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Theory of the correlation between capillary and free-flow zone electrophoresis and its use for the conversion of analytical capillary separations to continuous free-flow preparative processes
Application to analysis and preparation of fragments of insulin

Václav Kašička*, Zdeněk Prusík, Petra Sázelová, Jiří Jiráček, Tomislav Barth

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo 2, 166 10 Prague 6, Czech Republic

Abstract

A basic theoretical description of the correlation between capillary zone electrophoresis (CZE) and free-flow zone electrophoresis (FFZE) is presented. The theory of the correlation between CZE and FFZE results from the fact that both methods are based on the same separation principle, zone electrophoresis, and both are performed in the carrierless separation medium with the same composition of the background electrolyte. The equations describing the movement of the charged and noncharged particles in the d.c. electric field applied in the capillary and in the flow-through electrophoretic chamber are presented and used for the quantitative description of the correlation between CZE and FFZE. Based on the theory of the correlation between CZE and FFZE a procedure has been developed for conversion of analytical, microscale CZE separations into continuous preparative separation processes realized by FFZE. Practical application of the developed procedure is demonstrated by CZE analysis and FFZE preparation of an octapeptide fragment of human insulin. © 1998 Elsevier Science B.V.

Keywords: Electrophoresis; Preparative electrophoresis; Free-flow zone electrophoresis; Insulin

1. Introduction

Capillary zone electrophoresis (CZE) has developed during the last decade into a powerful analytical technique with a broad application potential [1–6]. Thanks to its high efficiency, sensitivity and speed of analysis it is becoming a recognized complement and/or counterpart of currently the most frequently used separation techniques, different variants of high-performance liquid chromatography (HPLC). The advantages of CZE over HPLC for analytical purposes are apparent: 10–100-times higher separation power achieving 10^5–10^7 theoretical plates, high sensitivity in the picomole–attomole range, short time of analysis (few minutes), low running costs and biocompatible carrierless separation medium suitable for separation of biologically active substances. However, for preparative purposes the application potential of CZE remains relatively limited. This is not only due to the more complicated adaptation of analytical CZE systems to preparative one than in the case of HPLC, but mainly due to the low preparative capacity of the capillary systems.

The more complicated conversion of the usual analytical CZE system to preparative one is caused by the fact that in CZE both ends of the capillary are

*Corresponding author.

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dipped in the electrode vessels and the electric field is applied during the whole time of experiment. This problem has been solved by different ways and some special devices for the fraction collection at the outlet end of the capillary have been developed [7–13].

The second problem, low preparative capacity of CZE, cannot be overcome, since due to the small dimensions of capillary tubes (typical I.D. 0.050–0.100 mm), the preparative capacity of CZE is limited to the nanogram to microgram amounts of substances. These amounts are sufficient only for some special highly sensitive, e.g., enzymatic or immunochemical tests of analytes or for their characterisation by other methods [mass spectrometry (MS), matrix-assisted laser desorption ionization (MALDI), sequence analysis] in biological and biomedical research. The capacity of capillary systems can be only partially increased by enlarging the inner diameter of the capillary up to 0.2–0.4 mm. Further enlarging of the inner diameter leads to the steep fall of separation power due to the loss of the anticonvective capillary effect and much worse Joule heat removal from the separation compartment.

An alternative way to enlarge the preparative capacity of zone electrophoresis (ZE) is to realize this separation principle in the continuous free-flow arrangement in the flow-through electrophoretic chamber, i.e., by free-flow zone electrophoresis (FFZE) [14–18]. The principle of FFZE is as follows: background (carrier) electrolyte (BGE) is laminary flowing between two narrowly spaced parallel plates and sample solution is continuously introduced into the carrier buffer as a narrow zone. The charged sample components are deflected from the straight direction of laminar flow by the d.c. electric field (applied perpendicularly to the direction of the flow) according to their electrophoretic mobilities at different angles and in such a way they are separated. At the outlet side of the chamber the separated sample components dissolved in the BGE are continuously collected in the fraction collector. In this instrumental format the preparative capacity of the separation can be increased up to the scale of tens to hundreds of milligrams per hour.

With respect to the fact that CZE and FFZE are based on the same separation principle, ZE, and both are performed in the carrierless medium with the same composition of the background electrolyte, a direct correlation exists between these two methods [19–21].

The aim of this article is to describe in more detail the theoretical basis of this correlation between CZE and FFZE and to show how this theory can be used in practice, namely for the conversion of analytical CZE separations to continuous free-flow preparative processes.

2. Theory

2.1. Correlation between CZE and FFZE

The diagram of the electrophoretic, electroosmotic and hydrodynamic movements in CZE and FFZE and the vector sum of these migration velocities in the capillary and in the flow-through electrophoretic chamber is shown in Fig. 1.

The resulting migration velocity of a charged component in the d.c. electric field in the capillary, v_r, is given by the sum of electrophoretic velocity, v_{ep}, and electroosmotic flow (EOF) velocity, v_{eo}, (see Fig. 1a):

$$v_r = v_{ep} + v_{eo} \tag{1}$$

The velocities in Eq. (1) can be expressed as the ratio of effective length of the capillary, l_{ef}, and the corresponding migration times, resulting migration time, t_r, electrophoretic migration time, t_{ep}, and the electroosmotic migration time, t_{eo}, respectively:

$$\frac{l_{ef}}{t_r} = \frac{l_{ef}}{t_{ep}} + \frac{l_{ef}}{t_{eo}} \tag{2}$$

From the combination of Eqs. (1) and (2) the following relation can be obtained for the electrophoretic velocity in the capillary:

$$v_{ep,c} = \frac{l_{ef}}{t_{ep}} = \frac{l_{ef}(t_{eo} - t_r)}{t_{eo} t_r} \tag{3}$$

Eq. (3) allows one to obtain the electrophoretic velocity from the data experimentally available from CZE analysis: t_r is resulting migration time of charged analyte which is moved both by electrophoretic movement and by EOF, t_{eo} is the migration time of the EOF marker (electroneutral compound

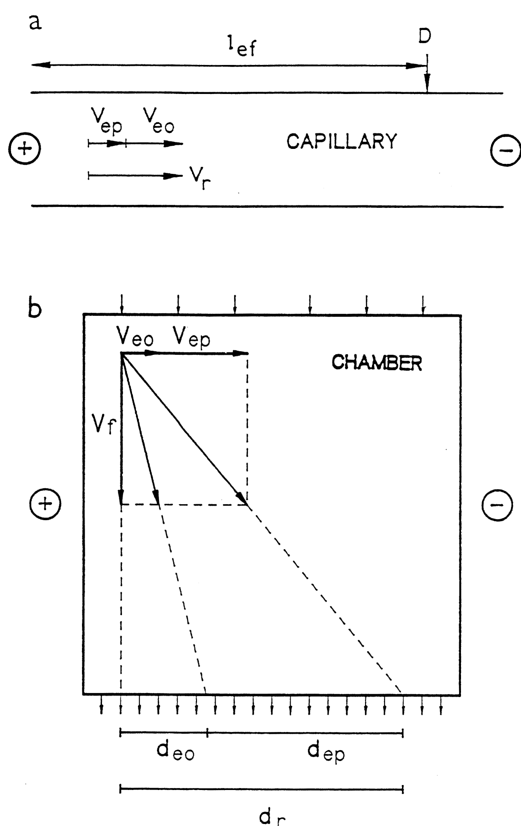

Fig. 1. Superposition of the migration velocities in capillary zone electrophoresis (a) and in free-flow zone electrophoresis (b). v_{ep} = Electrophoretic velocity, v_{eo} = electroosmotic velocity, v_r = resulting migration velocity, l_{ef} = effective length of the capillary, D = detection position, v_f = hydrodynamic flow velocity, d_{ep} = electrophoretic migration distance, d_{eo} = electroosmotic migration distance, d_r = resulting migration distance.

under given experimental conditions) and l_{ef} is the effective length of the capillary (from the injection end to the detector).

The velocity of EOF in the capillary, $v_{eo,c}$ can be calculated from the effective length of the capillary, l_{ef}, and the migration time of the EOF marker, t_{eo}:

$$v_{eo,c} = \frac{l_{ef}}{t_{eo}} \quad (4)$$

The resulting migration velocity of the charged component in the flow-through electrophoretic chamber is given similarly as in the capillary by the sum of electrophoretic velocity v_{ep} and EOF velocity v_{eo}, and in addition to it also by the vector sum of these two velocities with the velocity of hydrodynamic flow, v_f, which is perpendicular to the direction of v_{ep} and v_{eo} (see Fig. 1b).

The resulting migration distance in FFZE can be expressed as a sum of the electrophoretically migrated distance, d_{ep}, and the electroosmotically moved distance, d_{eo},

$$d_r = d_{ep} + d_{eo} \quad (5)$$

The electrophoretically migrated distance, d_{ep}, is given by the product of electrophoretic velocity in the flow-through chamber, $v_{ep,f}$, and the mean flow-through time of the BGE in the chamber, t_f:

$$d_{ep} = v_{ep,f} t_f \quad (6)$$

The electroosmotically migrated distance is given by the product of EOF velocity in the flow-through chamber, $v_{eo,f}$ and mean flow-through time of BGE, t_f.

$$d_{eo} = v_{eo,f} t_f \quad (7)$$

From Eqs. (5) and (6) the following relation for electrophoretic velocity in the chamber, $v_{ep,f}$, can be derived:

$$v_{ep,f} = \frac{d_r - d_{eo}}{t_f} \quad (8)$$

Using Eq. (8) the electrophoretic velocity of the charged analyte in the chamber, $v_{ep,f}$, can be calculated from the experimentally available data, namely from the resulting migration distance of the charged analyte, d_r, from the migration distance of the EOF marker, d_{eo} and from the flow-through time of BGE, t_f.

The EOF velocity in the chamber, $v_{eo,f}$, can be calculated from the migration distance of EOF marker, d_{eo}, and from the flow-through time of BGE, t_f:

$$v_{eo,f} = \frac{d_{eo}}{t_f} \quad (9)$$

For the description of the correlation between CZE and FFZE it is advantageous to express the ratio of electrophoretic velocities in the chamber and in the

capillary, r_{ep}, and the ratio of EOF velocities in the chamber and in the capillary, r_{eo}.

From Eqs. (3) and (8) the ratio r_{ep} can be expressed as

$$r_{ep} = \frac{v_{ep,f}}{v_{ep,c}} = \frac{t_r \cdot t_{eo}(d_r - d_{eo})}{l_{ef} \cdot t_f(t_{eo} - t_r)} \tag{10}$$

and from Eqs. (4) and (9) the ratio r_{eo} can be expressed as

$$r_{eo} = \frac{v_{eo,f}}{v_{eo,c}} = \frac{d_{eo}t_{eo}}{l_{ef}t_{ef}} \tag{11}$$

Provided that the adsorption of the sample components to the walls of the separation compartments (both capillary and flow-through chamber) can be neglected it is reasonable to assume that r_{ep} is approximately constant for different charged components separated by CZE and FFZE under the same separation conditions. This is a realistic assumption, since in fact it means, that if the electrophoretic velocity of component A in FFZE is r-times higher than in CZE, then the electrophoretic velocity of component B, analyzed under the same conditions as A, will be also r-times higher in FFZE than in CZE. Consequently, as follows from Eq. (10), coefficient r_{ep} (determined for standard component S) can be used to predict the electrophoretic velocities of sample components (A, B, C) in FFZE, if their electrophoretic velocities in CZE were determined under the same conditions as the electrophoretic velocity of standard S. Similar conclusion can be applied for the ratio of EOF velocities in CZE and FFZE, i.e., this ratio can be considered as a constant if the conditions of CZE and FFZE are the same as they were in the experiment when EOF velocity was determined.

Knowing the values of ratios, r_{ep} and r_{eo}, allows us to predict the migration velocities and migration distances of analytes in FFZE from the data obtained by their CZE analysis. Just this fact is the core of the procedure for conversion of analytical CZE separations into preparative FFZE separations.

2.2. Conversion of analytical CZE separations to preparative FFZE processes

Based on the above given relations and assumptions a procedure has been developed for the conversion of analytical microscale CZE separations into preparative continuous separation processes realized by FFZE.

The procedure consists of the following steps:

(1) Let us have a sample containing charged analyte A (analyte of our interest from both analytical and preparative point of view) and some charged admixtures (A1, A2....) and noncharged component(s) N (EOF marker). First, suitable conditions for CZE analysis of the given sample of analyte A have to be developed under which a good separation of the analyte A from the charged admixtures A1, A2 and noncharged component(s) N is achieved. From this experiment the electrophoretic velocity of the analyte A in the capillary, $v_{ep,c,A}$, is calculated according to Eq. (3), where $t_r = t_{r,A}$ and $t_{eo} = t_N$; $t_{r,A}$ and t_N are the resulting migration times of analyte A and EOF marker N, respectively.

(2) CZE separation of standard component(s) S (S1, S2...) and of EOF marker N is performed under the same condition as CZE analysis of the sample of analyte A and electrophoretic velocity of the standard components S in the capillary, $v_{ep,c,S}$, is calculated according to Eq. (3), where $t_r = t_{r,S}$ and $t_{eo} = t_N$; $t_{r,S}$ and t_N are the resulting migration times of components S and N, respectively. From the migration time of EOF marker N, t_N, the EOF velocity in the capillary, $v_{eo,c}$, is calculated according to Eq. (4), where $t_{eo} = t_N$.

(3) Standard component(s) S (S1, S2..) and EOF marker N are separated in the ‘‘standard’’ (empirically developed) FFZE regimen with the same BGE as that used in CZE. From this experiment the electrophoretic velocity of standard component S in the flow-through chamber, $v_{ep,f,S}$ is obtained according to Eq. (8), where $d_r = d_{r,S}$ and $d_{eo} = d_N$ ($d_{r,S}$ and d_N are resulting migration distances of components S and N, respectively) and EOF velocity in the chamber, $v_{eo,f}$ is calculated according to Eq. (9), where $d_{eo} = d_N$.

(4) From the results obtained in steps 2 and 3 the ratio, r_{ep}, of electrophoretic velocities of standard component S in the flow-through chamber and in the capillary is determined

$$r_{ep} = v_{ep,f,S}/v_{ep,c,S} \tag{12}$$

and similarly, the ratio of EOF velocities in the chamber and in the capillary, r_{eo}, is obtained:

$$r_{eo} = v_{eo,f,N} / v_{eo,c,N} \quad (13)$$

(5) From the electrophoretic velocity of analyte A (obtained in step 1) and from the coefficients r_{ep} and r_{eo} (obtained in step 4), the electrophoretic velocity of analyte A in FFZE, $v_{ep,f,A}$, and EOF velocity in FFZE chamber, $v_{eo,f}$, are calculated:

$$v_{ep,f,A} = r_{ep} v_{ep,c,A} \quad (14)$$

$$v_{eo,f} = r_{eo} v_{eo,c} \quad (15)$$

Then the predicted resulting migration distance of analyte A in the FFZE chamber, $d_{r,A,pre}$ can be obtained as the sum of electrophoretically moved distance, $d_{ep,A}$, and electroosmotically moved distance, d_{eo}:

$$d_{r,A,pre} = d_{ep,A} + d_{eo} = (v_{ep,f,A} + v_{eo,f}) t_f \quad (16)$$

(6) The resulting migration distances can be calculated also for the other components of the sample [admixtures A1, A2 and neutral component(s) N] and their separability in FFZE can be estimated. If the distances of the components of interest at the outlet side of the chamber are sufficient for their separation, then the FFZE separation can be performed under the same conditions as those used for separation of standard components.

If the predicted distances are not sufficient for the separation of sample components of interest, then the separation conditions of FFZE, namely clamp voltage and/or flow-through time, have to be further optimized. If the predicted distances are too small and the separation of sample components is not achieved then the voltage and/or flow-through time should be increased. If the predicted distances for the sample components are too long and there is a danger that the fastest component will reach the close vicinity of the ion-exchange membrane separating the separation chamber from the electrode compartment, where this component can be damaged or lost because of concentration, pH and conductivity nonhomogeneities occurring in this region, then the clamp voltage and/or flow-through time must be decreased. The migrated distance is approximately directly proportional to the voltage and to the flow-through time, i.e., p% prolongation of migration time and r% increasing of voltage will result in $(p+r)$% prolongation of migrated distance. Following this rule the suitable separation conditions can be selected.

The above described procedure allows to develop the suitable separation conditions in the more economical and faster microscale by CZE and only then the optimized conditions can be converted into the preparative scale realized by continuous FFZE separation.

3. Experimental

3.1. Instrumentation and methods

CZE was performed on the experimental device developed in our Institute [19]. It consists of the untreated fused-silica capillary with outer polyamide coating [310 mm (effective length 200 mm)×0.056 mm I.D.×0.200 mm O.D.], UV-photometric detector at 206 nm and high-voltage power supply. Acetic acid (0.5 mol/l, pH 2.6) was used as the BGE. Peptide samples and EOF marker (phenol) were dissolved in this BGE in the concentration range 0.1–0.8 mg/ml. The sample was introduced into the capillary manually forming a hydrostatic pressure (50 mm of water column) for the time period 5–20 s. High-voltage power supply was utilized in constant voltage mode (10.0 kV, 11–12 μA). Experiments were performed at ambient temperature 22–24°C without active cooling of the separation compartment.

FFZE experiments were performed in the apparatus developed in our Institute [22]. The core of this system is a flow-through electrophoretic chamber consisting of two parallel glass plates (500×500 mm) with a 0.5 mm gap between them. The BGE is introduced through six inlets by six-piston pump with a flow-through time 31 min. Sample solution was introduced by a peristaltic pump with a flow-rate of 1.5 ml/h. The effective length of the separation trajectory (from the sample inlet to the chamber outlet) was 440 mm. Both sides of the chamber were cooled by the air to the temperature −1°C. Separations were performed in the constant voltage regimen (3000 V, 122–125 mA). At the outlet side of the chamber the carrier electrolyte and sample components were collected in 48 fractions and periodically sucked-off into the fraction

collector. The fractions were evaluated by off-line UV-absorption measurement at 280 nm.

3.2. Chemicals

All chemicals were of analytical reagent grade. Diglycine and triglycine were obtained from Reanal (Budapest, Hungary), phenol and acetic acid were purchased from Lachema (Brno, Czech Republic), acetic acid was distilled before use. Octapeptide fragments of the B-chain (positions 23–30) of human insulin (HI), B23-30-HI, and *N*-Phenylacetyl derivative of this fragment *N*-Pac-B23-30-HI were synthesized in our Institute.

4. Results and discussion

The developed theory of the correlation between CZE and FFZE and the procedure for the conversion of analytical CZE separation to preparative FFZE process was applied to analysis and preparation of the synthetic derivative of octapeptide fragment of HI which is used for the development of new analogs of HI with higher stability and/or prolonged activity. The fragment originates from the active site of insuline molecule (C-terminal octapeptide of the B-chain of insulin, indicated as B23-30-HI) and has the following sequence: H–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Thr–OH. The derivative has a phenylacetyl (Pac) group attached to the *N*-terminal amino group of the octapeptide (Pac–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Thr–OH) and is indicated as *N*-Pac-B23-30-HI. The peptide was synthesized in our Institute by the usual Merrifield solid-phase method [23].

The analysis of the crude synthetic product of *N*-Pac-B23-30-HI by CZE is shown in Fig. 2a and the analysis of HPLC purified product is shown in Fig. 2b. In addition to the main synthetic product *N*-Pac-B23-30-HI (peak 2) in both preparation there is another charged component present (peak 1) which was identified as octapeptide with the sequence identical with B23-30-HI, but with cleaved Pac group. This is in agreement with the fact that cleavage of Pac group from the *N*-terminal amino group of the peptide will result in the increased effective charge (mobility) of the peptide and shorter migration time in CZE analysis. In addition to the charged admixture the sample contains also a small amount of noncharged component N which can serve as an EOF marker. Since even after HPLC purification the charged admixture (peak 1) was not completely removed (see Fig. 2b), it was decided to use FFZE for preparative separation of the crude product components. As follows from the above theory of the correlation between CZE and FFZE for the conversion of analytical CZE to preparative FFZE it is necessary to have not only the data from CZE analysis of the given sample but also to know the ratios r_{ep}, r_{eo} of electrophoretic and electroosmotic velocities in the FFZE chamber and in the capillary. The values of these coefficients were obtained from CZE and FFZE separation of the standard mixture of diglycine, triglycine and phenol by CZE and FFZE using the same background electrolyte, 0.5 mol/l acetic acid, in which successful separation of Pac-B23-30-HI and B23-30-HI was achieved. The data of CZE and FFZE separation of the standard mixture and the calculated values of electrophoretic and electroosmotic velocities and the coefficients r_{ep}, r_{eo} are presented in Table 1. From the migration times of the component 1 (B23-30-HI), and component 2 (Pac-B23-30-HI) and from the migration time of electroneutral component N (see Fig. 2a Table 1) the electrophoretic and electroosmotic velocities of these sample components in the capillary, $v_{ep,c}$ and $v_{eo,c}$ were calculated according to Eqs. (3) and (4), respectively (see the CZE columns in Table 2). From these velocities of the sample components in the capillary and using the values of coefficients r_{ep}, r_{eo} (see Table 1) the electrophoretic velocities, $v_{ep,f}$, and the predicted migration distances, $d_{r,pre}$, of the sample components 1, 2 and N in FFZE chamber were calculated (see FFZE columns in Table 2). These predicted migration distances (20–126 mm) and namely the differences of these distances between individual sample components at the outlet side of the chamber indicated that a sufficient separation will be achieved in the same FFZE regimen which was used for the separation of standard mixture.

For this reason, the conditions of FFZE, under which the standard mixture of diglycine, triglycine and phenol was separated (clamp voltage 3000 V, flow-through time 31 min), were applied also to the

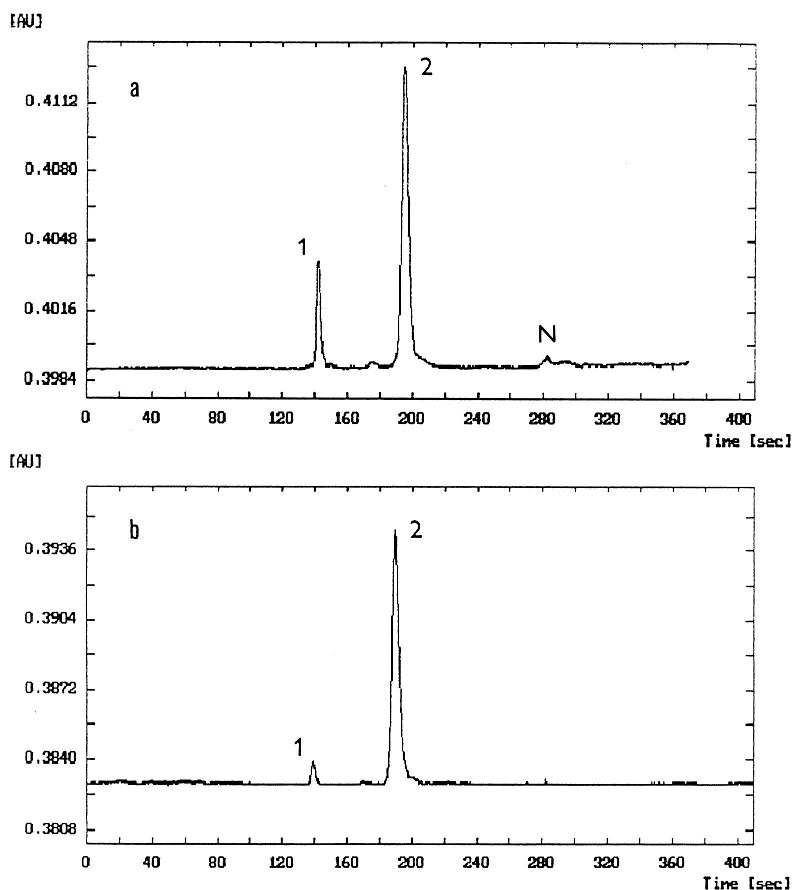

Fig. 2. CZE analysis of the octapeptide fragment of human insulin, *N*-Pac-B23-30-HI (0.8 mg/ml) (a) crude synthetic product, (b) product purified by HPLC. 1 = B23-30-HI, 2 = Pac-B23-30-HI, N = noncharged component(s), AU = absorbance at 206 nm.

Table 1
Migration times, migration distances, electrophoretic and electroosmotic velocities of standard components separated by CZE and FFZE

Standard component	CZE			FFZE			FFZE/CZE	
	t_r (s)	$v_{ep,c}$ (mm/s)	$v_{eo,c}$ (mm/s)	d_r (mm)	$v_{ep,f}$ (mm/s)	$v_{eo,f}$ (mm/s)	r_{ep}	r_{eo}
Diglycine	121.8	0.952	–	160	0.0753	–	0.0791	–
Triglycine	133.2	0.811	–	140	0.0645	–	0.0795	–
Phenol	289.8[a]	0	0.690	20[a]	0	0.0108	–	0.0156

[a] Resulting migration time, t_r, (migration distance, d_r) of phenol is equal to migration time, t_{eo}, (migration distance, d_{eo}) of electroneutral EOF marker.

t_r = Resulting migration time, $v_{ep,c}$ = electrophoretic velocity in CZE, $v_{eo,c}$ = electroosmotic velocity in CZE, d_r = resulting migration distance, $v_{ep,f}$ = electrophoretic velocity in FFZE, $v_{eo,f}$ = electrophoretic velocity in FFZE, r_{ep} (r_{eo}) = ratio of electrophoretic (electroosmotic) velocities in FFZE and CZE.

Table 2
Migration times, electrophoretic velocities and predicted and experimental migration distances of the sample components of CZE and FFZE separation of the crude product of octapeptide fragment of human insulin (*N*-Pac-B23-30-HI)

Sample component	CZE			FFZE			
	t_r (s)	$v_{ep,c}$ (mm/s)	$v_{eo,c}$ (mm/s)	$v_{ep,f}$ (mm/s)	$v_{eo,f}$ (mm/s)	$d_{r,pre}$ (mm)	$d_{r,exp}$ (mm)
1: B23-30-HI	141.0	0.711	–	0.0539	–	126	150
2: Pac-B23-30-HI	193.8	0.325	–	0.0240	–	68	75
N: electroneutral	282.8[a]	0	0.707	0	0.0110	20	15

[a] Resulting migration time, t_r, of electroneutral component N is equal to migration time, t_{eo}, of EOF marker.
t_r = Resulting migration time, $v_{ep,c}$ = electrophoretic velocity in CZE, $v_{eo,c}$ = electroosmotic velocity in CZE, $v_{ep,f}$ = electrophoretic velocity in FFZE, $v_{eo,f}$ = electrophoretic velocity in FFZE, $d_{r,pre}$ = predicted migration distance for FFZE, $d_{r,exp}$ = experimental migration distance in FFZE.

separation of the components of the crude product of Pac-B23-30-HI. The lyophilizate of the crude synthetic product of *N*-Pac-B23-30-HI (280 mg) was dissolved in 7 ml of BGE (0.5 mol/l acetic acid), centrifuged and applied to FFZE separation. The record of FFZE separation is shown in Fig. 3. Comparing Figs. 2 and 3, the "qualitative" similarity of separation profiles of CZE and FFZE can be observed. In addition to the main synthetic product (Pac-B23-30-HI, peak 2), the peak of the faster component (B23-30-HI, peak 1) and the peak of uncharged component N can be found on both records. The differences in relative peak heights are caused by the different detection wavelengths (206 nm in CZE and 280 nm in FFZE). Obviously, a better separation of sample components is achieved in CZE than in FFZE. The higher separation power of CZE than that of FFZE is given by the following differences in the experimental conditions of these two methods: more efficient anticonvective stabilization and Joule heat transfer in the capillary (I.D. 0.050 mm, wall thickness 0.07 mm) than in the flow-through chamber (gap between plates 0.5 mm, wall thickness 4 mm), one order lower separation time and sample concentration in CZE than in FFZE, absence of hydrodynamic flow in CZE.

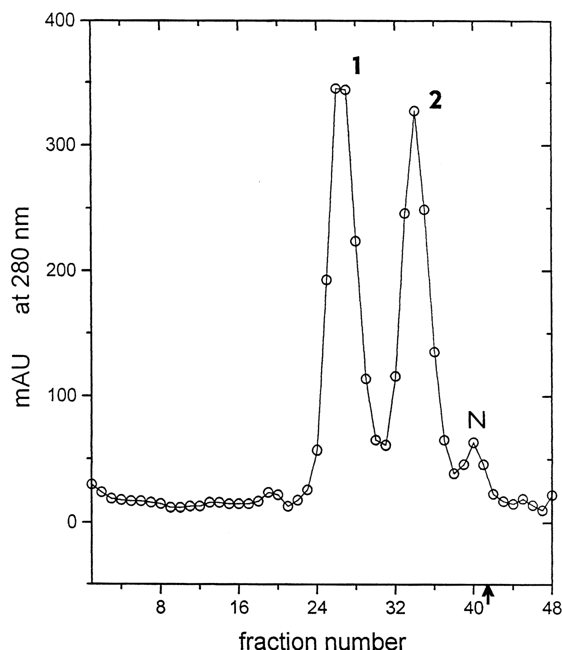

Fig. 3. FFZE separation of the crude synthetic product of the octapeptide fragment of human insulin, *N*-Pac-B23-30-HI (40 mg/ml). 1 = B23-30-HI, 2 = Pac-B23-30-HI, N = noncharged component(s), mAU = absorbance at 280 nm, ↑ = coordinate of the sample inlet in the chamber.

The UV record of FFZE separation in Fig. 3 shows relatively good separation of all three sample components, but this pattern gives only an approximate estimation about the peptide purity in individual fractions. For this reason all fractions with UV-positive absorption were analyzed by CZE. The complete set of CZE analyses of FFZE fractions showed that the main synthetic product (peak 1) is completely free from the faster component 2 and from the noncharged component(s) N. Fractions 33–36 contained pure main synthetic product (Pac-B23-30-HI) and fractions 25–29 contained pure component 1 (B23-30-HI) as demonstrated by single peaks in the CZE analyses of the lyophilizate of the pooled fractions 33–36 and 25–29, respectively (see

Fig. 4a and 4b). The high purity degree of both synthetic products was confirmed also by other methods, particularly HPLC and amino acid analysis. The advantage of utilization of acetic acid as BGE is, that peptide is obtained in acetate, i.e., physiologically tolerable form, that can be directly applied to biological tests.

The relatively good agreement between the predicted migration distances and the experimentally found distances of the sample components (see Fig. 3 Table 2) confirms the quantitative correlation between CZE and FFZE. The discrepancy between the predicted and experimental values is probably caused by the differences in partial adsorption of sample components to the fused-silica capillary wall and to the glass wall of the flow-through electrophoretic chamber.

5. Conclusions

This paper shows the usefulness of combination of CZE and FFZE to the analysis and preparation of synthetic peptides and/or other analytes. First, CZE is used for analysis of the peptide preparation and suitable conditions for its analysis are developed in a microscale minimizing time and material expenses for the development of the optimized separation conditions. Then, based on the theory of the correlation between CZE and FFZE, the optimized con-

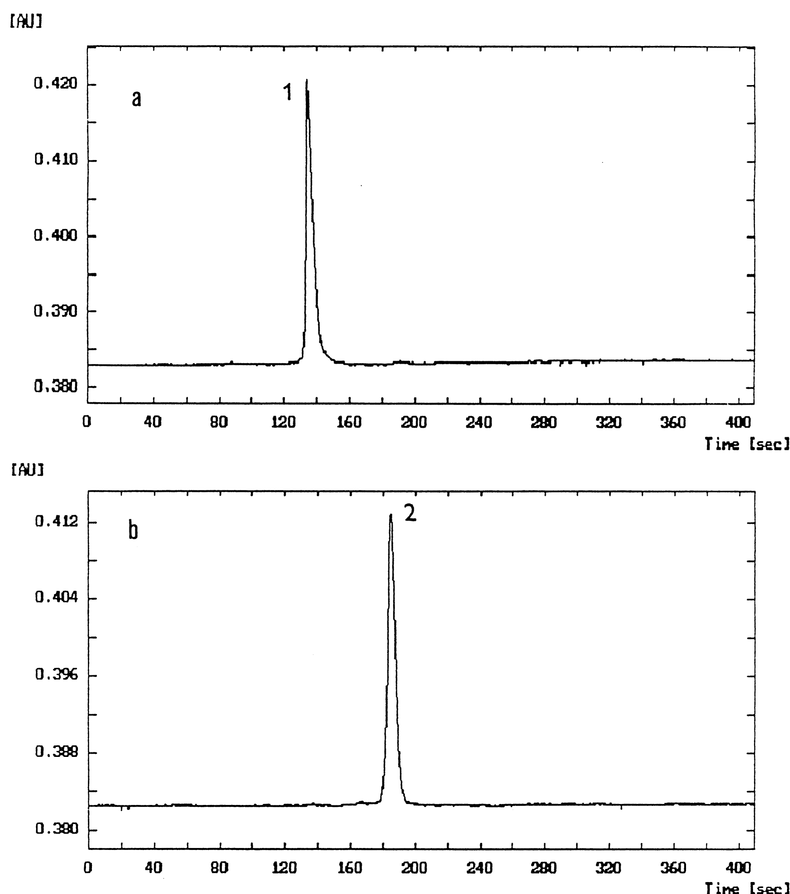

Fig. 4. CZE analysis of the fractions of the FFZE separation of human insulin fragments presented in Fig. 3. (a) Lyophilisate of the pooled fractions 25–29 (peak 1) of FFZE separation, (b) lyophilisate of the pooled fractions 33–36 (peak 2) of FFZE separation. 1 = B23-30-HI, 2 = Pac-B23-30-HI, AU = absorbance at 206 nm.

ditions of CZE separation are converted to FFZE separation. FFZE separation of peptide preparation provides an efficient tool for preparative purification of peptide sample with the preparative capacity of tens to hundreds of milligrams per hour. The advantage of this method is that it works continuously, in a carrierless medium, in mild conditions under which the biological activity of separated peptides is retained and the loss of material is minimized. The purity of peptide fractions separated by FFZE can be then checked by CZE and/or other methods. Combination of more methods based on different separation principles should be preferred since in such a way more complete information about the peptide and its purity can be obtained.

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References

[1] R.L. St. Claire, Anal. Chem. 68 (1996) 569R.
[2] S. Hjerten, Methods Enzymol. 270 (1996) 296.
[3] P. Camilleri, J. Chem. Soc., Chem. Commun. 16 (1996) 1851.
[4] K.D. Altria, Capillary Electrophoresis Guidebook, Principles, Instrumentation, Operation and Applications, Humana Press, Totowa, 1995.
[5] P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1995.
[6] V. Kašička and Z. Prusík, in A. Townshend et al. (Editors), Encyclopedia of Analytical Sciences, Academic Press London, 1995, p. 1096.
[7] A. Guttman, A.S. Cohen, D.N. Heiger, B.L. Karger, Anal. Chem. 62 (1990) 137.
[8] N.A. Guzman, M.A. Trebilcock, J.P. Advis, Anal. Chim. Acta 249 (1991) 247.
[9] N. Banke, K. Hansen, I. Diers, J. Chromatogr. 559 (1991) 325.
[10] Y.F. Cheng, M. Fuchs, D. Andrews, W. Carson, J. Chromatogr. 608 (1992) 109.
[11] A. Cifuentes, X. Xu, W.T. Kok, H. Poppe, J. Chromatogr. A 716 (1995) 141.
[12] F. Foret, O. Muller, J. Thorne, W. Gotzinger, B.L. Karger, J. Chromatogr. A 716 (1995) 157.
[13] H.F. Yin, C. Keely-Templin, D. McManigill, J. Chromatogr. A 744 (1996) 45.
[14] Z. Prusík, in Z. Deyl (Editor), Electrophoresis, Part A: Techniques, Elsevier, Amsterdam, 1979, p. 229.
[15] H. Wagner, V. Mang, R. Kessler and W. Speer, in C.J. Holloway (Editor), Analytical and Preparative Isotachophoresis, Walter de Gruyter, Berlin, 1984, p. 347.
[16] K. Hannig and H.K. Heidrich, Free-Flow Electrophoresis, GIT, Darmstadt, 1989.
[17] H. Wagner and J. Heinrich, in H. Tschesche (Editor), Modern Methods in Protein and Nucleic Acid Research, Walter de Gruyter, Berlin, 1990, p. 69.
[18] M.C. Roman, P.R. Brown, Anal. Chem. 66 (1994) 86A.
[19] Z. Prusík, V. Kašička, P. Mudra, J. Štěpánek, O. Smékal, J. Hlaváček, Electrophoresis 11 (1990) 932.
[20] V. Kašička, Z. Prusík, J. Pospíšek, J. Chromatogr. 608 (1992) 13.
[21] V. Kašička, Z. Prusík, O. Smékal, J. Hlaváček, T. Barth, G. Weber, H. Wagner, J. Chromatogr. B 656 (1994) 99.
[22] Z. Prusík, J. Chromatogr. 91 (1974) 867.
[23] J. Ježek, J. Velek, J. Jiráček and T. Barth, manuscript in preparation.