility of the pattern obtained by cIEF. Run 15 and 16 (black traces) are indistinguishable; electropherograms that overlap so perfectly are not easily obtained, since a large number of parameters determines the location of the protein bands in the interior of the capillary and their rate of mobilization, thus affecting the migration time of each of the sample components. As stated in the methodology section, all reagents should be replaced after a few runs (e.g., every five runs) to achieve maximum reproducibility. It is especially important to replace the catholyte (40 m*M* NaOH) and mobilizer, because they lack buffering capacity, and their pH changes continuously during the analyses. When performing multiple analyses from the same sample vial, the sample solution can be contaminated by carryover of solutes on the electrode and capillary external surfaces. When the sample is contaminated by anolyte or catholyte, the migration time during mobilization is affected, thus decreasing reproducibility. Fig. 1 also shows that after 500 continuous runs using the same capillary (gray trace) the peaks have shifted towards the detector (shorter migration times), but the reproducibility of the separation profile is still excellent.

3.3. The use of buffers as catholytes and mobilizers

Reproducibility is also affected by continuous use of reagents lacking buffering capacity. In a model for the IEF process [19], the anolyte and catholyte solutions provide a pH cage from which zwitterions (including ampholytes) can not escape either by electromigration or diffusion, but this cage only works for molecules with lower (higher) p*I* than the catholyte (anolyte). If the pH of the catholyte (anolyte) decreases (increases) all molecules with a higher (lower) p*I* will migrate out of the capillary towards the electrode. Sodium hydroxide is widely used in both gel IEF and cIEF, and dilute solutions of this compound lack pH buffering capacity. Using a simple system (hemoglobin) it is easy to observe a systematic drift of migration time as a function of time (Fig. 2). Migration time (t_m) drift is accompanied by loss of resolution (Fig. 3), but t_m can be restored almost to its original value (see last data point of Fig. 2), and resolution improved by simply replacing the catholyte. One difficulty in the use of compounds other than strong bases as catholytes is

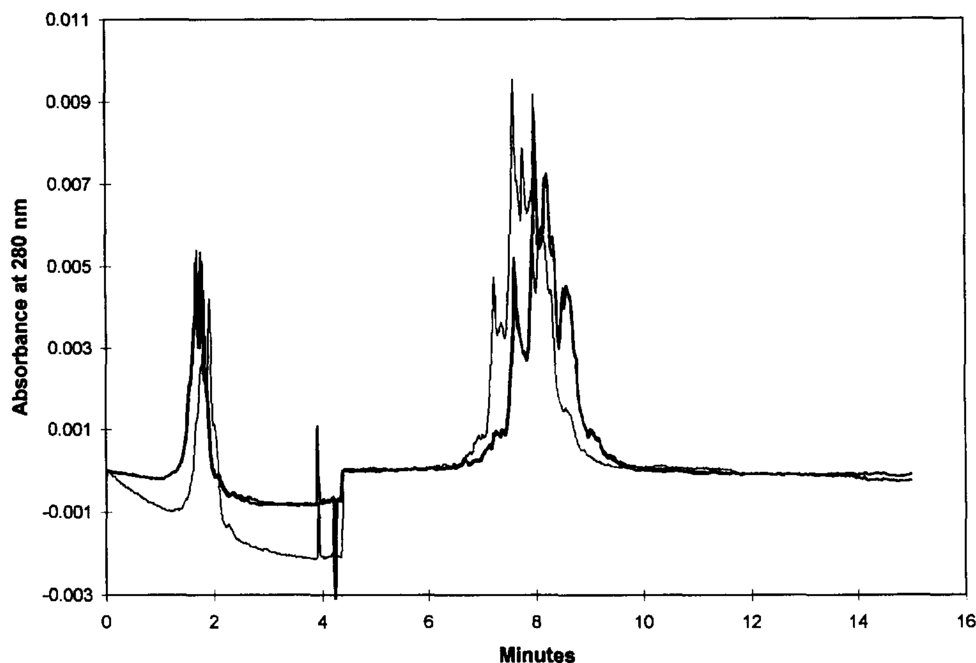

Fig. 1. Reproducibility of cIEF analyses of human transferrin. The black trace is an overlay of runs 16 and 17, and gray trace is an overlay of run 500. Although the peaks' elution times are shorter in the 500th run, the integrity of the pattern is still very apparent. Ampholytes spanning a pH range from 3.0 to 8.0 were used. A zwitterionic spacer (pI=8.7) was added to block the blind side of the capillary. Analysis conditions: capillary 24 cm×25 μm, coated with linear polyacrylamide; focusing: 4 min at 15 kV; mobilization at 15 kV; detection was carried out at 280 nm. Sample and capillary were thermostated at 20°C.

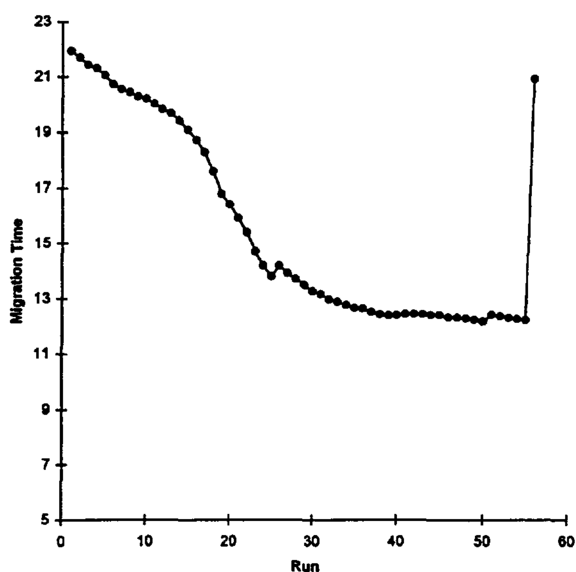

Fig. 2. Plot of migration time vs. analysis number when using NaOH. Sample: bovine hemoglobin, 1 mg/ml in 3-10 Biolyte 2%. All analysis conditions identical to those described in Fig. 3.

that most of them are salts. Although in theory a pH cage can be formed between any two buffers of different pH [16], the use of most buffers interferes with the focusing process, and in many cases it is impossible to achieve equilibrium. It is well known that most salts are actually used to disrupt the focusing equilibrium by a process termed ion-addition mobilization. According to Hjertén's equation [18] for cIEF, at steady state the electroneutrality condition in the capillary can be expressed as

$$C_{H^+} + \sum C_{NH_3^+} = C_{OH^-} + \sum C_{COO^-}$$

where C_{H^+}, C_{OH^-}, $C_{NH_3^+}$, and C_{COO^-} are the concentrations of protons, hydroxyl ions, and positive and negative groups in the ampholytes, respectively. In anodic mobilization, addition of a non-proton cation X^{n+} to the anolyte introduces another term to the left side of the equation:

$$C_{X^{n+}} + C_{H^-} + \sum C_{NH_3^+} = C_{OH^-} + \sum C_{COO^-}$$

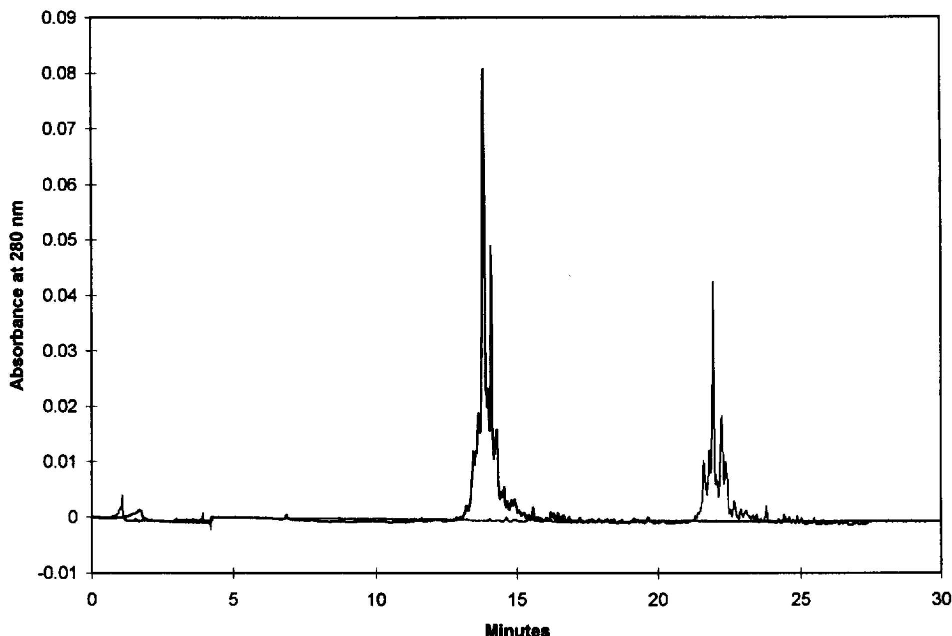

Fig. 3. Effect on resolution of bovine hemoglobin by continuous usage of NaOH as catholyte in cIEF. The same reagents were used to perform 25 continuous runs. Gray trace: run 1, the presence of a high number of minor components surrounding the main hemoglobin band is easily observed. Black trace: 25th analysis, minor components exhibit decreased resolution. Notice the discrepancy in migration time between the two electropherograms. Analysis conditions: 24 cm×50 μm, coated capillary; focusing at 15 kV for 4 min; mobilization by ion-addition at 15 kV. Capillary and reagents were thermostated at 27°C.

Migration of the non-proton cation into the capillary will result in a reduction in proton concentration, i.e., an increase in pH. Similarly, addition of a non-hydroxyl anion Y^{m-} to the catholyte in cathodic mobilization yields a similar expression:

$$C_{H^+} + \sum C_{NH_3^+} = C_{OH^-} + \sum C_{COO^-} + C_{Y^{m-}}$$

indicating that migration of a non-hydroxyl anion into the capillary results in a reduction in hydroxyl concentration, i.e., a decrease in pH. Progressive flow of non-proton cations (anodic mobilization) or non-hydroxyl anions (cathodic mobilization) causes a progressive pH shift along the capillary, resulting in mobilization of proteins in sequence past the detector point. So any buffer solution containing a competing ion will cause mobilization (or drift). On the other hand, one way to maintain electroneutrality during cIEF would be to add a neutral (zwitterionic) compound to any of the reagents (as long as it is neutral under the conditions of the solution to which it is added):

$$C_{H^+} + \sum C_{NH_3^+} = C_{OH^-} + \sum C_{COO^-} + {}_{+X^n}C_{Y^{m-}}$$

where ${}_{+X^n}C_{Y^{m-}}$ represents an electrically neutral molecule, and if this compound possesses buffering capacity it could eliminate or reduce t_m drift. Notice that a neutral compound could be added to either side of the equation maintaining electroneutrality. When this ideal situation can not be implemented, other compounds can be used as buffers, even if they are not neutral, as long as they allow the focusing of proteins to occur. In principle, this means that any ion with low mobility could be used. Fig. 4 displays t_m over 50 consecutive analysis using 0.5 *M* taurine buffer pH 8.8 as catholyte solution. The same vial (0.5 ml) was used for all analysis. Although there is still some variability, the general downward trend was eliminated when using taurine buffer as catholyte, and resolution was not affected over time (Fig. 5). The concept of using neutral molecules as catholyte has been explored in the past, but in most cases the catholyte consisted of amino acids (e.g., arginine, lysine) or related molecules (e.g., lysyl–

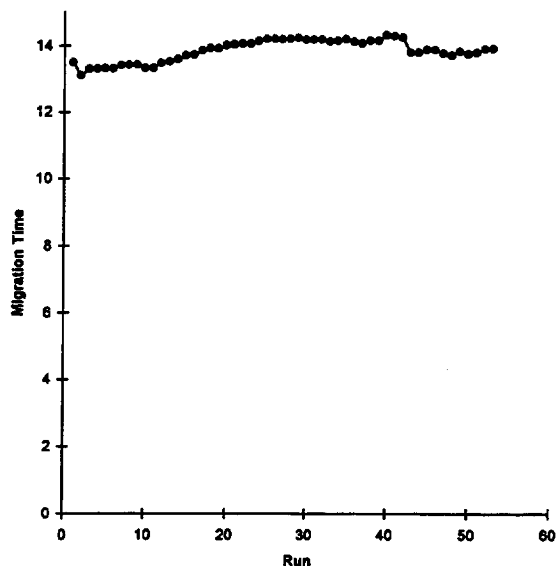

Fig. 4. Migration time plot for Hb when using taurine buffer as catholyte. Sample: bovine hemoglobin, 1 mg/ml in 3-10 Biolyte 2%. All analysis conditions identical to those described in Fig. 3.

lysine). Using the same concept, we have been able to use buffers other than taurine (results not shown), e.g., 2-(N-morpholino)ethanesulfonic acid (MES), 3-cyclohexylamino-1-propanesulfonic acid (CAPS), acetate, 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPS), etc. An almost identical approach had been described previously by Nguyen and Chrambach [15]. Besides the advantage of higher reproducibility, the use of buffers will also minimize the deleterious exposure of coated capillaries to the harsh conditions of extreme pH typical of NaOH solutions. An interesting feature of the catholyte buffers so far used in our laboratory is that the pH can be varied within at least a small range (we have used taurine buffers ranging in pH from 8.5 to 8.9) without noticeable adverse effects. This characteristic can be important when a limited range of pH inside the capillary is desired, since ampholytes with p*I* above (below) the pH of the catholyte (anolyte) will readily exit the capillary. This technique can also potentially improve results when single step

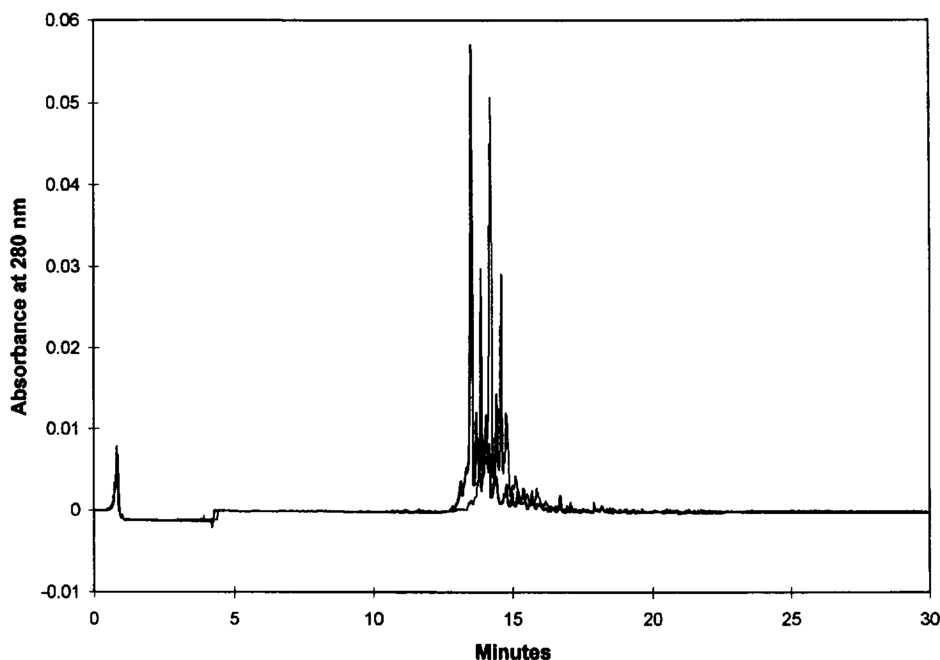

Fig. 5. Resolution over extended usage time when using buffered catholyte. The same reagents were used to perform 25 continuous runs. Gray trace: first run, the presence of a high number of minor components surrounding the main hemoglobin band is easily observed. Black trace: 25th run, all sample components are clearly resolved even after 25 analyses with use of the same reagents. Analysis parameters identical to those of Fig. 3, except that the catholyte used was 0.5 *M* taurine buffer, pH 8.8, instead of NaOH.

cIEF is used, since in this technique the catholyte is used to fill a significant portion of the capillary, and the column contents are transported into the outlet reagent (typically the catholyte) during the analysis.

When non-zwitterionic compounds are used as catholytes, true equilibrium is not reached, and focused protein bands eventually migrate towards the detector if the voltage is maintained (results not shown). Thus, this method is a variation of single-step cIEF. When the migration of proteins is too slow or when acidic proteins are analyzed, the transport can be accelerated by switching to a mobilization stage after focusing has been achieved.

Proteins focused using buffer catholyte can be mobilized using ion-addition techniques or hydraulic forces. Cathodic mobilization by ion-addition mobilization using buffers is basically an identical equation to the one described before

$$C_{H^+} + \sum C_{NH3^+} = C_{OH^-} + \sum C_{COO^-} + {}_{+X^n}C_{Y^{m-}} + C_{Y^{m-}}$$

where $C_{Y^{m-}}$ represents the added competing ion. During ion-addition mobilization, we have obtained maximum reproducibility by preparing the mobilizer by adding salts to the same type of catholyte buffer.

3.4. Effect of sample salt concentration

Fig. 6 shows a dramatic sample salt content-

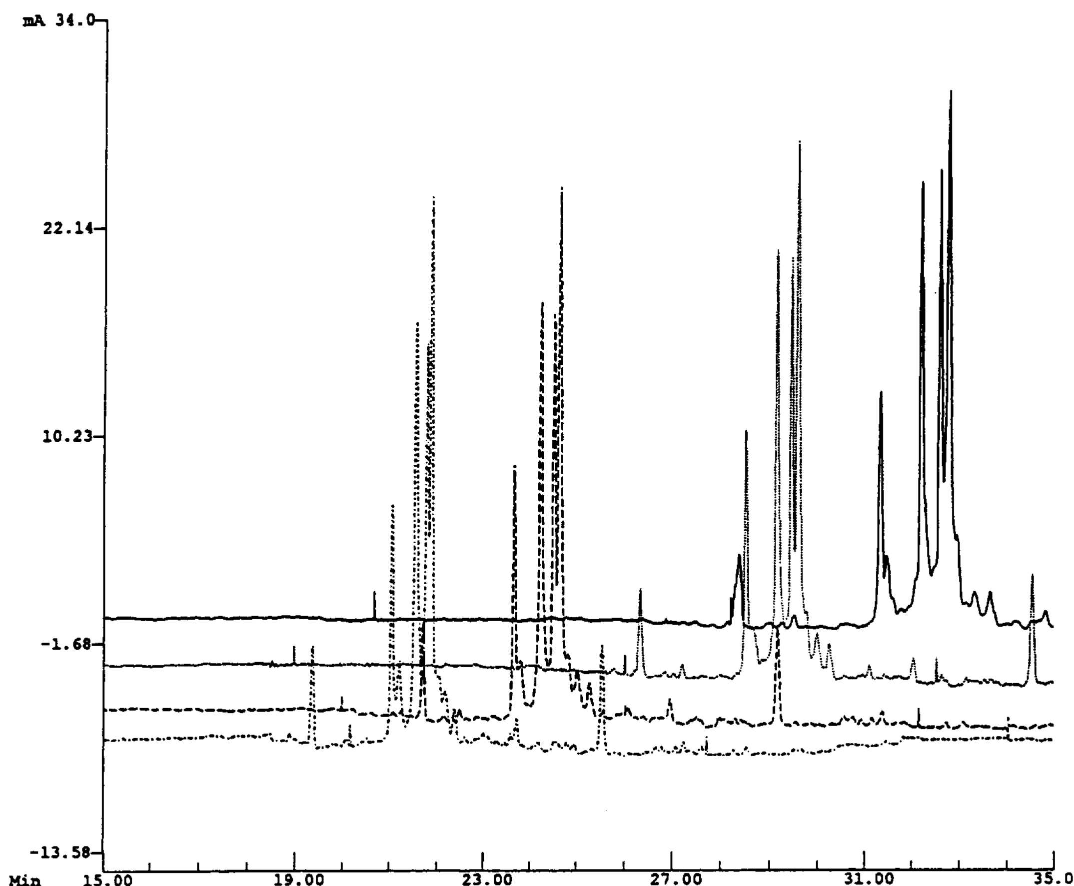

Fig. 6. Effect of sample salt concentration on the migration time of human hemoglobins A, F, S, and C. A significant shift in migration time is easily observed, with samples containing higher amounts of salt eluting faster. Conditions: 24 cm×25 μm capillary, coated; focusing at 15 kV for 4 min; ion addition (zwitterion) mobilization at 15 kV; detection was carried out at 280 nm. The capillary and sample were thermostated at 20°C. Upper trace, 20 m*M* Tris phosphate; upper middle trace, 30 m*M* Tris phosphate; lower middle trace, 40 m*M* Tris phosphate; lower trace 50 m*M* Tris phosphate.

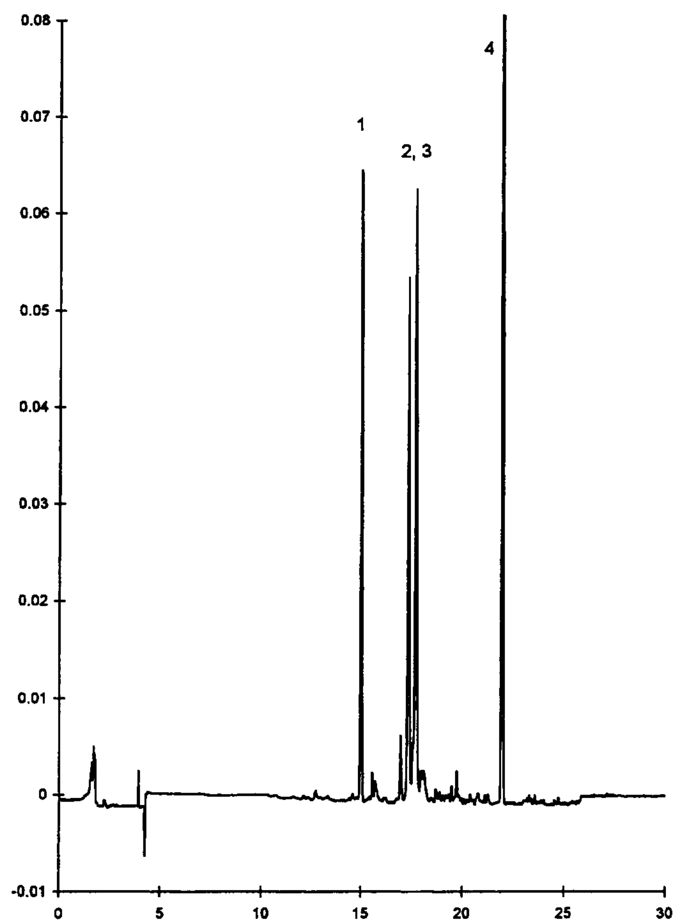

Fig. 7. Analysis of human hemoglobins A and S using internal standards of known isoelectric points. After the capillary was filled with sample dissolved in ampholytes, the standards (also dissolved in ampholytes) were introduced into the capillary as a second injection (1 s at 0.033 MPa) that occupied only a small fraction of the tube. The advantage of this procedure is that the unused sample remains free of standards. Peak identities: 1, BioMark p*I* 5.3 marker; 2, hemoglobin S; 3, hemoglobin A; 4, BioMark p*I* 8.4 marker.

Table 1
Reproducibility table of five consecutive cIEF analyses of hemoglobins A and S

Run	Migration time reproducibility (t_m, min)		Isoelectric point reproducibility (p*I*)	
	HbA	HbS	HbA	HbS
1	19.16	18.77	7.39	7.22
2	19.01	18.63	7.38	7.23
3	18.88	18.5	7.38	7.22
4	18.74	18.36	7.38	7.22
5	18.63	18.26	7.38	7.22
Average	18.88	18.5	7.38	7.22
S.D.	0.21	0.2	0.004	0.004
R.S.D. (%)	1.11	1.1	0.06	0.06

The same runs were analyzed using migration times and isoelectric points. The migration times of the two internal standards seen in Fig. 7 (flanking peaks) were used to produce a p*I* calibration plot for each run and the migration times of the hemoglobins were interpolated from the plot. The p*I* values obtained were then subjected to statistical analysis.

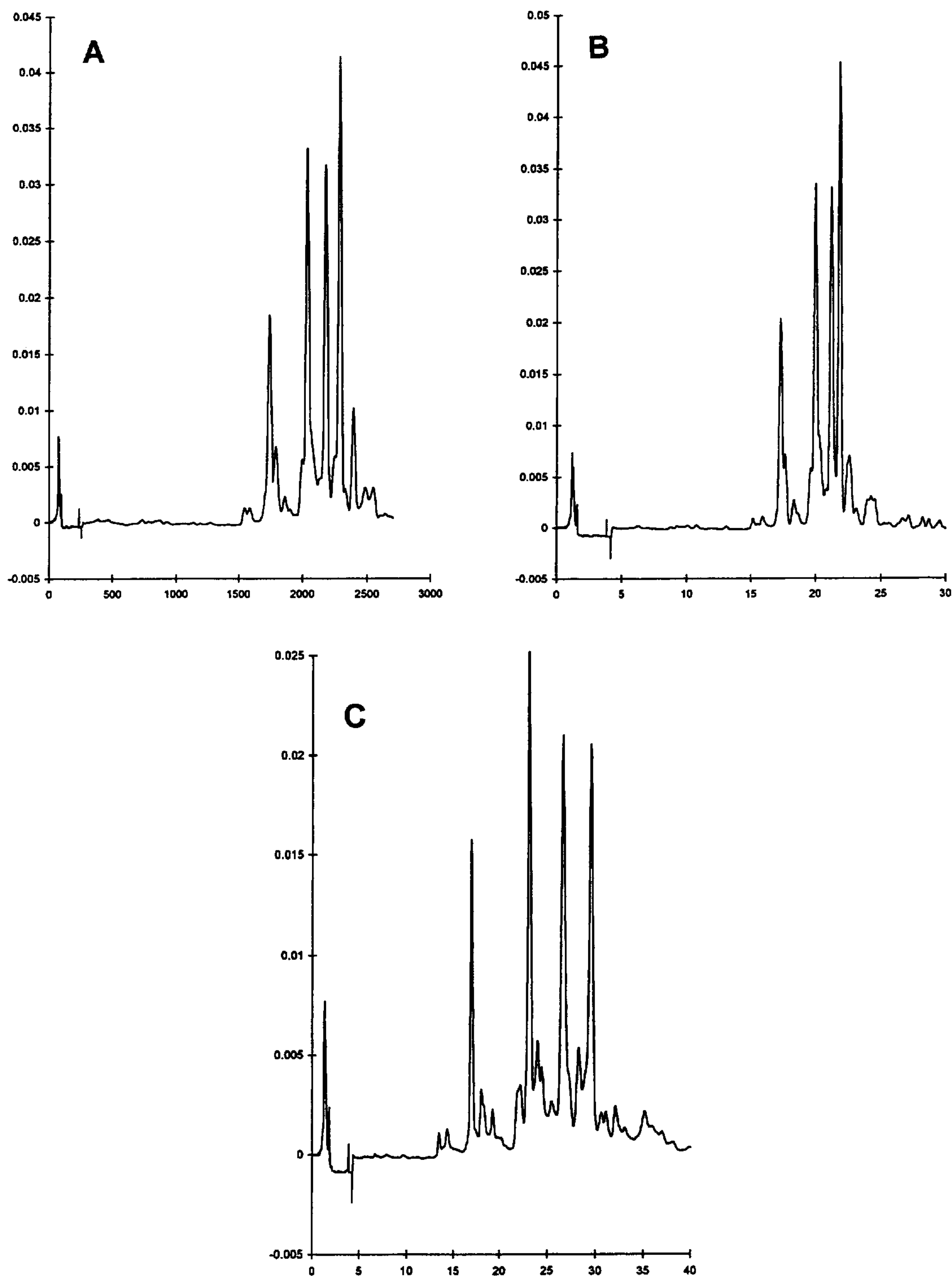

Fig. 8. Effect of the type of salt used for ion-addition mobilization. Displayed are the electropherograms obtained when mobilizing by addition to the catholyte of one of the following salts: (A) NaCl, (B) sodium acetate, and (C) sodium tetraborate. Capillary: 24 cm×50 μm, coated; focusing at 15 kV for 4 min; mobilization at 15 kV. Analysis conditions were identical for all electropherograms.

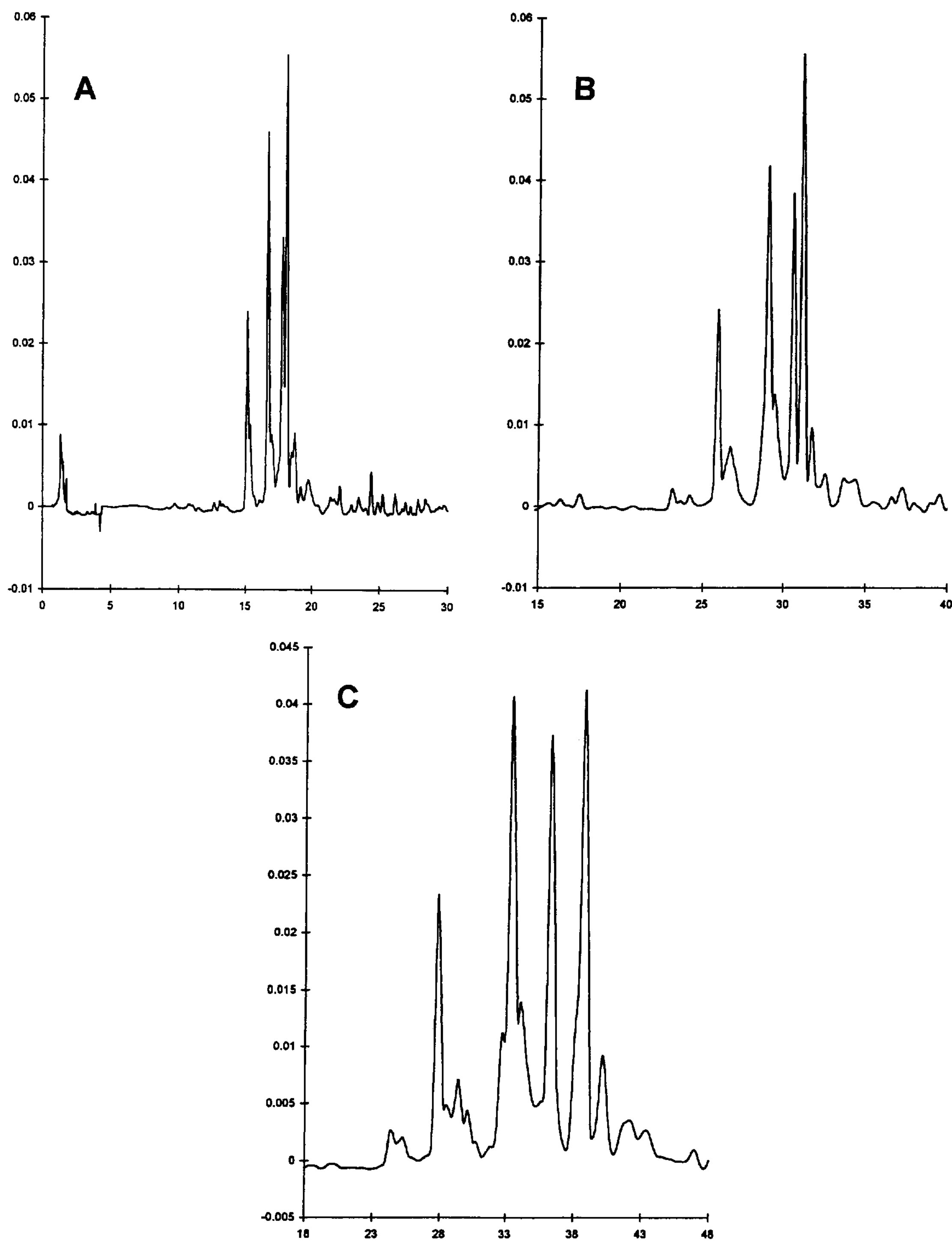

Fig. 9. Effect of mobilizer concentration. Na_2HPO_4 was used to assess the effect of salt concentration. Displayed are the electropherograms at Na_2HPO_4 concentrations (A) 100, (B) 50, and (C) 25 m*M*. Resolution improves as the salt concentration is reduced, but the analysis time is increased.

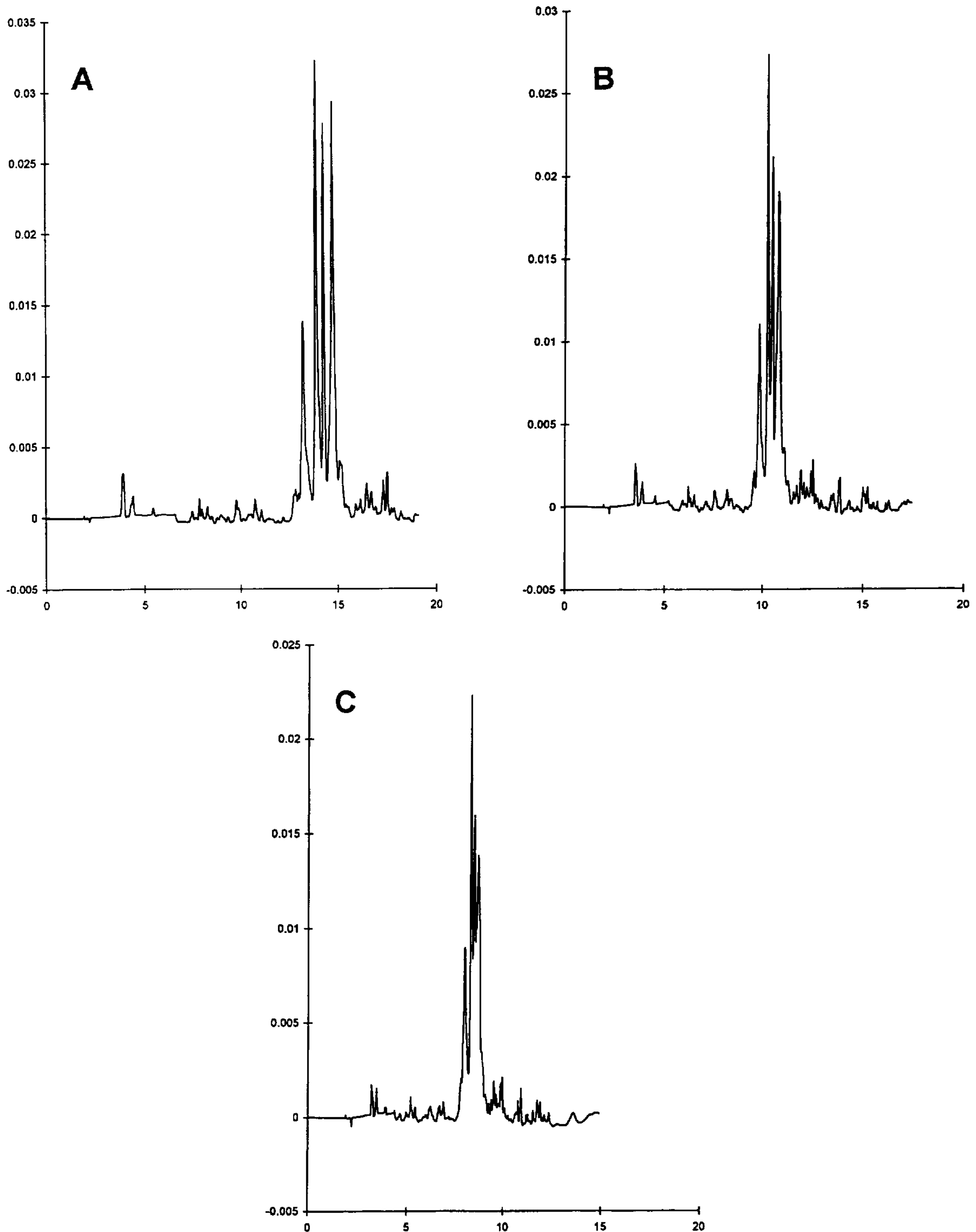

Fig. 10. Effect of applied pressure in resolution of hemoglobin variants. Electropherograms using (A) 0.003, (B) 0.005 and (C) 0.007 MPa at 0.4% methylcellulose concentration are displayed. Methylcellulose was added to the anolyte, catholyte and sample + ampholyte solutions. A 27 cm × 50 μm, LPA coated capillary was used. The column was filled first with catholyte at 0.133 MPa of pressure, the sample was then introduced for 24 s also at 0.133 MPa. Focusing was performed for 2 min at 13.5 kV. The same field strength was maintained during mobilization. Sample zones were detected by UV absorption at 280 nm.

dependent systematic drift of migration times for hemoglobins A, F, S, and C. Although the amount of salt ions present in the sample mixture was not excessive (40 m*M* Tris), the migration time differed as much as 50%. The main effect of salts in the sample is gradient compression, and in this electropherogram the distance between the first and last peak is diminished, but the hemoglobin variants are still clearly resolved. At higher levels of salt, resolution can be seriously compromised. The present example shows a decreased analysis time for samples containing the most salt, but we have also observed increased migration times, especially when salt content is so high that it impairs the focusing process. Since total salt content is difficult to control, reproducibility problems are common when different samples are compared. This fact prevents the use of external standards for p*I* determination.

3.5. Use of internal p*I* markers

Due to the high number of parameters affecting migration time in cIEF analyses, the use of internal standards is highly desirable. Protein standards, including those developed for slab gel IEF, are not suitable for use as internal standards, because they normally produce multiple peaks during cIEF, which may interfere with the identification of sample components. Important characteristics of p*I* standards include high purity, low rate of degradation, high solubility (under focusing conditions), high absorption, and no interaction with sample and ampholytes. A number of p*I* markers with all the desirable characteristics has been synthesized [9]. Analysis of human hemoglobins A and S flanked by synthetic p*I* standards with values of 5.3 and 8.4 is depicted in Fig. 7. The reproducibility report for five consecutive analyses of hemoglobin is presented in Table 1. To obtain this data, migration times were calculated for all peaks. Using the same data, p*I* values were calculated using the internal markers to create a calibration plot, and then the p*I* values of the hemoglobin peaks were obtained by interpolation (linear regression). Although the t_m R.S.D.% value obtained was good (about 1%), precision of calculated p*I* values was even better (0.06%). Considering the complexity of the cIEF process, normalization of protein migration times to internal standards can be advantageous in accurate determination of protein identity.

3.6. Effect of salt type and concentration in ion-addition mobilization

The effect of salt type in the overall resolution of hemoglobin variants during ion-addition mobilization is depicted in Fig. 8. Mobilization is clearly affected by the type of salt used to disrupt the chemical equilibrium attained during focusing. Sodium chloride was the first salt used for mobilization, and is still one of the most widely used compounds for ion-addition mobilization, but it is evident that other salts provide better results; in particular, the use of 50 m*M* sodium tetraborate greatly increased resolution. Higher concentration of sodium tetraborate was not used due to solubility problems. The total analysis time fluctuated between 25 and 40 min.

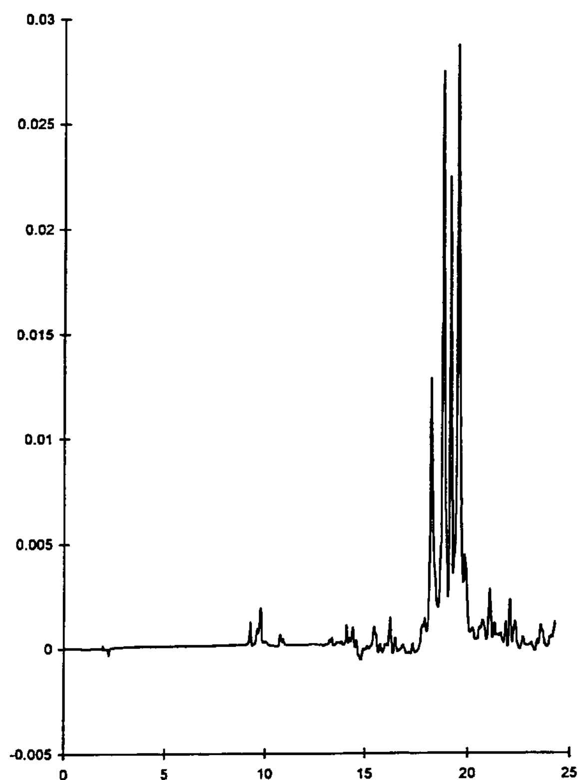

Fig. 11. Resolution of Hb variants at 0.007 MPa with increased methylcelluslose concentration (0.6%).

Other salts produced modest improvements, and in some cases resolution was diminished.

The effect of the concentration of the salt (dibasic sodium phosphate) used for mobilization is shown in Fig. 9. High resolution was not obtained when using sodium phosphate at a concentration of 100 m*M*, and separation was better at lower concentrations (especially at 25 m*M*), at the expense of total analysis time. Manipulation of salt concentration is obviously an alternative for analysis where high resolution is required.

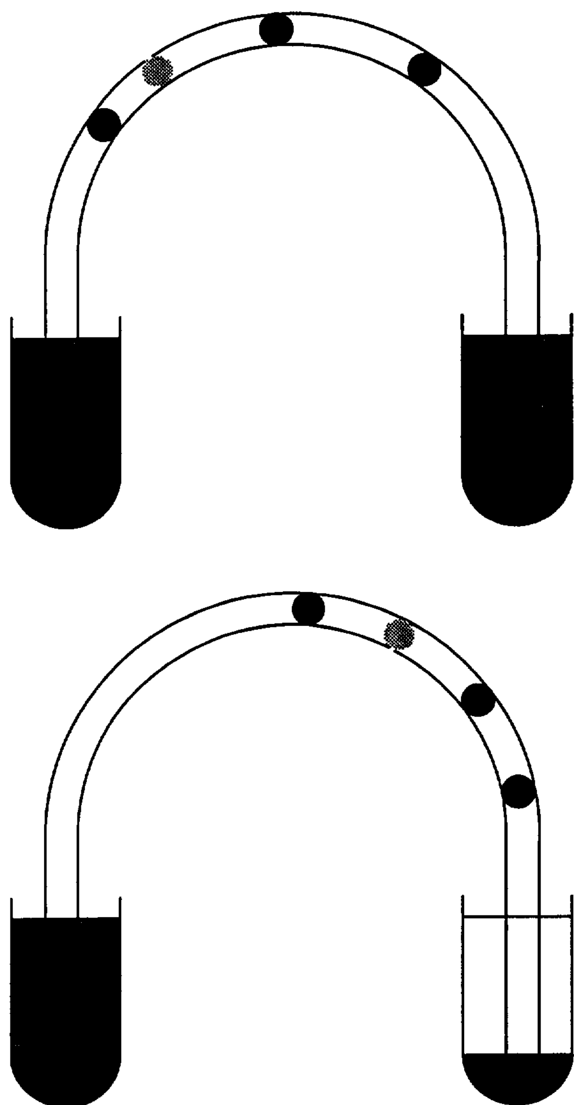

Fig. 12. Schematic gravity mobilization. After protein zones reached equilibrium during focusing, they can be transported by lowering the destination vial, or by replacing the original vial for one containing a lower volume. This method can be used to mobilize towards the anode or towards the cathode.

3.7. Hydraulic mobilization

3.7.1. Pressure

Fig. 10 shows resolution as a function of the pressure applied during mobilization. These electropherograms demonstrate that resolution decreases as the pressure is increased. When the pressure is 0.003 MPa the hemoglobin variants are practically baseline resolved, but at 0.007 MPa the sample components were detected as a group of fused peaks, and most minor bands were totally lost. If the same analysis was performed at a lower polymer concentration, the separation was also diminished (re-

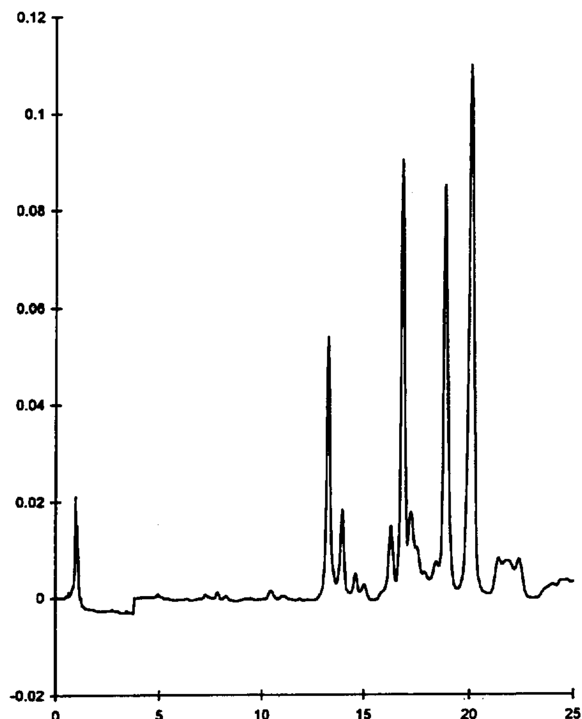

Fig. 13. The electropherogram depicts cIEF of human hemoglobin variants A, F, S, and C mobilized by gravity using an approximately 2 cm differential between the anolyte and catholyte solutions. Capillary: 24 cm×50 μm, coated with linear polyacrylamide. Focusing for 4 min at 15 kV; mobilization at 15 kV. Catholyte: 500 m*M* taurine buffer, pH 8.8.

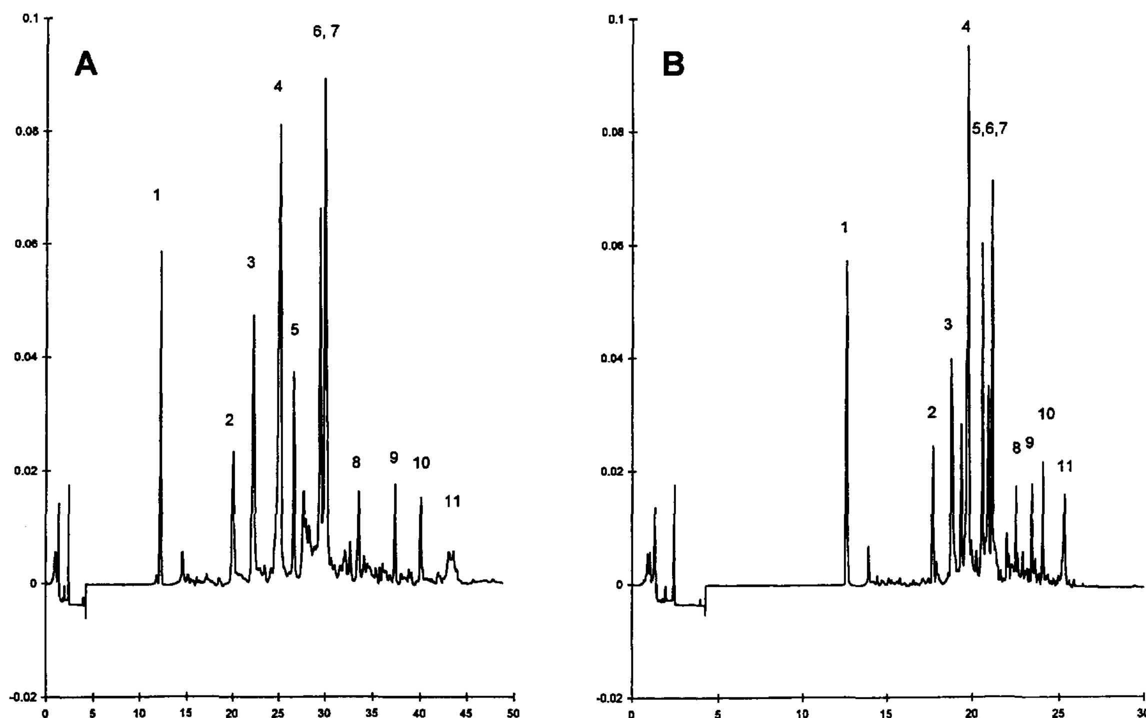

Fig. 14. (A) Gravity vs. (B) ion addition mobilization. 24 cm×75 μm coated capillary; focusing at 15 kV for 4 min; mobilization at 15 kV; sample injected into the capillary by applying 0.667 MPa for 20 s. Ion-addition mobilization employed a proprietary zwitterion used as competing ion. Gravity mobilization consisted of replacing the catholyte solution (40 m*M* NaOH) for one containing the same reagent at a lower volume. Total height differential was approximately 2 cm. Notice that with gravity mobilization, phycocyanin (peak 11) was resolved into more than one peak.

sults not shown). To determine if higher viscosity could counter the negative effects of higher applied pressure, mobilization at 0.007 MPa was performed with increased polymer concentration. Increasing the methylcellulose concentration to 0.6% (Fig. 11) could not achieve the resolution obtained at 0.003 MPa, and at this high polymer concentration mixing of sample and ampholytes was difficult and the risk of entrapped bubbles was increased.

3.7.2. Gravity

As shown above, resolution during hydrodynamic mobilization is inversely related to the pressure applied. Gravity mobilization (Fig. 12) as a means to mobilize focused protein zones has been suggested [20] and demonstrated [21] previously. Advantages of this method include its simplicity (no valves, no pressure sources, no regulators), and its universality (it can be implemented in almost any CE instrument). The force applied can easily be controlled by adjusting the height difference of the two reservoirs. Flow velocity can be manipulated by adding viscosity enhancers such as cellulose derivatives or other polymers. When the method is optimized, high resolution is achieved (Fig. 13). Fig. 14 shows that hydraulic mobilization increases resolution for acidic proteins as compared to ion-addition mobilization. During hydraulic mobilization, phycocyanin was resolved into two bands.

4. Conclusions

Some important topics considered in this study include: robustness of cIEF analyses, the effect of buffering solutions, reproducibility of peak patterns, and the advantages of protein identity by isoelectric point to correct for t_m variations. It was shown that

by limiting the pH range of the ampholytes to 8.5 or less, hundreds of runs on an individual capillary are possible, which is a necessity for routine analysis. The use of internal standards facilitates peak identity by isoelectric point, and also corrects variations due to the multiple parameters that can influence the migration times of the protein zones. It was also shown that small variations in the sample composition (e.g., salt content) can have a significant adverse effect on reproducibility.

Mobilization parameters profoundly affect the performance of cIEF. Resolution was shown to be affected by the type and concentration of the salt added to chemically mobilize focused protein zones, with low salt providing higher resolution at the expense of time. During hydraulic mobilization, resolution is inversely related to the magnitude of applied pressure. Gravity mobilization provides a simple and universal means to perform hydraulic mobilization, and the height differential can be chosen to generate extremely small forces.

References

[1] S. Hjertén, M. Zhu, J. Chromatogr. 347 (1985) 265.

[2] J.M. Hempe, R.D. Craver, Clin. Chem. 40 (1994) 2288.

[3] T. Wehr, M. Zhu, R. Rodriguez, D. Burke K. Duncan, Am. Biotech. Lab., September (1990) 22.

[4] K.W. Yim, J. Chromatogr. 559 (1991) 401.

[5] M. Zhu, R. Rodriguez, T. Wehr, C. Siebert, J. Chromatogr. 608 (1992) 225.

[6] M. Zhu, T. Wehr, V. Levi, R. Rodriguez, K. Shiffer, Z.A. Cao, J. Chromatogr. A. 652 (1993) 119.

[7] S. Molteni, W. Thormann, J. Chromatogr. 638 (1993) 187.

[8] G. Küllertz, G. Fischer, J. Chromatogr. A. 684 (1994) 329.

[9] K. Slais, Z. Friedl, J. Chromatogr. A. 661 (1994) 249.

[10] P.G. Righetti, G. Tudor, K. Ek, J. Chromatogr. 220 (1981) 115.

[11] R. Rodriguez-Diaz, M. Zhu, T. Wehr C. Siebert, presented at the 13th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, San Francisco, CA, 1993, Paper 611.

[12] S. Hjertén, J. Chromatogr. 347 (1985) 191.

[13] T.L. Huang, P.C.H. Shieh, N. Cooke, Chromatographia. 39 (1994) 543.

[14] S.-M. Chen, J.E. Wiktorowicz, Anal. Biochem. 206 (1992) 84.

[15] N.Y. Nguyen, A. Chrambach, Anal. Biochem. 79 (1977) 462.

[16] M.J. Swanson, B.E. Sanders, Anal. Biochem. 67 (1975) 520.

[17] M. Zhu, R. Rodriguez, T. Wehr, J. Chromatogr. 559 (1991) 479.

[18] S. Hjertén, J.-L. Liao, K. Yao, J. Chromatogr. 387 (1987) 127.

[19] Righetti, P.G., Isoelectric focusing, Elsevier, Amsterdam, 1989.

[20] S.F.Y. Li, Capillary Electrophoresis (Journal of Chromatography Library, Vol. 52), Elsevier, Amsterdam, 1992.

[21] R. Rodriguez, C. Siebert, Presented at the 6th International Symposium on High-Performance Capillary Electrophoresis, San Diego, CA, 1994, Paper P-639.