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Journal of Chromatography A, 813 (1998) 349–360

JOURNAL OF CHROMATOGRAPHY A

# New set-up for capillary isoelectric focusing in uncoated capillaries[1]

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Received 8 July 1997; received in revised form 6 November 1997; accepted 10 November 1997

**Abstract**

Substituted aminomethylphenol dyes, low-molecular-mass isoelectric point (p*I*) markers and hemoglobin samples from normal individuals and diabetic patients were used to test a new set-up of capillary isoelectric focusing (cIEF) in uncoated capillaries. In previous cIEF methods, a mixture of sample components and carrier ampholytes was applied in the capillary and analyzed. In the new set-up a fractionated injection protocol is used to apply a 'sandwich' ampholyte–sample–ampholyte plug in the capillary for analysis. This new set-up allows the separation of amphoteric compounds having p*I* values outside the pH region of the ampholytes applied in the capillary with high precision. The high resolution power of this technique was proven with the analysis of hemoglobin variants. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Isoelectric focusing; Sandwich injection; Injection methods; Haemoglobin; Dyes; Nitrophenols

## 1. Introduction

Capillary electrophoretic techniques have the advantage of providing qualitative and quantitative data that are important for clinical diagnosis [1]. Isoelectric focusing (IEF) is an electrophoretic technique for separation of amphoteric compounds in a pH gradient that is formed by ampholytes in an electric field. IEF can be performed in gel format or in free solution, e.g., in capillaries. Capillary isoelectric focusing (cIEF) has the advantages of automation and on-line detection of the separated components. cIEF has been applied successfully for protein analysis, e.g., separation of transferrin isoforms, hemoglobin variants and other proteins in coated [2–11] and uncoated [12–22] capillaries. Special conditions are necessary when coated capillaries are used for the mobilization of the pH gradient in the capillary in order to move the substances towards the detection point [23]. In uncoated capillaries, however, mobilization of focused zones is accomplished by the electroosmotic flow ('dynamic cIEF'). In this case the EOF is controlled with methylcellulose (MC) which also prevents the protein interaction with the capillary wall [12,15].

IEF experiments necessitate the use of reference substances [isoelectic point (p*I*) markers] for qualitative analysis. A good choice is the application of aminomethylated nitrophenols (dyes) that are highly soluble in water and have a high absorbance at low wavelengths [24,25].

In previous cIEF set-ups, sample substances and carrier ampholytes were introduced in mixtures. However, the possible interactions between analytes

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[1]Paper presented at the 10th International Symposium on High-Performance Capillary Electrophoresis, Kyoto, 8–11 July 1997.

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PII: S0021-9673(98)00377-X

and ampholytes may disturb the analysis if the sample cannot be applied immediately after the preparation of this mixture [6]. Another limitation is that the p*I* values of the separands must fall in the same range as the ampholytes. We developed a new set-up, in which the separands and the ampholyte solutions are applied consecutively. The results show that this set-up is as good as or better than the cIEF methods used earlier, and with this technique separation of amphoteric compounds can be accomplished by ampholyte solutions with pH ranges not including the isoelectric points of the separands.

## 2. Experimental

### *2.1. Chemicals and samples*

All chemicals used were of analytical reagent grade. MC with a viscosity of 4000 cP (2%, w/v, aqueous solution in 25°C) was from Sigma (St. Louis, MO, USA). A stock solution of 1.5% (w/v) MC was prepared and stored in the refrigerator for a maximum of 2 months. Carrier ampholytes, such as Ampholine (LKB, Bromma, Sweden), Pharmalyte (Pharmacia, Uppsala, Sweden), BioLyte (Bio-Rad, Richmond, CA, USA) and Servalyt (Serva Feinbiochemica, Heidelberg, Germany) covering narrow or broad pH ranges, were used in 2% solutions unless otherwise indicated. Aminomethylated nitrophenol dyes having isoelectric points of 5.3, 6.4, 6.6, 7.2, 7.9, 8.6, 10.4, were kindly supplied by K. Šlais, (Brno, Czech Republic). One mg/ml stock solutions in water were prepared from the dye powders.

Preparation of the hemoglobin from blood was made as published by Molteni et al. [18]. Briefly, whole blood was centrifuged for 10 min at 3600 rpm. The supernatant was removed and the red blood cells (RBC) were washed three times with 0.9% NaCl solution followed by centrifugation for 5 min at 3600 rpm. The RBCs were lysed by adding 800 μl of 1 m*M* KCN in an Eppendorf tube containing 200 μl of RBCs. The hemolysate was then centrifuged at 11 000 rpm for 3 min and the clear supernatant was stored at 4°C for a maximum 10 days prior to injection. Standard solutions of hemoglobin variants, AFSC – hemoglobin electrophoresis control (Isolab, Akron, OH, USA) were prepared according to the instructions of the manufacturer.

### *2.2. Instrumentation and running conditions*

cIEF was performed on a PrinCE (Lauerlabs, Emmen, The Netherlands) modular capillary electrophoresis system combined with a Lambda 1000 UV-Vis detector (Bischoff, Leonberg, Germany). On-line detection was used at 280 or 415 nm. Data collection and evaluation of the data were performed by the Axxiom Chromatography 717 system, v3.91 (Moorpark, CA, USA) and Ceasar Evaluation version 3.0 software (Prince Technologies, Emmen, The Netherlands), respectively.

Bare fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and used in uncoated form [60 cm (effective length 45 cm)×50 or 75 μm I.D.].

The capillary was conditioned with 0.015% MC in 20 m*M* NaOH using 2500 mbar before the first run every day and between the experiments. Injection of samples and carrier ampholytes were performed with 50 or 100 mbar in three consecutive steps (see Section 3). Applied voltage was 20 or 30 kV. NaOH (20 m*M*) and $H_3PO_4$ (10 m*M*) were used as catholyte and anolyte, respectively. Every experimental set-up (with its unique parameters) was tested with runs repeated 3–5 times, in order to prove reproducibility.

## 3. Results

### *3.1. New injection protocol for cIEF*

Aminomethylated nitrophenols and hemoglobin variants were analyzed by cIEF separations in uncoated capillaries. A new injection protocol was developed for the experiments, where the ampholytes and analytes were injected separately in three steps (Fig. 1), i.e., ampholyte solutions (zones 4 and 2 in Fig. 1) were injected before and after the sample (zone 3). The same type or different types of ampholyte solutions were applied as zones 4 and 2, and the lengths of the zones were also varied. Approximately 20–30% of the whole capillary was filled with the ‘sandwich’ plug. Isoelectric-focusing

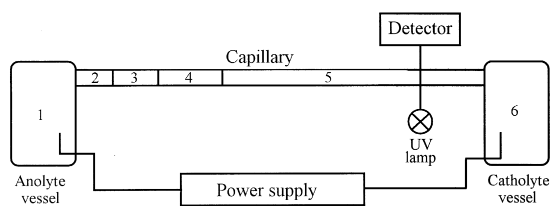



Fig. 1. Schematic representation of the injection set-up in cIEF. Anolyte (1) 10 m$M$ $H_3PO_4$, catholyte (6) 20 m$M$ NaOH. The capillary is purged with 20 m$M$ NaOH containing 0.015% methylcellulose (5) before sample application. The injection of ampholytes (zones 2 and 4) and sample (3) is performed in three consecutive steps filling ca. 20–30% of the whole capillary. The composition of the ampholyte solutions and the lengths of the zones can be varied easily in this protocol.

analysis of the samples was then accomplished by applying the current and the pH gradient moved towards the cathode with the electroosmotic flow.

### *3.2. Experiments with 'homologue' ampholytes in zones 2 and 4*

Aminomethylated nitrophenols were used in experiments to optimize the conditions for the 'sandwich injection'. In the first series of the experiments, the same ampholytes were applied as zones 2 and 4. The lengths of the injected zones (2, 3 and 4) were varied by the injection pressure and time. Figs. 2–5 show results using Ampholine, Pharmalyte, BioLyte and Servalyt solutions, respectively. Narrow and broad pH ranges were tested for comparison. The components in the isoelectropherograms were confirmed by spiking the sample and also by their spectral properties. Although the pH ranges of the ampholytes did not cover the p$I$ values of the seven dyes in several cases (see the runs with narrow pH ranges), we obtained separation of the analytes to a certain extent in every experiment. The use of capillaries with different inner diameters, 75 μm or 50 μm, acquired different experimental conditions as is presented in Figs. 2–5, respectively. The worst resolution was obtained with the ampholyte solutions covering the lowest narrow pH range, pH 3–5 (Fig. 2a, Fig. 4a). Better separation was obtained with ampholytes covering higher narrow or broad pH ranges. Surprisingly, the resolution and the migration times of the dyes were different when ampholyte solutions covering similar pH ranges obtained from different sources were applied (compare the results in Fig. 2b, Fig. 3a, Fig. 4b or in Fig. 2c, Fig. 3b, Fig. 4c, Fig. 5a). The two dye compounds having very close p$I$ values, 6.6 and 6.4, were resolved in only a few runs (Fig. 2b, Fig. 3a, Fig. 4d, Fig. 5a and b). It must be noted, that a direct comparison of the data of Figs. 2 and 3 to Figs. 4 and 5 is not obvious due to the different capillary geometry. However, for comparison, the injection parameters were calculated for the capillaries with different I.D.s to provide the same lengths of the zones.

### *3.3. Combination of ampholyte solutions*

To improve the resolution, combinations of different ampholyte solutions were tested. Two different ampholyte solutions have been used in dynamic cIEF previously but always in mixtures with sample components. Fig. 6a shows the isoelectropherogram of the dyes applying the experimental conditions from Ref. [18]. The mixture contained a broad (1% Ampholine pH 3.5–10) and a narrow (2% Pharmalyte pH 5–8) ampholyte solution. For comparison the new injection protocol was applied (Fig. 6b) where the same amounts of every component were injected in the capillary, but in separate zones according to Fig. 1. The appropriate experimental parameters were calculated from the concentrations of sample and ampholytes, length and diameter of capillary, duration and pressure of injection. Sharper peaks were observed for all the seven dyes with the exception of the dye component with p$I$ 5.3 using the sandwich injection protocol (compare Fig. 6a and b). Significantly better resolution was obtained using other concentrations of the ampholytes (Fig. 6c). As was obtained with homologue ampholytes in zones 2 and 4 (see Figs. 2–5) we found that with a combination of two narrow pH-range solutions, the seven dyes can similarly be separated even if the pH ranges do not cover every p$I$ value (Fig. 6d and e). Although the separation occurred within considerably shorter analysis time, the resolution was better when a combination of the ampholytes was used.

The plots of p$I$ values of standards versus migration times make the determination of the isoelectric points of unknown substances possible. These plots are inserted in Fig. 6 showing the linearity or nonlinearity of the separation of the standards. The

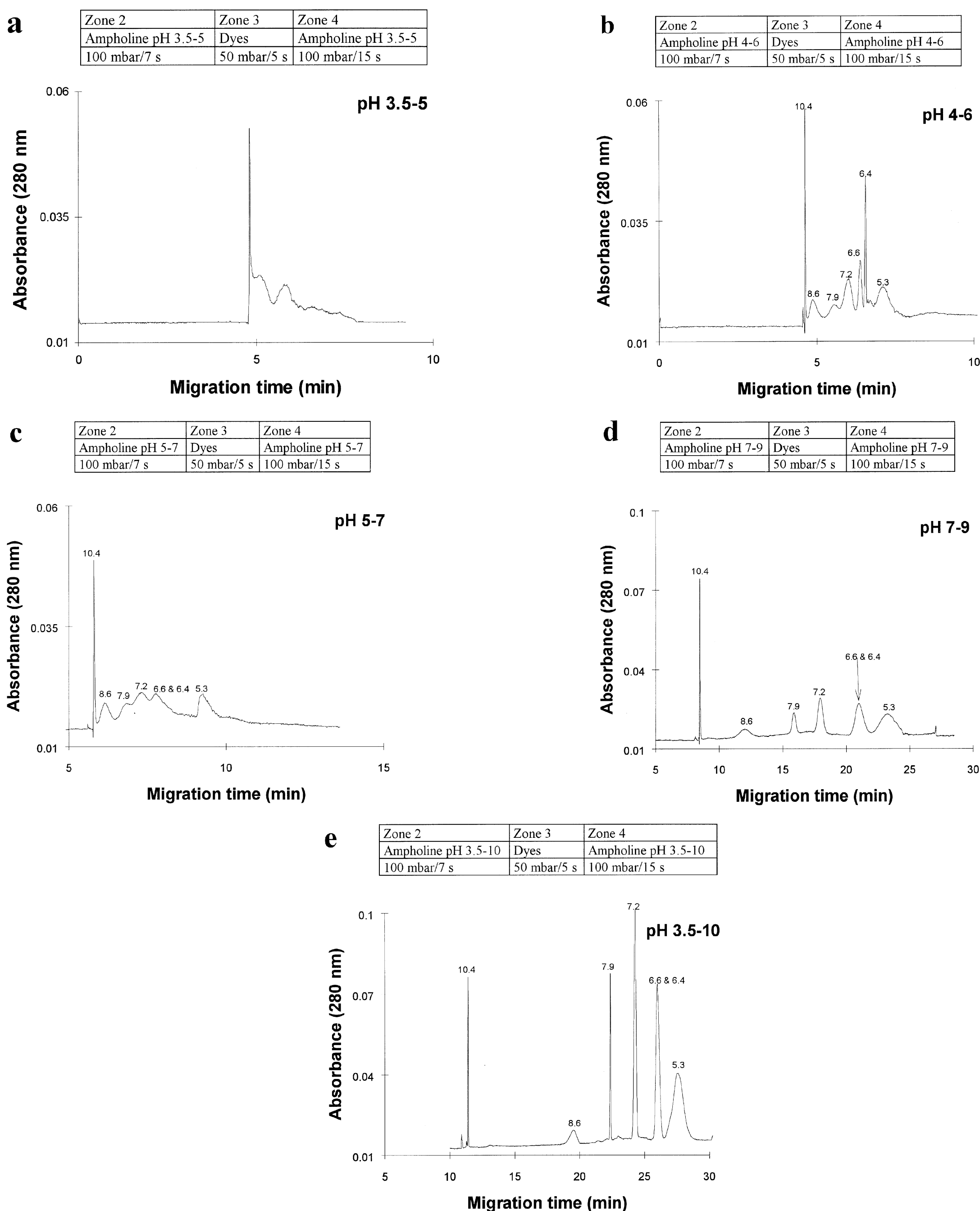



Fig. 2. cIEF of a mixture of aminomethylated nitrophenols (dyes) with 2% 'homologue' Ampholine solutions as zones 2 and 4 according to the injection set-up in Fig. 1. (a) pH 3.5–5, (b) pH 4–6, (c) pH 5–7, (d) pH 7–9, (e) pH 3.5–10. The sample consisted of dyes having p*I* values: 10.4, 8.6, 7.9, 7.2, 6.6, 6.4 and 5.3; concentration 0.143 mg/ml each. The peaks are assigned in the isoelectropherogram by their p*I* values. Injection parameters for zones 2–4 (injection presssure in mbar and length of injection in s) are given in the tables inserted. Experimental conditions: applied voltage 20 kV, detection wavelength 280 nm, capillary 60 cm (effective length 45 cm)×75 μm I.D.

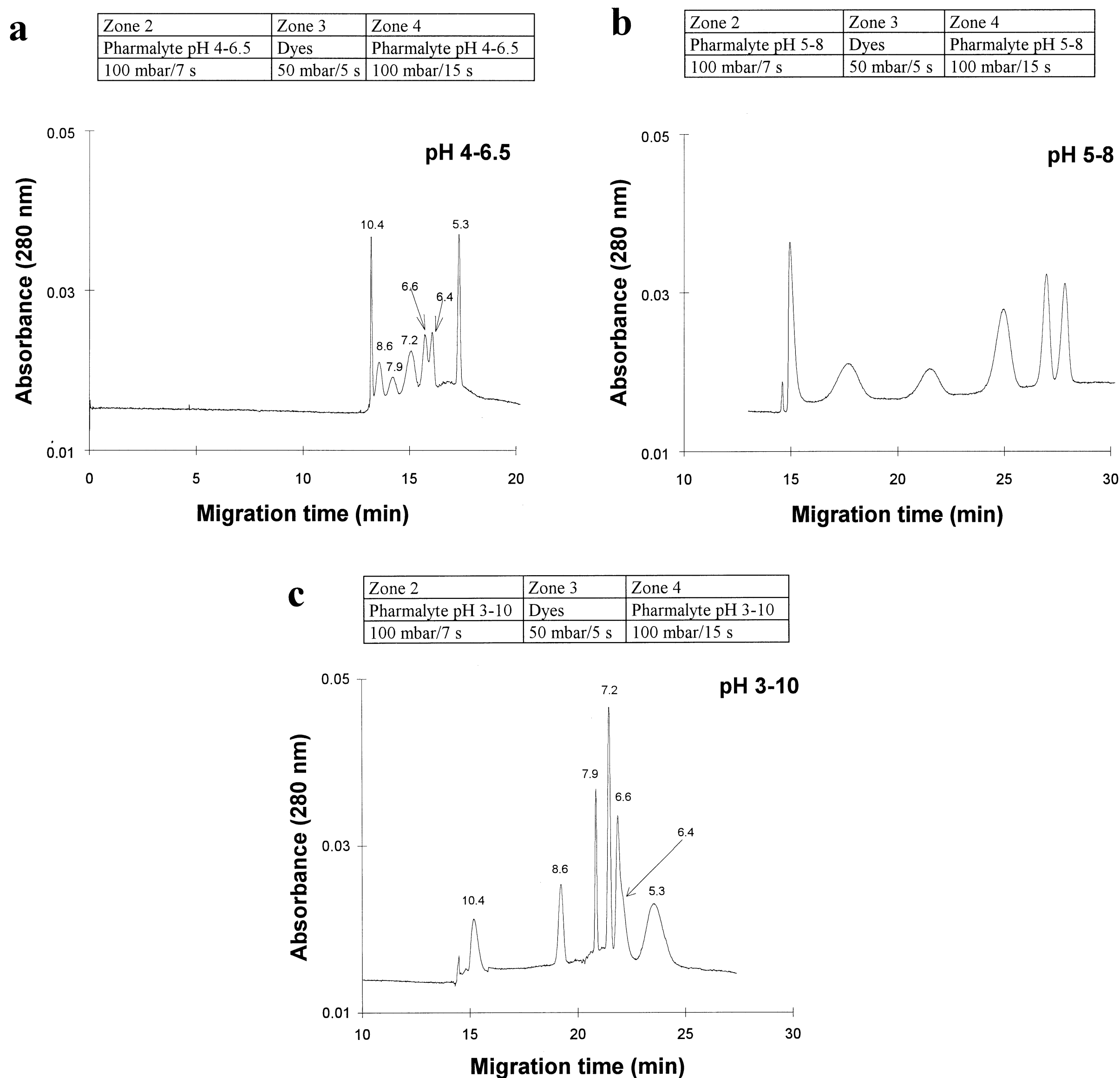



Fig. 3. cIEF of a mixture of aminomethylated nitrophenols (dyes) with 2% 'homologue' Pharmalyte solutions as zones 2 and 4 according to the injection set-up in Fig. 1. (a) pH 4–6.5, (b) pH 5–8, (c) pH 3–10. Experimental conditions and composition of sample as in Fig. 2. Injection parameters for zones 2–4 (injection presssure in mbar and length of injection in s) are given in the tables inserted.

migration times, peak shapes and resolution of the dyes were reproducible and the experimental error was not bigger than 3% in runs repeated several times within a given day or day-to-day.

### 3.4. Separation of hemoglobin variants

In order to test the method we performed experiments with hemoglobin standard solutions, and

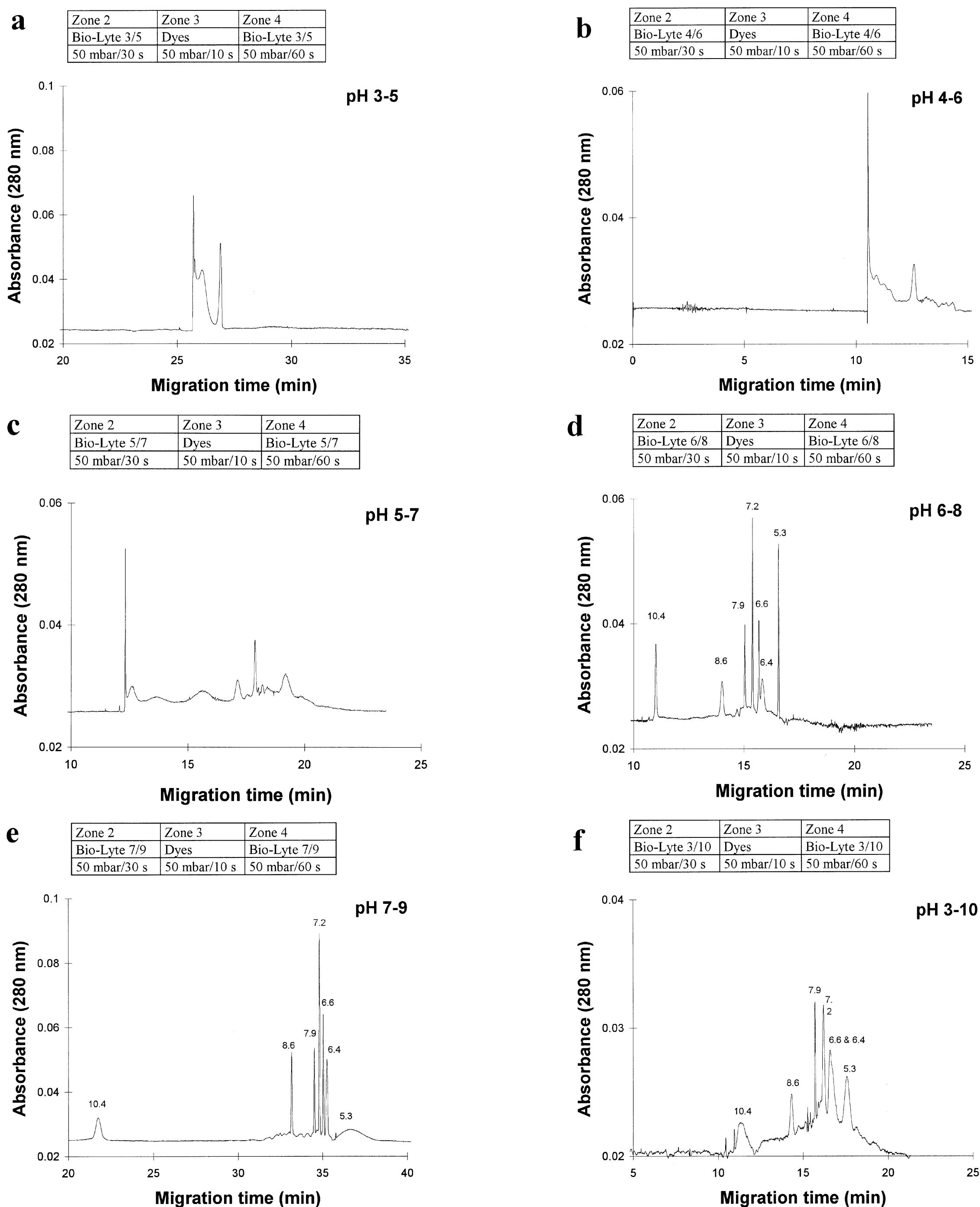
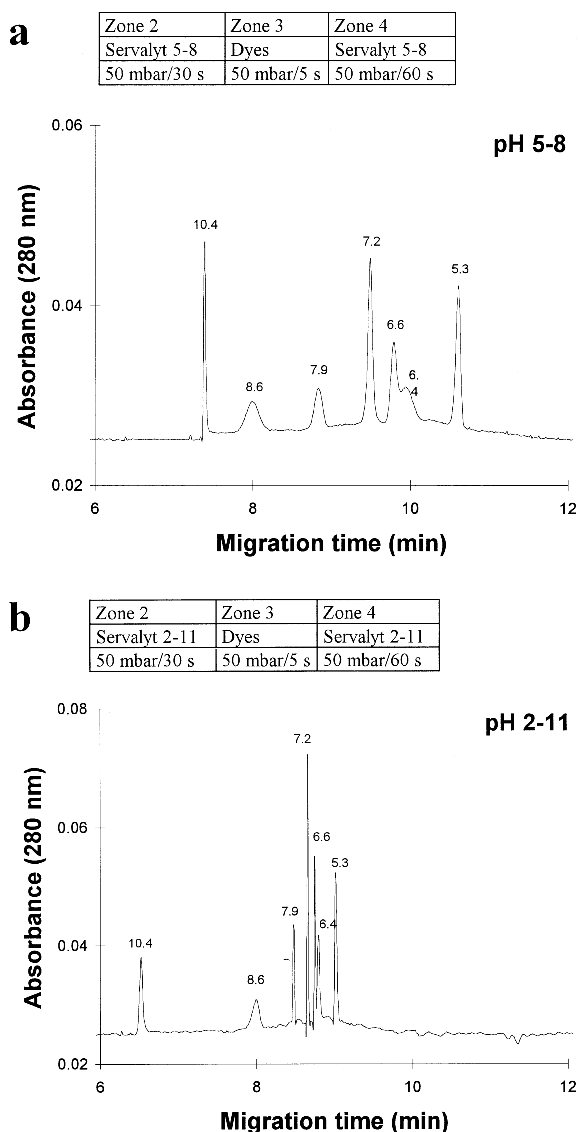



Fig. 4. cIEF of a mixture of aminomethylated nitrophenols (dyes) with 2% 'homologue' BioLyte solutions as zones 2 and 4 according to the injection set-up in Fig. 1. (a) pH 3–5, (b) pH 4–6, (c) pH 5–7, (d) pH 6–8, (e) pH 7–9, (f) pH 3–10. Experimental conditions and composition of sample as in Fig. 2, except the capillary I.D. was 50 μm. Injection parameters for zones 2–4 (injection presssure in mbar and length of injection in s) are given in the tables inserted.



a

| Zone 2 | Zone 3 | Zone 4 |
|---|---|---|
| Servalyt 5-8 | Dyes | Servalyt 5-8 |
| 50 mbar/30 s | 50 mbar/5 s | 50 mbar/60 s |

b

| Zone 2 | Zone 3 | Zone 4 |
|---|---|---|
| Servalyt 2-11 | Dyes | Servalyt 2-11 |
| 50 mbar/30 s | 50 mbar/5 s | 50 mbar/60 s |



Fig. 5. cIEF of a mixture of aminomethylated nitrophenols (dyes) with 2% 'homologue' Servalyt solutions as zones 2 and 4 according to the injection set-up in Fig. 1. (a) pH 5–8, (b) pH 2–11. Experimental conditions and composition of sample as in Fig. 4. Injection parameters for zones 2–4 (injection presssure in mbar and length of injection in s) are given in the tables inserted.

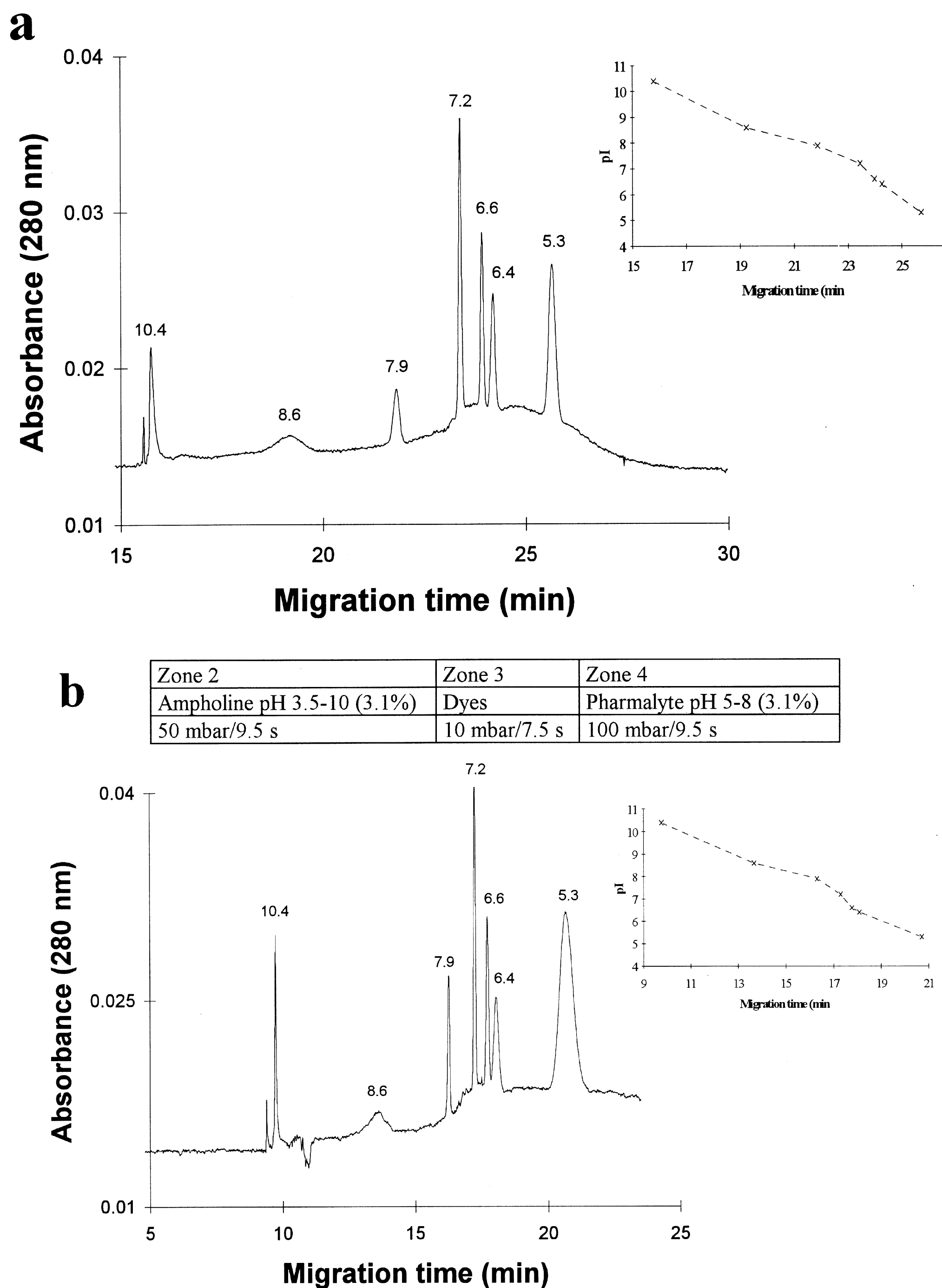
hemoglobin preparations from blood of controls and patients with diabetes. The electropherograms show the nice separation of all the major variants and their glycosylated forms (Fig. 7).

## 4. Discussion

cIEF has the advantages of automation over the common gel electrophoretic methods. However, the on-line detection of the components makes the method more powerful, since small amphoteric molecules, such as the nitrophenol dyes can be used as p*I* markers in the experiments providing efficient internal standardization of the pH gradient [24]. Two main techniques were developed in the past using coated and uncoated capillaries for the separation. The amphoteric compounds, i.e., sample components and ampholytes, have been applied in mixtures in those techniques. The pH gradient formed separates all the sample components having p*I* values inside the respective pH region of the ampholytes. Components having p*I* values outside this pH range migrate out from the pH gradient and will not show up in the isoelectropherogram. Since the mixing of the separands and ampholytes may cause unexpected reactions in the experiments [6] and due to some other practical reasons, we tried to avoid the unnecessary mixing of those components, and developed a new, three-step injection protocol for cIEF in uncoated capillaries. The first results immediately showed that this technique gives new possibilities and therefore a more detailed study with low-molecular-mass aminophenylated dyes was performed for the evaluation. Although the experiments' reproducibility tests were satisfactory, the purpose of this study was not to make any tangible, quantitative comparisons of data to prove that this new method is better than other methods in the literature.

The automated capillary electrophoretic equipments provide good opportunities to apply several zones in the capillary before the run. In our method we have tried several set-ups, in which the order and number of zones were varied, and the zones separately contained the analytes and ampholytes of various concentrations. We found that the set-up presented in Fig. 1 was the most effective for the separation. The variation of the experimental parameters, injection pressure, length of injection, capillary diameter, temperature, makes this method very flexible. In the evaluation procedure the type, concentration and amount (length) of the ampholytes were varied and the results compared.

It was found that isoelectric focusing is a complex

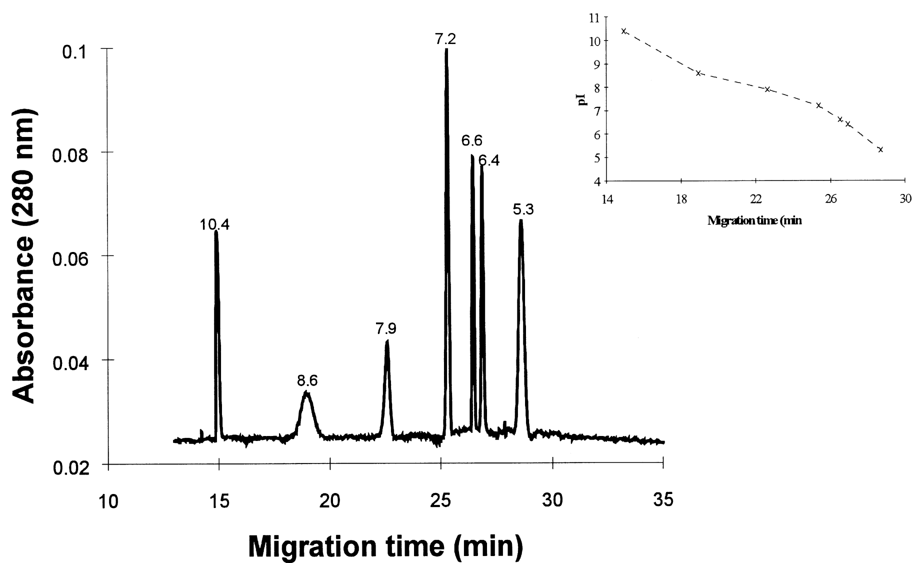



| Zone 2 | Zone 3 | Zone 4 |
| --- | --- | --- |
| Ampholine pH 3.5-10 (3.1%) | Dyes | Pharmalyte pH 5-8 (3.1%) |
| 50 mbar/9.5 s | 10 mbar/7.5 s | 100 mbar/9.5 s |

Fig. 6. cIEF of a mixture of aminomethylated nitrophenols (dyes) with a combination of ampholyte solutions. (a) the sample and the ampholytes were mixed as in Ref. [17] and injected, 100 mbar, 15 s; (b) the same amounts of ampholytes and sample were injected in the capillary as in (a) using appropriate injection pressure and time (see table inserted); (c) to (e) ampholytes from different sources and covering broader and/or narrower pH ranges, were applied in zones 2 and 4. Other experimental conditions and composition of the dyes as in Fig. 2. The plots of p*I* values versus migration times show the possibility of determination of isoelectric points in this new set-up.

**c**

| Zone 2 | Zone 3 | Zone 4 |
|---|---|---|
| Ampholine pH 3.5-10 (2%) | Dyes | Pharmalyte pH 5-8 (2%) |
| 50 mbar/30 s | 50 mbar/10 s | 50 mbar/60 s |




**d**

| Zone 2 | Zone 3 | Zone 4 |
|---|---|---|
| Ampholine pH 5-7 | Dyes | Pharmalyte pH 6.7-7.7 |
| 100 mbar/10 s | 50 mbar/5 s | 100 mbar/15 s |

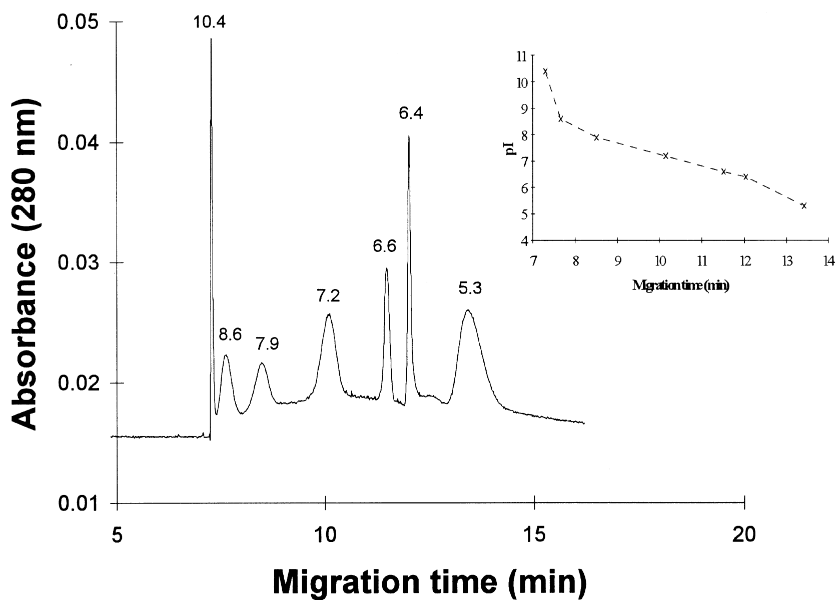
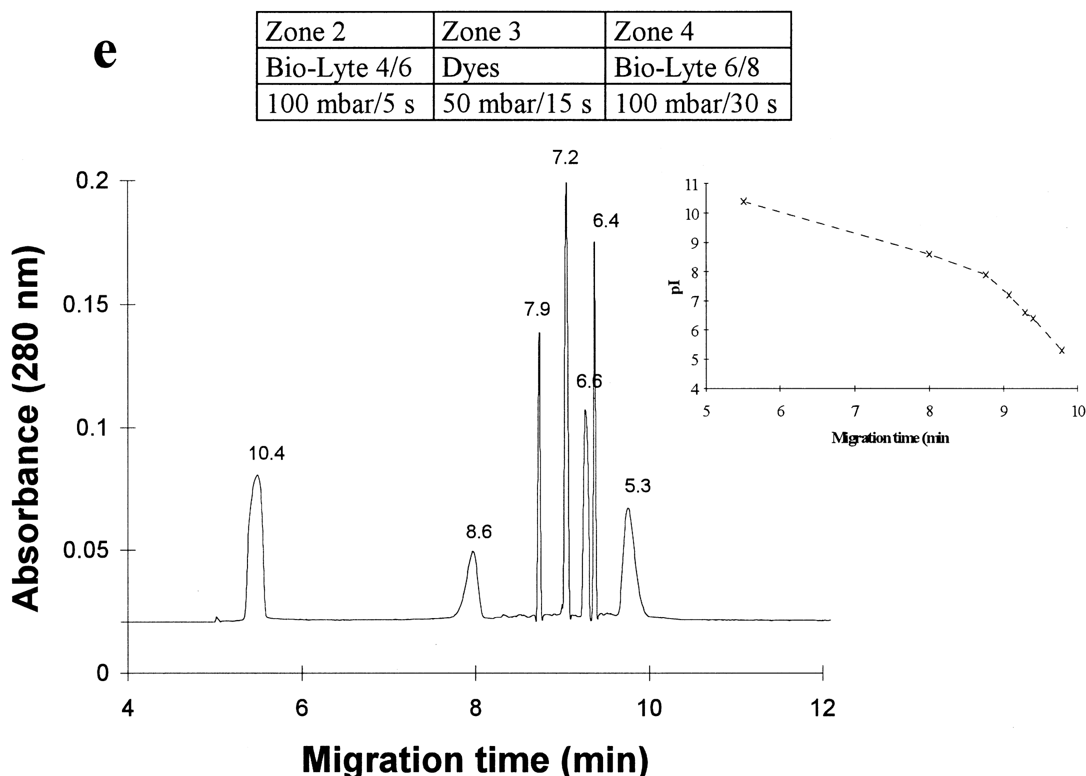



Fig. 6. (*continued*)



| Zone 2 | Zone 3 | Zone 4 |
|---|---|---|
| Bio-Lyte 4/6 | Dyes | Bio-Lyte 6/8 |
| 100 mbar/5 s | 50 mbar/15 s | 100 mbar/30 s |



Fig. 6. (*continued*)

process in this new set-up. Ampholyte components positioned originally at the two sides of the sample analytes move through the middle zone, resulting in a pH gradient, but at the same time the sample components migrate through the developing pH gradient. It became obvious from the experiments, that the cross migration of the components separates the separands irrespective of the pH range covered by the ampholytes (see experiments with narrow pH range ampholytes). In this new set-up, the components having p*I* values outside the actual pH range leave the pH gradient, but with different migration speeds, and therefore, a separation occurs. For example, the dye with p$I$=10.4 appears as a sharp peak in several cases, and always separated from the other dyes with lower p*I* values.

It is not possible to state what are the best conditions for the separation, due to different practical considerations, e.g., what types of ampholytes are available or what are the characteristics of the analytes, etc. In the case of the dyes, conditions in Fig. 5b seemed to be superior, when homologue ampholytes were applied, but with the combinations of the ampholytes, other experimental conditions resulted in better separation (see Fig. 6c or e).

The p*I* calibration curve constructed from the migration times showed nonlinearity in several cases. This is due to the special formation of the pH gradient by two fusing zones which influences the migration of the dye components. Several effects should be also considered, such as, the differences of the pH ranges and concentrations of the two ampholyte solutions in the initial zones (zone 2 and 4), the homogeneity of the ampholyte components, the length of the sample between the two separated ampholyte zones, etc. Since these effects cause different conditions along the capillary and/or along the forming pH gradient, the migration times plotted in the calibration curves might deviate from linearity, as it was observed (see insertions of the figures). In

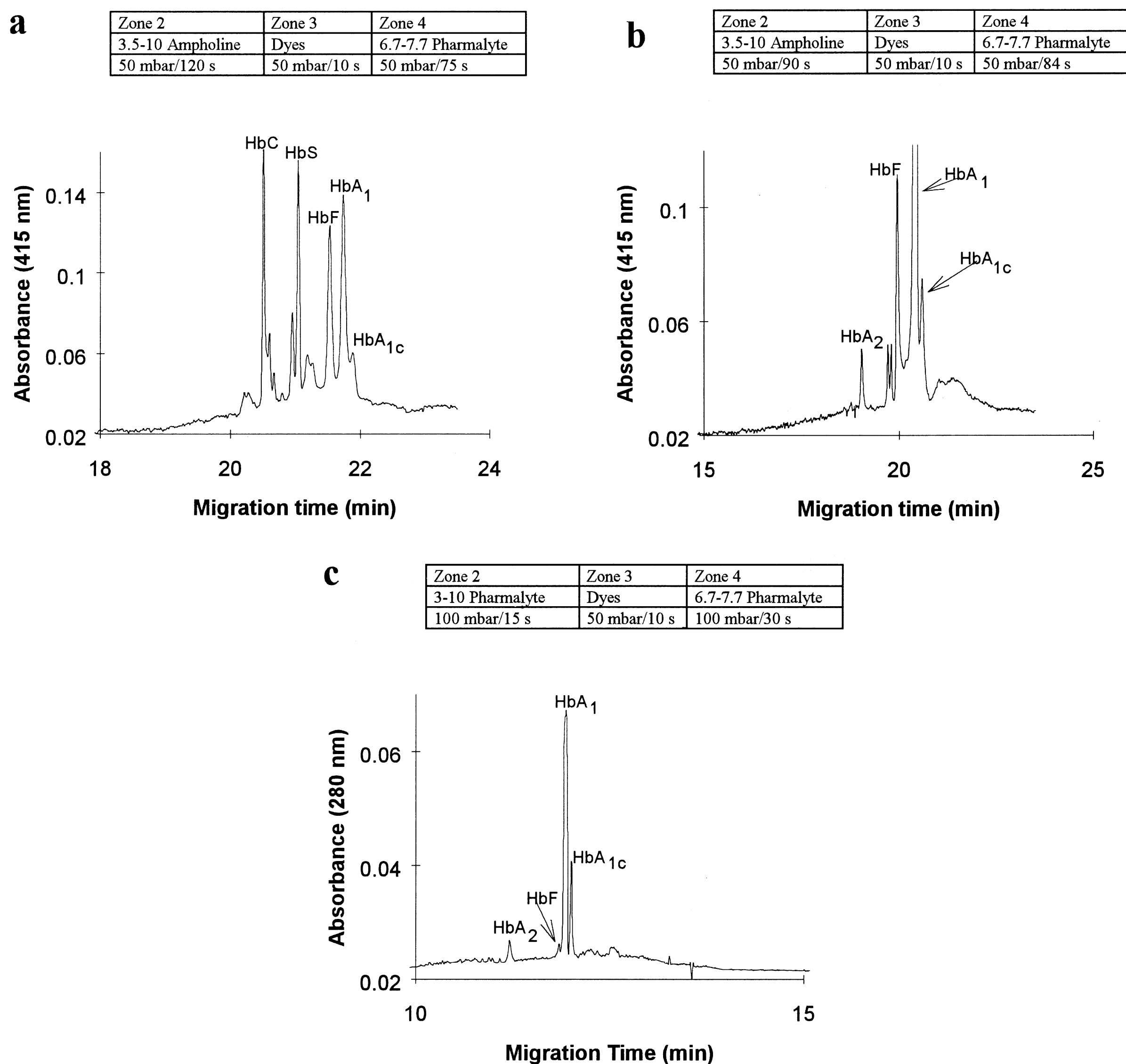



Fig. 7. cIEF of the hemoglobin variants. Experimental conditions: applied voltage 20 kV, capillary 60 cm (effective length 45 cm)×50 μm I.D. Injection parameters for zones 2–4 (injection presssure in mbar and length of injection in s) are given in the tables inserted. (a) Hemoglobin variants in an AFSC control solution (1 mg/ml), (b) hemoglobin from normal adult blood (1 mg/ml), (c) hemoglobin from a diabetes patient (1 mg/ml).

order to clarify the real tendency of these calibration curves further studies will be necessary.

Experiments with ampholytes from different sources covering the same or similar pH ranges (see Figs. 2–5), did not provide identical results, indicating differences in the distribution of the components and/or chemical composition, as it has been shown earlier [23].

When analyzing the hemoglobin variants of normal and diabetic patients we used a combination of two different ampholyte solutions covering a narrow (pH 6.7–7.7) and a broad pH region (pH 3.5–10).

From the results, we can conclude that separation of the hemoglobin variants was observed to be as efficient as other methods found in the literature. The precision makes this method a possible alternative tool for clinical diagnosis of hemoglobin variants.

## 5. Conclusion

This new set-up of capillary isoelectric focusing giving an alternative method for separation of amphoteric compounds with high effectivity appears to be a potentially very useful alternative approach to performing cIEF. We found that the sandwich injection of samples and ampholytes provides excellent results that are similar or better compared to previously used methods in coated or uncoated capillaries [15,18], and the use of ampholytes of different p*I* values in the 'bracketing' zones appears to be very useful and practical. Since the migration of the components, both, ampholytes and samples, is a very complex process the optimization approach in finally choosing different ampholyte mixtures is – at this stage – all trial and error. To describe a more systematic and smarter approach in optimization we need a detailed modeling of this set-up in the future. Simulations of a somewhat similar set-up have been done previously [26]. Such modeling studies will also make it possible to obtain data about peak asymmetry values, plate counts and other (customary) performance characteristics in CE and cIEF, and consequently, to clarify, in a quantitative sense, whether these newer results are indeed an improvement in performance over any or all of the already described methods. The experimental set-up was also evaluated with the analysis of hemoglobin variants of normals and diabetic patients. Since the migration of the components (ampholytes and samples) is a very complex process, a description of it needs detailed modelling in the future.

## Acknowledgements

The work was supported by grants OTKA T16657, T25527, and TKT T-07/624/93, T-08/394/96, MKM 473/95, 554/96, AMFK 861/96 and F/61-95.

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