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Improved sensitivity by on-line isotachophoretic preconcentration in the capillary zone electrophoretic determination of peptide-like solutes☆

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Abstract

Isotachophoresis (ITP) was studied as an on-line preconcentration technique in combination with capillary zone electrophoresis for the determination of positively charged peptide-like solutes. This paper shows the effects of different electrolyte compositions, different ITP times and various injection volumes on the time necessary for stacking and destacking. Injection volumes as large as 1.4 μl resulted in a resolution between the test peptides similar to that of pure capillary zone electrophoresis. Calibration graphs were linear in a range from 15 μmol/l down to 30 nmol/l after injections of 1.4 μl. Initial studies of plasma extracts appeared promising, although in the long term the overall efficiency decreased.

1. Introduction

Capillary electrophoresis (CE) is a rapidly developing separation technique with a high separation power [1]. Recently a review of quantitative aspects of CE with regard to the analysis of pharmaceuticals was published [2]. However, CE is still considered insensitive in terms of concentration when compared with the more established method of high-performance liquid chromatography (HPLC). This is one of the major limitations with regard to the applicability of CE for bioanalytical purposes. The insensitivity is largely caused by the small volume of the detection cell used in CE and the small injection volume, nanolitres compared with microlitres in HPLC, which can be applied without sacrificing the separation efficiency [3–5]. It should be noted that when measured in terms of mass quantified, CE is capable of extremely low detection limits, as much as 2–3 times better than HPLC [6].

To improve the sensitivity on the detector side, different approaches can be followed. One of them is based on laser-induced fluorescence (LIF) detection [7]. Another approach was the development of a Z-shaped flow cell for UV detection [8,9]. Since Olivares et al. [10] published the first paper in 1987 on the coupling of CE with mass spectrometry (MS), much work has been done to make this detection technique applicable for CE [11–13].

Apart from optimization on the detector side,

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a highly efficient way to improve the sensitivity of CE is the injection of larger volumes in combination with on-line preconcentration techniques [14]. One of the advantages of on-line versus off-line preconcentration techniques is that the complete process can be easily automated. Different electrophoretic analyte focusing techniques can be used to increase the sample volume in CE. These are based on local differences in the electrical field strength to permit stacking of the analyte ions [15,16].

Field-amplified injection is a promising approach for preconcentration where the analyte is dissolved in a sample matrix, usually water, which has a lower conductivity than the background electrolyte. Owing to this difference in conductivity, stacking takes place at the boundary between the injected sample and the background electrolyte [17].

Isotachophoresis (ITP) is another stacking technique which can be used on-line with CE [15,16,18–21]. ITP is carried out in a discontinuous buffer system. The analytes are injected between a leading electrolyte (higher mobility than the analytes) and a terminating electrolyte (lower mobility). When a voltage drop is applied over the capillary, a steady-state migration configuration will be established in which the analytes migrate as consecutive zones which are not diluted by the background electrolyte as in capillary zone electrophoresis (CZE). During ITP, the concentration of the analyte zones will adapt to the higher concentration of the leading ion [22] and therefore stacking of the analytes will occur. As ITP results in consecutive zones of analytes, on-line ITP can be used as a preconcentration stacking technique before destacking and CZE separation take place.

In this work, the on-line combination of ITP with CZE in a single capillary was investigated for the determination of peptide-like solutes. The experiments were based on a combined ITP–CZE system initially described by Foret et al. [16], which was also studied for the determination of positively charged solutes with injection volumes up to 700 nl [23]. The effects of different electrolyte compositions, ITP times and injection volumes on the time necessary for stacking and destacking are described. Theoretical models for different ITP–CZE systems have been presented, which show that both stacking and destacking can be controlled [24,25]. One of these models [25] is compared with results obtained in practice.

The present study demonstrates that injection volumes as large as 1.4 μl result in a resolution similar to that with small sample volumes in CZE, and that even larger injection volumes can be analysed by using recently published techniques [26,27] based on back-pressure. Preliminary results show that the combination of ITP–CZE with a back-pressure system can be used for bioanalytical work in the analysis of plasma extracts obtained through solid-phase extraction.

2. Experimental

2.1. Equipment

Experiments were performed with a PRINCE programmable injector for CE equipped with a high-voltage power supply (Lauerlabs, Emmen, Netherlands). Injections were made hydrodynamically and the volume was calculated according to Poiseuille with viscosities taken from the literature [28]. For detection a Spectra 100 UV detector (Spectra-Physics, San Jose, CA, USA) with an on-column cell (Linear Instruments, Reno, NV, USA) was used at 216 nm. For integration an SP-4270 integrator (Spectra-Physics) was used. The I.D. of the fused-silica capillary was 100 μm and the effective and total lengths were 57.5 and 72.5 cm, respectively (Polymicro Technologies, Phoenix, AZ, USA) unless stated otherwise. The capillary was thermostated by means of a forced air flow at 30°C unless other temperatures are indicated. To prevent electroosmotic flow, the capillary was coated with linear polyacrylamide [29], which also hindered the adsorption of the peptides on the capillary wall.

2.2. Chemicals

Ultra-high-purity grade water (18 MΩ cm resistivity) was obtained from an ELGA (High Wycombe, UK) purification system. Ammonium

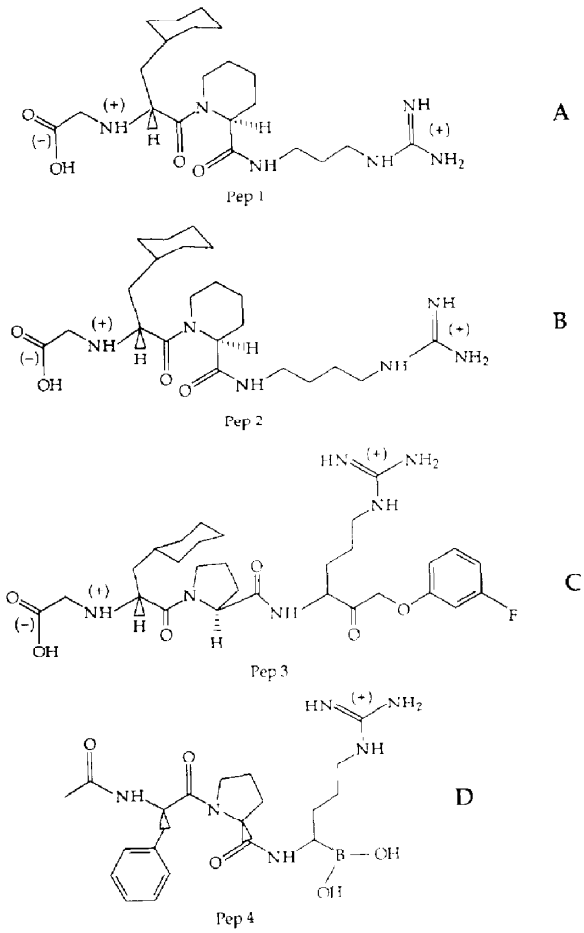

Fig. 1. Structures of test substances. (A) Pep1; (B) Pep2; (C) Pep3; (D) Pep4; (+) and (−) denote the actual charge at pH 3.6. The net charge is +1.

acetate (MicroSelect grade) and 6-aminocaproic acid (EACA) (puriss) were purchased from Fluka (Buchs, Switzerland). Acrylamide, ammonium peroxodisulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED), used for the preparation of the coating, were obtained from Bio-Rad (Richmond, CA, USA). 3-Methacryloxypropyltrimethoxysilane, used to silylate the capillary before coating, was obtained from ABCR (Karlsruhe, Germany). Glacial acetic acid (HAc) was purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) from Rathburn (Walkerburn, UK).

Fig. 1 shows the structures of the peptide-like test substances. Two of the standard solutes (Pep1 and Pep2) were supplied by the Department of Medicinal Chemistry at Astra Hässle (Mölndal, Sweden). Pep3 was synthesized at

Table 2
pK_a values together with the absolute and effective mobilities (μ) of the ions present in the electrolytes at pH 3.6

Electrolyte	pK_a	Absolute μ ($m^2/V \cdot s$)	Effective μ ($m^2/V \cdot s$)
LE[a]	9.2 [31]	$72 \cdot 10^{-9}$ [31]	$72 \cdot 10^{-9}$
TE[a]	4.7 [23]	$362 \cdot 10^{-9}$ [23]	$7 \cdot 10^{-9}$
BGE[a]	4.3 [32]	$30 \cdot 10^{-9}$ [32]	$28 \cdot 10^{-9}$

[a] See Table 1.

Table 3
Effective mobilities for Pep 1–4

Analyte	Voltage (kV)	Effective mobility (10^{-9} $m^2/V \cdot s$)
Pep1	20	14.4
	30	21.3
Pep2	20	13.9
	30	20.7
Pep3	20	15.0
	30	19.7
Pep4	20	17.0
	30	22.2

Capillary bore, 100 μm; effective length, 57.5 cm; total length, 72.5 cm; electrolyte, BGE; temperature, 30°C for Pep1 and Pep2, 35°C for Pep3 and Pep4.

Table 1
Composition of electrolyte solutions

Electrolyte	Composition
Leading electrolyte (LE)	10 mmol/l ammonium acetate (NH_4Ac), pH 3.6[a]
Terminating electrolyte (TE)	50 mmol/l acetic acid (HAc), pH 3.1
Background electrolyte (BGE)	20 mmol/l 6-aminocaproic acid (EACA), pH 3.6[a]

[a] The BGE and LE were adjusted to pH 3.6 with acetic acid.

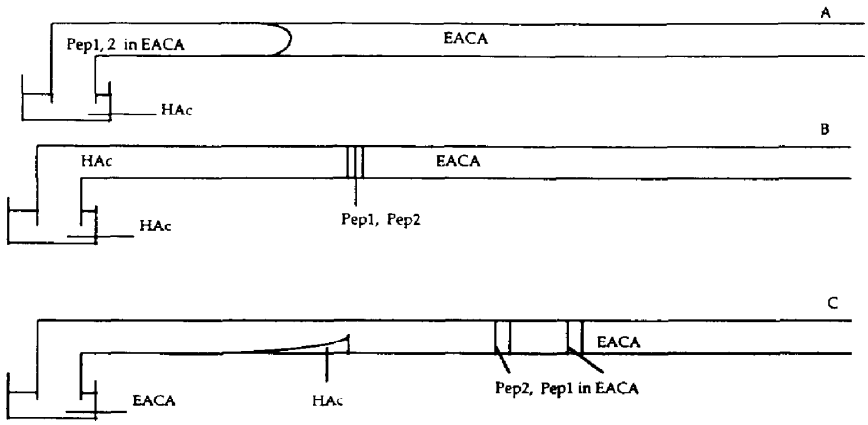

Fig. 2. ITP–CZE system with HAc as a terminating electrolyte and EACA as a background and leading electrolyte. (A) Start of ITP; (B) stacking complete; (C) controlled desacking followed by CZE.

Ferring Research Institute (Chilworth, Southampton, UK) [30] and Pep4 (DuP 714) at BACHEM Feinchemikalien (Bubendorf, Switzerland). It should be mentioned that the UV absorbances of Pep1 and Pep2 are lower than those for Pep3 and Pep4.

Tables 1 and 2 show the compositions of the electrolytes studied. Acetate was used as the counter ion and has an absolute mobility of $-42 \cdot 10^{-9}$ $m^2/V \cdot s$ [22].

2.3. Calibration

A calibration graph for Pep3 with Pep4 (1.5 μmol/l) as an internal standard was obtained by injections of 1.4 μl into a capillary with an I.D.

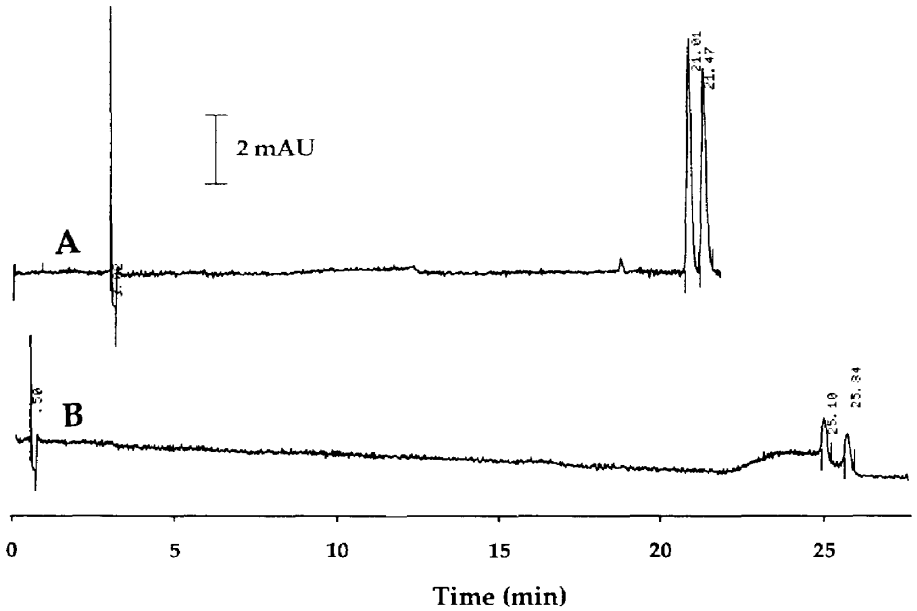

Fig. 3. Electropherograms obtained with HAc as a terminating electrolyte. Injection volume, 636 nl. ITP for (A) 3 and (B) 0.5 min; CE at 30 μA. The UV trace starts at the start of the ITP. ITP–CZE system as in Fig. 2.

of 100 μm and effective and total lengths of 47 and 64 cm, respectively. The studied samples contained Pep3 in a concentration range from 15 μmol/l down to 30 nmol/l. This injection volume results in a sample plug length of 18 cm. Three repetitive injections were made at each concentration level. The combined system, with HAc as terminating ion and NH_4^+ as leading ion, was used and ITP was initiated for 2 min at a constant current of 30 μA with the capillary end placed in a vial with terminating electrolyte at the anode side. The CZE separation took place at a constant voltage of 20 kV.

3. Results and discussion

3.1. Capillary zone electrophoresis

The electrophoretic behaviour of Pep1, Pep2, Pep3 and Pep4 in zone electrophoresis was studied with 18 mmol/l EACA buffer as background electrolyte. The differences in mobility between Pep1–4 are small, as shown in Table 3 and the differences observed between 20 or 30 kV might be due to heating of the capillary at the higher voltage. A voltage difference of 30 kV generated a current of approximately 60 μA and the total power generated was almost 2 W. This may result in heating in relatively wide-bore capillaries (100 μm) which are only cooled by an air-driven system such as that in our system. This results in a lower viscosity of the electrolyte and a higher mobility of the analytes. However, in general it also results in a higher diffusion and therefore a decrease in overall efficiency [31,32]. The decrease in efficiency is the main reason to avoid heating of the capillary. The effect of different injection volumes on the resolution of Pep1 and Pep2 was studied and showed that an increase in injection volume from 3.6 to 18 nl results in a decrease in resolution from 1.5 to almost 1.1.

3.2. Acetic acid as terminating electrolyte

Pep1 and Pep2 were dissolved in an aqueous solution of EACA, which was the background electrolyte (BGE) in these experiments. The steps involved in the ITP–CZE sequence are depicted in Fig. 2. The main advantage of this system is that the time needed to collect the zone easily can be calculated according to Eq. 1 as proposed in literature [25]. The major disadvantage is that the vial at the inlet side of the capillary has to be replaced during the run, which prolongs the run time.

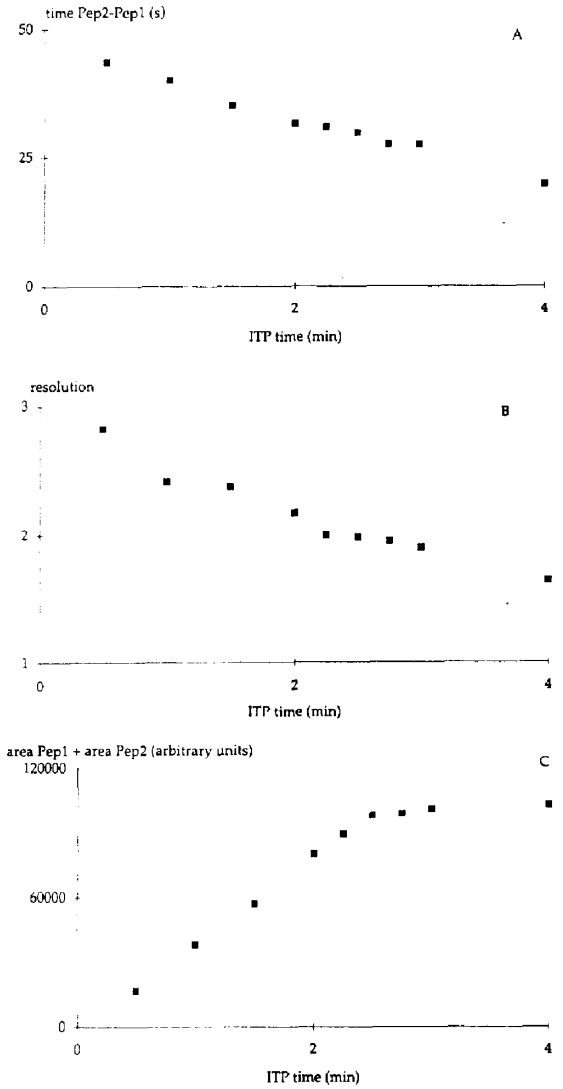

Fig. 4. Effect of different ITP times at a constant current of 30 μA on (A) difference in migration time between Pep1 and Pep2, (B) resolution between Pep1 and Pep2 and (C) total peak area. Injection volume, 636 nl of 7.5 μmol/l of Pep1 and Pep2. ITP–CZE system as in Fig. 2.

The fact that time and place of stacking and destacking need to be controlled can be seen in the Figs. 3 and 4. If destacking is initiated too early the whole plug will not be collected (Fig. 3B). If the destacking is too late the separation of the linked zones, which takes place during the CZE, is not complete. Fig. 4A shows that the difference in migration time (Δt) between Pep1 and Pep2 decreases if the stacked zones move further through the capillary. The same can be seen in Fig. 4B with regard to the resolution. The total migration time (ITP + CZE) of the analytes decreased on increasing the ITP time, i.e., the zones moved faster in the ITP mode than in the CZE mode.

The speed of the boundary between the leading and terminating electrolytes was measured at a constant current of 30 μA. The capillary was filled with leading electrolyte with the anode side placed in the terminating electrolyte. From the time it takes for the boundary to reach the detector one can calculate the speed of this boundary. At a fixed current this speed is assumed to remain constant. The observed speed of the zones during ITP was about 6.4 mm/s at a constant current of 30 μA. This means that a plug of 636 nl (8.1 cm) should be collected in about 2 min, but Fig. 4C shows that it takes longer than 2 min for the whole plug to be collected. This long collection path may be due to at least two reasons: the injected plug has a parabolic profile, which makes it longer than 8 cm, or already during the ITP those molecules of Pep1 and Pep2 which have not yet been collected move towards the cathode. For the second reason it is better to use Eq. 1 to calculate the time it takes to collect the whole sample. This equation takes into account the speed of the peptide-like solutes. The calculated time to collect the zone is 3.0 min at a constant current of 30 μA, which is in good agreement with our observations. From this equation, it can also be seen that a leading ion with a higher mobility, e.g., NH_4^+, results in a shorter collection time compared with a leading ion with a lower mobility, e.g., $EACAH^+$.

$$t = F[N_{le}\mu_{le}(1 - \mu_r/\mu_{le})]/I(\mu_{le} - \mu_s) \quad (1)$$

where t = time to collect the injected plug (s); F = Faraday constant, 96 500 C/mol; N_{le} = number of moles of the leading ion present in the injected plug; μ_{le} = absolute mobility of leading ion ($cm^2/V \cdot s$); μ_r = absolute mobility of counter ion ($cm^2/V \cdot s$); μ_s = effective mobility of sample ion ($cm^2/V \cdot s$); I = current (A).

3.3. Ammonium as leading ion

Injections were made of mixtures of Pep1 and Pep2 in 10 mmol/l ammonium acetate in a capillary which contained EACA as a background electrolyte. The advantage of this system

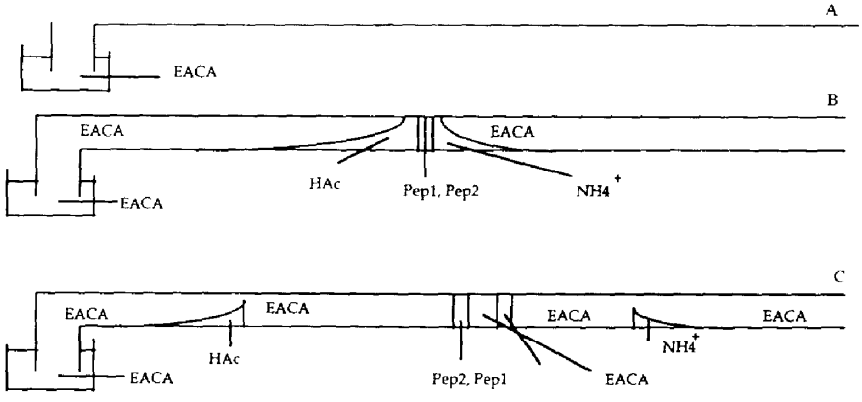

Fig. 5. ITP–CZE system with HAc as a terminating electrolyte, NH_4^+ as a leading electrolyte and EACA as a background electrolyte. (A) Start of ITP; (B) stacking complete; (C) uncontrolled destacking followed by CZE.

is that the inlet vial does not have to be replaced during the run and that the leading ion is fast, making the analysis quicker. The main disadvantage is that it is more complicated to estimate the position of the zone inside the capillary, as Eq. 1 is not valid. The ITP–CZE transition takes place smoothly. The basic steps in this system are outlined in Fig. 5. Fig. 6 shows electropherograms obtained after injections of different volumes. Δt (Fig. 7A) and the resolution (Fig. 7B) decrease at larger volumes because a larger part of the capillary is used for stacking. An increase in the volume injected was accompanied by a decrease in migration time. The separation length (Table 4) was calculated from Δt, assuming that the solute mobilities were constant during CZE. Table 4 also shows that the sum of the length of the injected plug and the calculated capillary length used for separation is smaller than the effective length of the capillary. This means that the destacking takes place after collection of the whole plug and also that the plug length directly influences the place of and time for destacking.

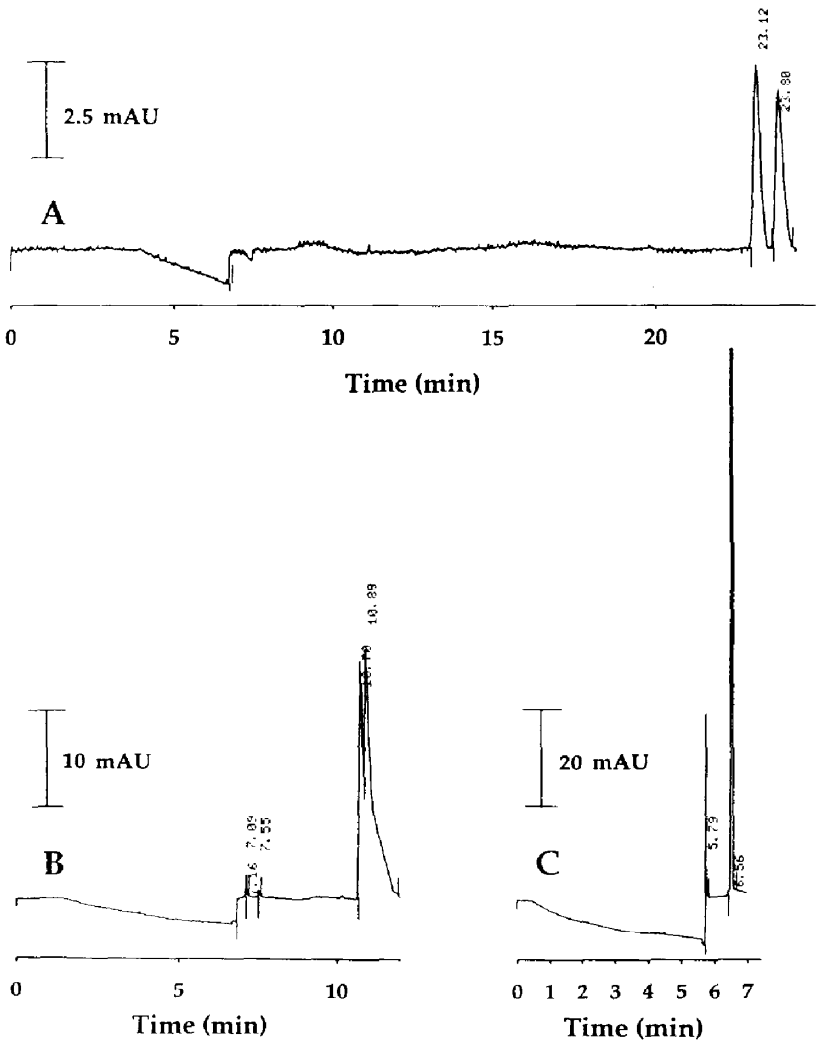

Fig. 6. Electropherograms after injections of (A) 636, (B) 3180 and (C) 4134 nl of a mixture of 7.5 μmol/l of Pep1 and Pep2 dissolved in 10 mmol/l ammonium acetate. ITP–CZE system as in Fig. 5.

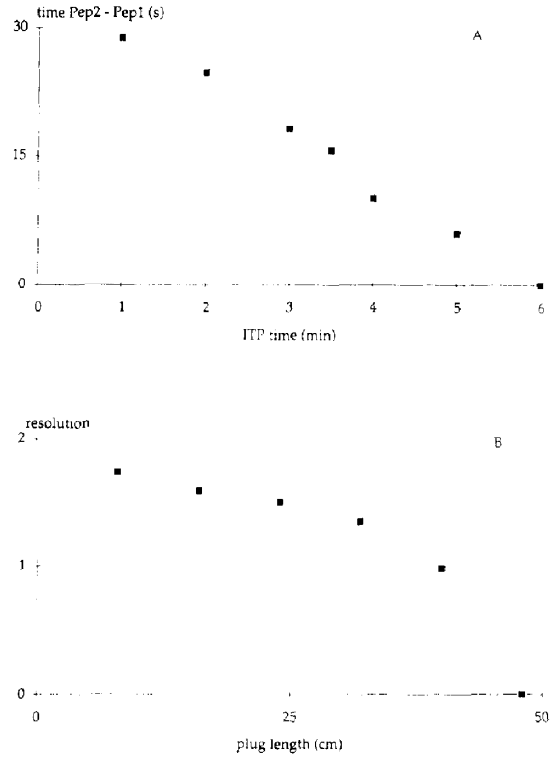

Fig. 7. Effect of different plug lengths (different injection volumes) on (A) difference in migration time between Pep1 and Pep2 and (B) resolution between Pep1 and Pep2. ITP–CZE system as in Fig. 5.

3.4. Combined ammonium acetate–acetic acid system

A system containing the combined electrolytes, as outlined in Fig. 8, would have the benefits from both systems described above. If NH_4^+ and HAc are used the system will be fast owing to the presence of a leading ion with a high mobility. In a combined system a volume of 636 nl will take 0.7 min to be collected instead of almost 3 min in the system without NH_4^+ owing to the faster moving leading ion present in the injected plug (Eq. 1). Owing to the presence of acetic acid, the concentrated zone will be easy to locate inside the capillary, as the ITP–CZE transition will not take place as long as HAc is present at the anode side. During zone electrophoretic separation the analytes will move through the capillary with EACA as a background electrolyte, which has a mobility much closer to that of the analytes compared with NH_4^+. With regard to peak shape and efficiency, it is favourable to choose a background electrolyte having a mobility close to that of the analytes of interest [33]. Still the main disadvantage of this combined system will be that the inlet vial has to be changed during the run. Fig.

Table 4
Calculated length left for separation at different injection volumes

Injection volume (nl)	Plug length[a] (cm)	Migration time (min)		Δt[b] (s)	CZE length[c] (cm)	Calculated length[d] (cm)
		Pep1	Pep2			
636	8	23.06	23.72	39.6	54	62
1272	16	17.58	18.00	25.2	35	51
1908	24	15.09	15.40	18.6	25	49
2544	32	12.61	12.84	13.8	19	51
3180	40	9.89	10.01	7.2	10	50
3816	49	7.36	7.39	1.8	2	51

Constant total amount of 4.8 pmol of each of Pep1 and Pep2; electrolyte system as in Fig. 5.
[a] Length of the injected plug.
[b] Δt = time(Pep2) − time(Pep1).
[c] The length used for separation during CZE after ITP, calculated from $57.5\Delta t/\Delta t_{CZE}$, assuming a constant selectivity. Δt_{CZE} = time(Pep2) − time(Pep1) measured in a CZE separation on a capillary with effective length of 57.5 cm (Δt_{CZE} = 42 s).
[d] Plug length + CZE length; effective capillary length = 57.5 cm.

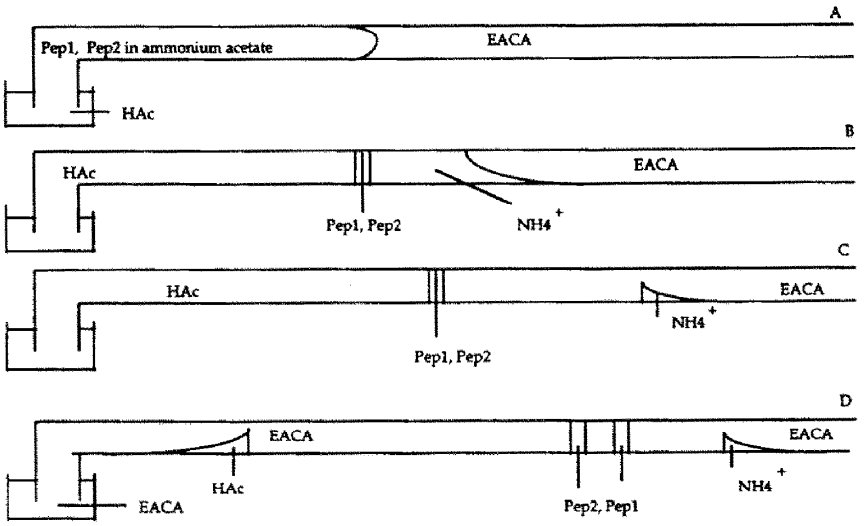

Fig. 8. ITP–CZE system with HAc as a terminating electrolyte, NH_4^+ as a leading electrolyte (LE) and EACA as a background electrolyte (BGE). (A) Start of the ITP; (B) stacking complete; (C) stacking continues; fast LE moves into slower BGE; (D) controlled destacking followed by CZE.

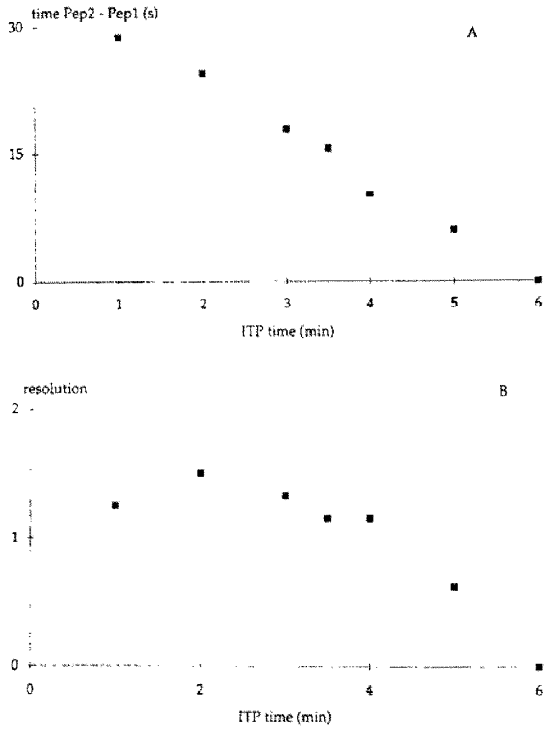

Fig. 9. Effect of different ITP times at a constant current of 30 μA on (A) difference in migration time between Pep1 and Pep2 and (B) resolution between Pep1 and Pep2. Injection volume, 1055 nl of 7.5 μmol/l of Pep1 and Pep2. ITP–CZE systems as in Fig. 8.

9A shows the influence of different ITP times on Δt and Fig. 9B shows a corresponding decrease in resolution. The total migration time decreased as the ITP period was prolonged.

3.5. Calibration graph

A calibration graph was obtained with the combined NH_4^+–HAc system showing the ratio of the area of Pep3 to that of Pep4 at different Pep3 concentrations. Fig. 10 shows electropherograms of the highest (15 μmol/l) and lowest (30 nmol/l) concentration of Pep3 studied. At the lowest concentration, 30 nmol/l with a signal-to-noise ratio of about 4, the R.S.D. of the ratio of the peak area of Pep3 to that of Pep4 was 11% ($n = 3$). The equation for the calibration graph is given in Table 5.

3.6. Analysis of plasma extracts

The plasma samples were extracted with a simple solid-phase extraction (SPE) method. A volume of 500 μl of the elution solvent from the SPE was evaporated under vacuum in a laboratory-made evaporation centrifuge. The residue was dissolved in 500 μl of EACA buffer–ace-

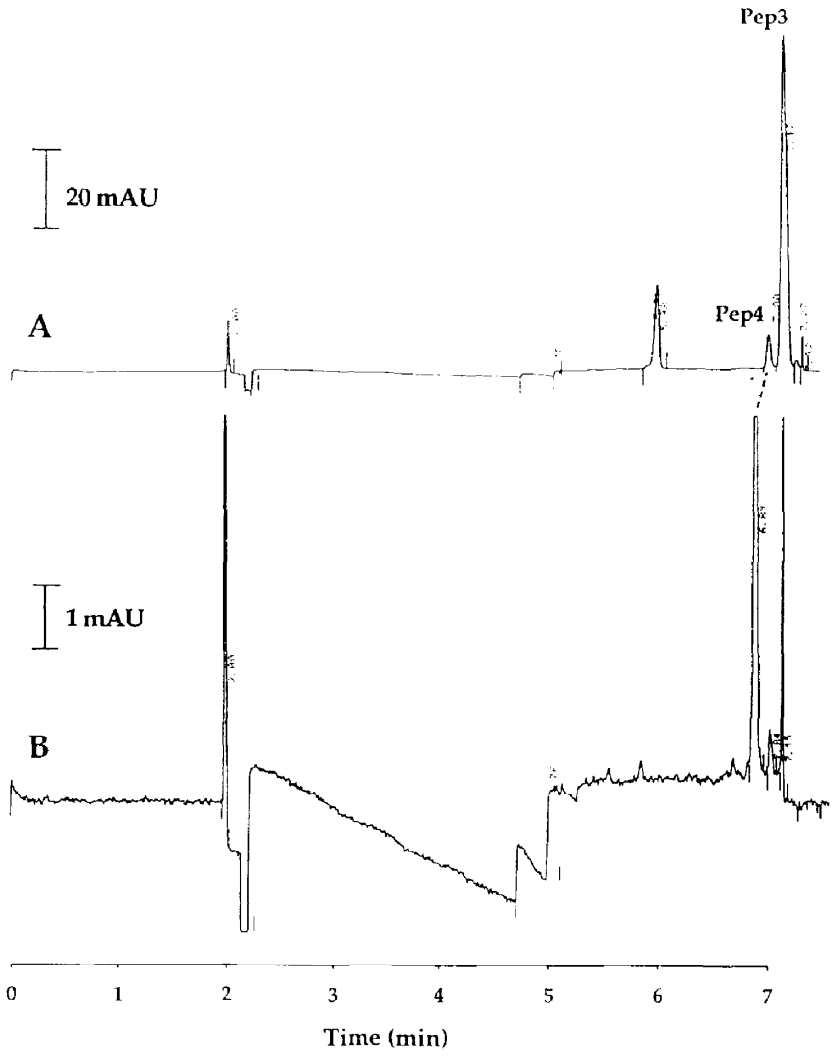

Fig. 10. Electropherogram of (A) 15 μmol/l and (B) 30 nmol/l of Pep3. Concentration of internal standard, 1.5 μmol/l. The UV trace starts at the start of the ITP. ITP–CZE system as in Fig. 8.

Table 5
Calibration graph

Equation	$y = ax + b$
Slope	$a = 1.076$
y-Intercept	$b = 0.004$
Correlation coefficient	0.9995

Equation of the calibration graph for Pep3 with Pep4 as internal standard (y = area counts in 0.50 μV·s; x = μmol/l). Concentration range, 30 nmol/l–15 μmol/l; injection volume, 1.4 μl; plug length, 18 cm. ITP–CZE system as in Fig. 8.

tonitrile (90:10, v/v). The acetonitrile was added to the electrolyte to prevent plasma components from sticking to the capillary. Without an organic modifier the efficiency of the CZE separation deteriorated seriously after only a few injections. Between runs the capillary was first rinsed for 2 min with acetonitrile followed by rinsing for 2 min with electrolyte.

To be able to measure pharmacological concentrations of Pep1, large volumes have to be injected. Therefore, injections of 1.5 μl were

Table 6
Instrumental sequence for the analysis of plasma extracts

Event	Time/action	Position	Inlet vial
Injection	2 min/75 mbar	33 cm[a]	Sample[b]
ITP	10 min/ 10 μA	36 cm	TE[c]
Δp	4 min/ −30 mbar	10 cm	Water
CZE	15 min/ 25 kV	–	BGE[d]
Clean 1	2 min/ 2000 mbar	–	Acetonitrile
Clean 2	2 min/ 2000 mbar	–	BGE
Replenish outlet	2 min/ 2 ml/min	–	BGE

Injection volume, 1500 nl; capillary bore, 75 μm; effective length, 50 cm; total length, 65 cm; temperature, 35°C; BGE, 10 mmol/l 6-aminocaproic acid (pH 3.6)–acetonitrile (90:10, v/v).
[a] Calculated front of the sample plug, measured from inlet side of the capillary.
[b] 3.18 μmol/l Pep1 and 2.18 μmol/l Pep2 in BGE.
[c] 50 mmol/l acetic acid (pH 3.1).
[d] 10 mmol/l 6-aminocaproic acid (pH 3.6)–acetonitrile (90:10, v/v).

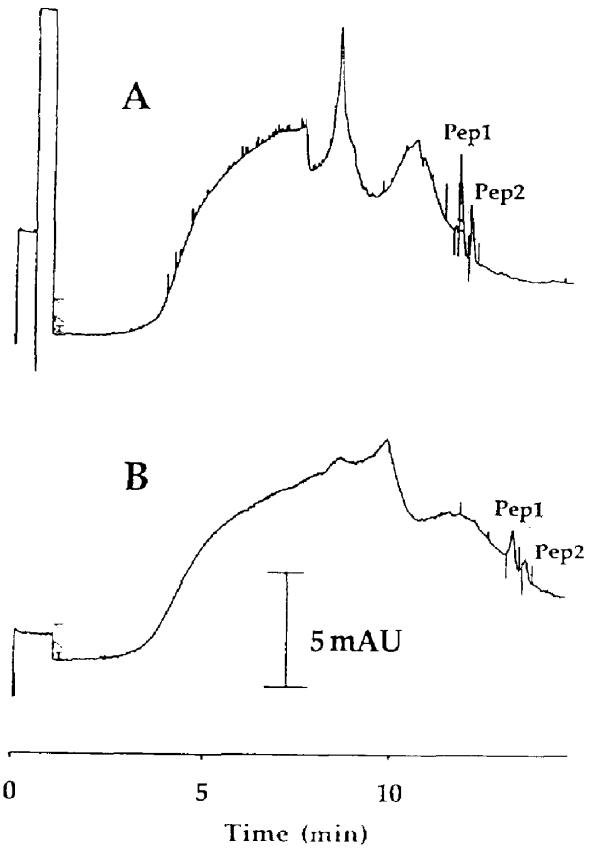

Fig. 11. Electropherograms (A) for the first injection and (B) after more than 100 injections of a plasma extract. The UV trace starts at the start of the CZE. ITP–CZE system as in Table 6.

made. After stacking of this long plug the resulting zones were too close to the detector to achieve sufficient separation during CZE. The zones needed to be transferred towards the inlet side of the capillary and under-pressure was therefore applied at the inlet side [26,27]. During the air-driven transfer, the sharp boundaries are disturbed by the parabolic flow profile. However, after moving the sample zone backwards, there is still a small zone of terminating electrolyte present in the capillary end. This facilitates refocusing of the zones, just before the CZE separation starts. Extension of the capillary length would result in a lower field strength and a longer operating time. The exact instrumental sequence used for the plasma extracts is given in Table 6. Fig. 11 shows the first injection and an injection after more than 100 runs. The differences in peak height and peak shape can be seen.

4. Conclusions

On-capillary ITP preconcentration permits the application of larger injection volumes for CE. All electrolyte systems presented in this paper were useful for the test substances. The main limitation with regard to the injection volume is the capillary length left for the separation after

the stacking. When destacking is initiated too close to the detector no separation, or insufficient separation, takes place if the stacked zones were not transferred towards the inlet side of the capillary. Early studies of plasma extracts with the combined system and a back-pressure technique appeared promising. Future work will focus on the improvement of the separation of analytes from plasma components and on the stability of the coating. We shall also try to decrease the overall run time, which is more than 37 min for the plasma samples in the system described. One should keep in mind that the injection of larger volumes in combination with the back-pressure system results in longer run times. Therefore, a balance between the required minimum detectable concentration and maximum acceptable run time should be found for each individual application.

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