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Capillary electrophoresis of peptides

Analysis of adrenocorticotropic hormone-related fragments

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ABSTRACT

Capillary electrophoresis can be used succesfully to analyse small peptides to give additional information to that obtained using high-performance liquid chromatography (HPLC). The separation of a modified adrenocorticotropic hormone (4–9) fragment (Org 2766) and several of its fragments was investigated using capillary zone electrophoresis. Prediction of migration in aqueous systems using pK_a-related data and the migration behaviour using sodium dodecyl sulphate in the buffer are discussed, as is the choice of buffer systems. The electrophoretic patterns are compared with the HPLC separation.

INTRODUCTION

Adrenocorticotropic hormone (ACTH) has long been known for its stimulation of the adrenal cortex to produce and release steroid hormones. In addition to extra-adrenal activities, *e.g.*, lipolytic and melanotropic effects, ACTH also influences behaviour in animals and man [1]. In recent years several studies have shown that ACTH and related peptides stimulate the recovery of sensorimotor function after nerve damage [2]. Org. 2766, a modified ACTH-(4–9) fragment (I), also active in this respect, was found to prevent neuropathies induced by cystostatic drugs in both animals and man [3].

Part of the development work of a compound involves finding conditions to separate the parent compound from its fragments, either synthetic products or metabolites. High-performance liquid chromatography (HPLC) is an established technique for the separation of peptides. Capillary electrophoresis is a rapidly expanding separation method in which automation common in chromatography is combined

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with the separation power of electrophoresis [4,5]. Capillary zone electrophoresis (CZE) has been shown to be an additional technique to HPLC in the analysis of small peptides [6–11]. Small differences in peptide charge and size can lead to separation with a different selectivity as compared with HPLC

In the development of an adequate separation of I from five of its possible fragments, we investigated the use of CZE in aqueous systems and tried to predict the optimum separation using a simple program for the prediction of peptide charge and mobility. The performance in several buffers was also investigated.

To obtain a better resolution for compounds with an overall charge of zero, sodium dodecyl suplhate (SDS) can be added to the buffer at a concentration above its critical micelle concentration (CMC). This leads to the formation of an extra micellar phase in the system. Because these micelles are charged and migrate with a different velocity than the electroosmotic flow (EOF), separations are based on the difference in affinity for the micellar phase. This technique, known as micellar electrokinetic capillary chromatography (MECC) [12], can lead to a different selectivity. The influence of the SDS concentration on the separation was investigated.

For reasons of comparison we also examined the separation of the peptides by HPLC.

EXPERIMENTAL

Instrumentation

CE experiments were performed on a P/ACE System 2000 capillary electrophoresis instrument (Beckman, Palo Alto, CA, U.S.A.) using UV detection at 200 and 214 nm. The capillary used was a 57 cm (50 cm to the detector) × 75 μm I.D. untreated fused-silica tube from Poly Micro Technology (Phoenix, AZ, U.S.A.), mounted in the Beckman capillary cartridge. After installation, the capillary was treated for 45 min with 0.1 *M* KOH and subsequently rinsed for 45 min with water. Data were collected using the P/ACE System 2000 software and analysed with CAESAR capillary electrophoresis software, which was developed in our laboratory.

HPLC experiments were carried out on an HP 1090 M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, U.S.A.) provided with a ternary solvent-delivery system, an autoinjector, an autosampler and a diode-array detector. The apparatus was equipped with a computer workstation and printer/plotter facilities. As supporting material a reversed-phase octadecylsilica column (Supelcosil LC-18 DB, 5-μm particles, 250 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) was used. A guard column (20 × 4.6 mm I.D.) filled with the same material preceded the analytical column.

Materials

Fig. 1 shows the amino acid composition of the hexapeptide I and its fragments which were synthesized by the peptide chemistry group of Organon. Part of the sequence of ACTH is also shown.

All other chemicals used were of analytical-reagent grade and obtained from Merck (Darmstadt, Germany), except for SDS, which was of electrophoresis grade from Polysciences (Warrington, PA, U.S.A.). All buffers and samples were prepared using ultrapurified water (Milli-Q system; Waters–Millipore, Bedford, MA, U.S.A.) with a resistance of better than 10 MΩ/cm.

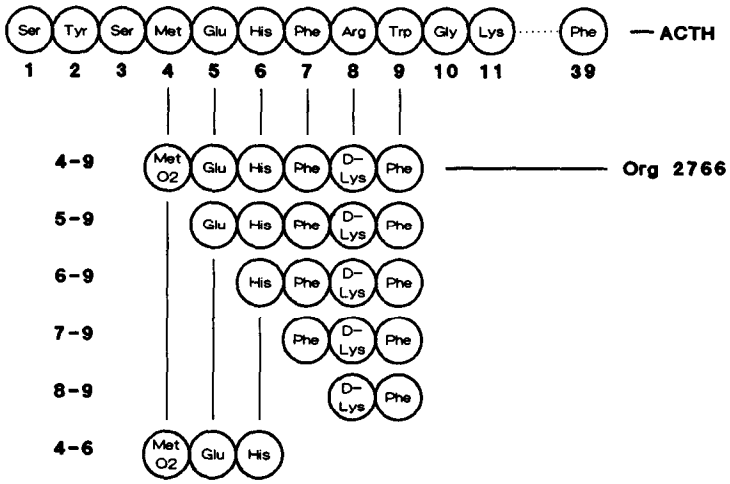

Fig. 1. Primary structure of I (Org 2766) and several of its fragments.

Methods

CE analyses were performed in the following way. Prior to each analysis the capillary was washed successively for 2 min with 0.1 *M* KOH, 2 min with water and 2 min with the buffer system, to obtain reproducible conditions in the separation system. Samples were dissolved in water unless stated otherwise and were analysed immediately. Injection was performed using pressure and the injection volume was in the range 5–25 nl (a 1-s pressure injection represented about 5 nl). The temperature was preset to 25°C and kept constant during the analyses. All analyses were performed using a potential difference of 25 kV.

The choice of electrolyte systems is a critical issue in CZE. To illustrate the effect of some essential parameters, characteristics of the aqueous buffers used are given in Table I.

Buffers used in MECC separations were prepared by mixing appropriate amounts of the aqueous buffer of pH 8.3 with the same buffer containing 100 m*M* SDS.

TABLE I

OPERATIONAL BUFFER SYSTEMS

No.	pH	Concentration[a]	Conductivity (mS/cm)	EOF · 10^{-5} (cm^2/V s)	UV absorbance (AU at 214 nm)
1	2.2	25 m*M* phosphate + KOH	4.03	<3.2	0.000
2	3.8	20 m*M* formate + alanine	0.95	16.4	0.002
3	4.4	20 m*M* ε-aminocaproate + acetic acid	0.84	29.7	0.007
4	6.2	20 m*M* histidine + MES	0.39	52.8	0.059
5	7.5	40 m*M* imidazole + MOPS	0.73	57.6	0.057
6	8.3	100 m*M* borate + KOH	1.90	67.9	0.000

[a] MES = 2-(N-morpholino)ethanesulphonic acid; MOPS = 3-(N-morpholino)propanesulphonic acid.

TABLE II

GRADIENTS APPLIED IN HPLC

Gradient No.	Time (min)	Elution profile[a]			Temperature (°C)
		A (%)	B (%)	C (%)	
1	0	20	65	15	35
	60	20	55	25	
2	0	20	65	15	35
	30	20	55	25	
3	0	20	62	18	45
	20	20	54	26	

[a] A, 0.5 *M* NaH_2PO_4 and H_3PO_4 to pH 2.1; B, water; C, acetonitrile–water (60:40).

For the HPLC separation a phosphate buffer–acetonitrile gradient system [13] was applied: solvent A, 0.5 *M* NaH_2PO_4 and H_3PO_4 to pH 2.1; solvent B, water; and solvent C, acetonitrile–water (60:40). Three different linear gradient runs were applied (Table II). All separations were performed at a flow-rate of 1.0 ml/min. Prior to use the mobile phases were filtered and degassed with helium. Peptide samples were dissolved in the initial mobile phase and 100 μl of this solution, corresponding to 10–15 μg of each peptide, were injected. Detection was at 210 nm. The retention times and peak areas were recorded.

Calculations of the titration curves and mobility for the several peptide fragments were performed using CAS, peptide mobility software, also developed in our laboratory.

RESULTS AND DISCUSSION

Using the animo acid composition of the peptide it is possible to calculate an imaginary titration curve for this compound using the pK_a values of the various amino acids [14]. The charge of the peptides under various pH conditions can then be easily calculated. In Fig. 2 the charge *versus* pH curves are given for the different peptide fragments calculated. For $MetO_2$ the same pK_a values are used as for methionine.

Charge is, of course, one of the main parameters that determine the electrophoretic migration behaviour of peptides in an aqueous buffer system. Several workers have tried to propose a model including other parameters, *e.g.*, molecular weight or number of animo acids for accurate determination of the migration characteristics.

By plotting charge divided by the two-thirds power of the molecular weight against pH, an estimate for the relative electrophoretic mobility can be obtained [9] and these data are given in Fig. 3. Measurement of the real effective mobilities under different pH conditions and comparison of the data with the calculated mobilities are necessary in order to validate this model.

Fig. 4 shows the electropherograms of I and its fragments using the six different buffer systems. A baseline separation in less than 12 min is shown in Fig. 4A. The peak performance is poor, because the buffer has a high conductivity, resulting in

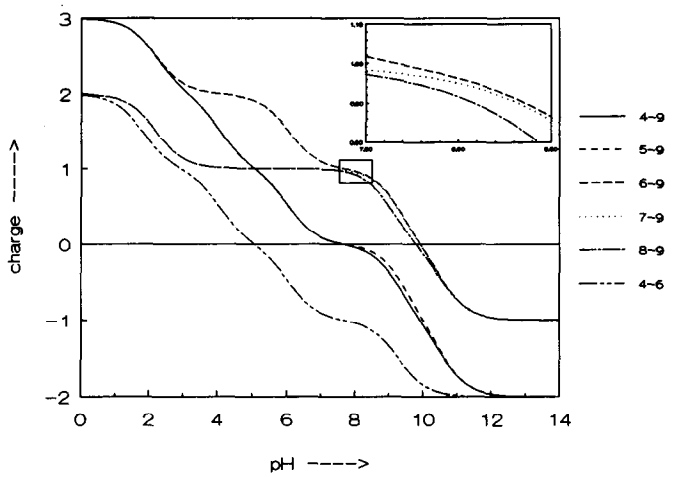

Fig. 2. Calculated titration curves for I and fragments. The inset shows the minor differences around pH 8.

extensive Joule heating. This was also clear from the changes in migration time, which were high. It should be emphasized that the solutes are migrating as positive ions.

In Fig. 4B and C the separations show very good peak shapes. Plate numbers of 200.000–300.000 were measured for all peaks. That minor changes in pH can cause a different migration order is clear from the position of peak 4. Clearly all compounds migrate as positive ions, which indicates that hydrostatic effects on the negatively charged capillary surface play only a minor role. The negative dips in the electropherograms mark the osmotic flow.

Fig. 4D and E shows the influence of highly UV-absorbing buffers. A clear negative peak marks the osmotic flow and the peak heights are considerably smaller. The detection limits in these systems are therefore higher than in the other systems.

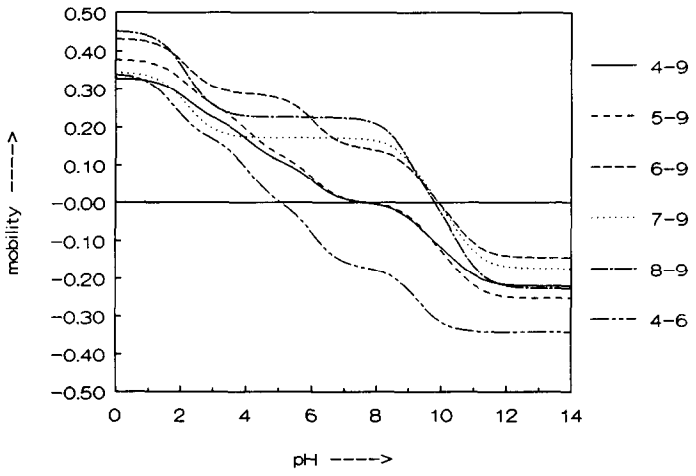

Fig. 3. Calculated relative mobilities ($\cdot 10^{-1}$) for I and fragments.

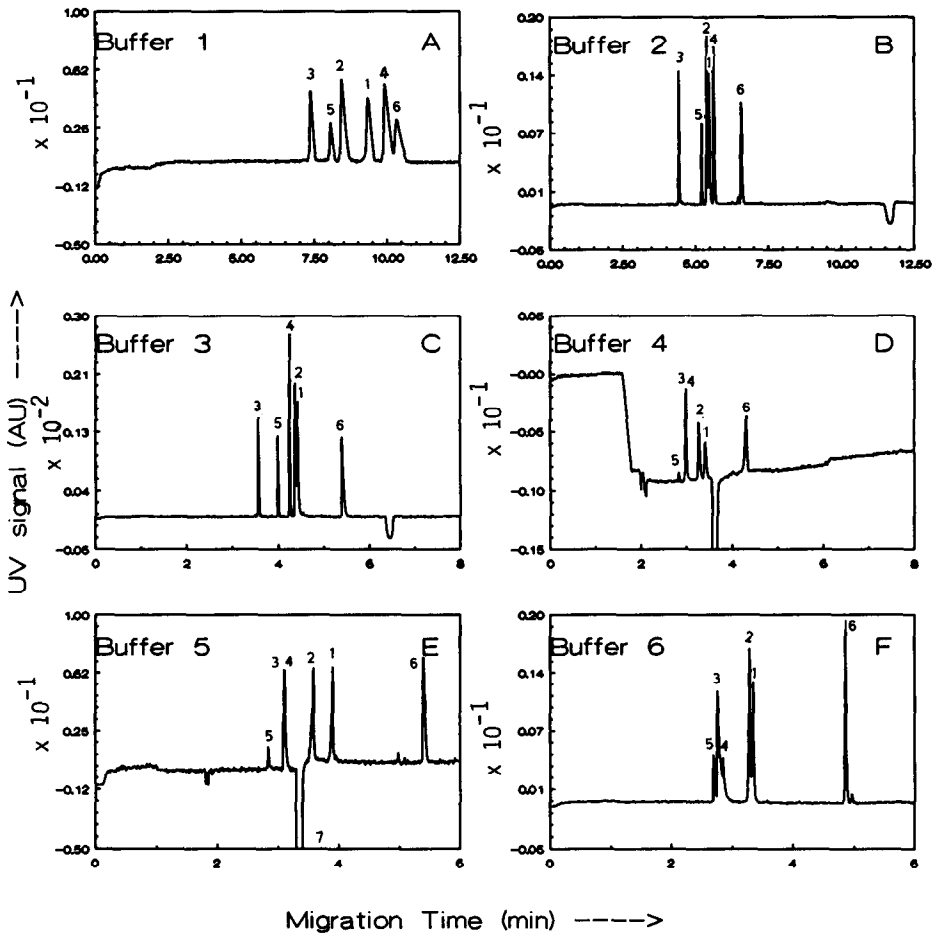

Fig. 4. Electropherograms of I and fragments separated using buffers 1–6. UV detection at 214 nm. Fragments: (1) 4–9; (2) 5–9; (3) 6–9; (4) 7–9; (5) 8–9; (6) 4–6.

Note that at this moderate pH, part of the compounds is positively and part negatively charged. Both electropherograms show a triplet of small negative dips, which was present in every analysis. Considering the migration time it can be that we are dealing with small metal ions, detected through exclusion of the positive UV-absorbing buffer ions.

When we compare the observed migration order of the compounds in the different systems with the calculated migration order presented in Fig. 3, we can conclude that the migration order can be predicted exactly. A group of three peaks as in Fig. 4F is also found in the predicted migration behaviour. However a correction factor should be used to obtain the same absolute values. When we plot $1.1391 \times$ (calculated mobility $\times 10^{-2}$) $- 0.7252$ against (measured mobility $\times 10^{-8}$) we obtain Fig. 5. A good correlation with a regression coefficient of 0.94 was found, although six different buffer systems were used. Comparing data obtained within one system we

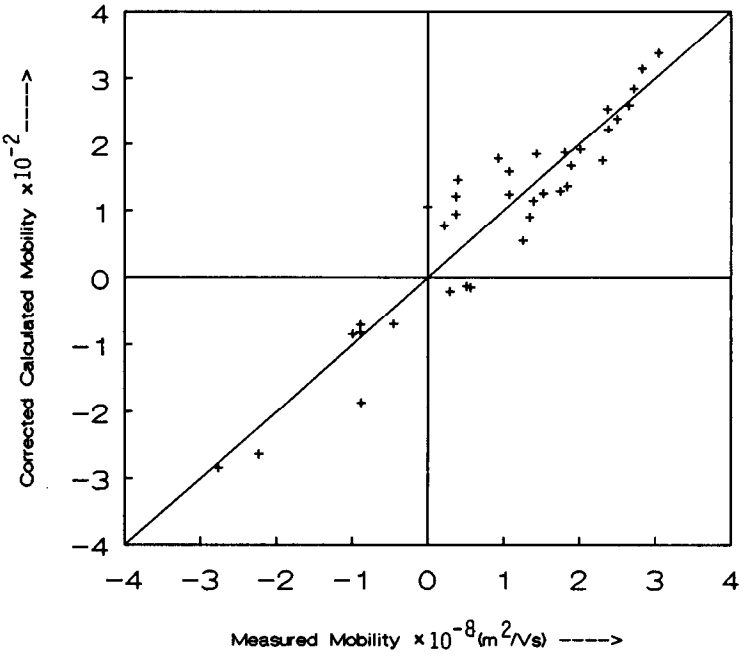

Fig. 5. Comparison of calculated and measured mobilities.

find an even better correlation with a regression coefficient of 0.98 for high pH to 0.99 for the low and middle pH region.

In order to use CE for the purity control of synthetic peptides, optimization of the separation of the compound from its fragments or impurities has to be performed. To determine possible impurities also, calibration graphs have to be constructed and detection limits have to be established.

In Fig. 6 the calibration graph for I using buffer systems 1 and 6 is given. The

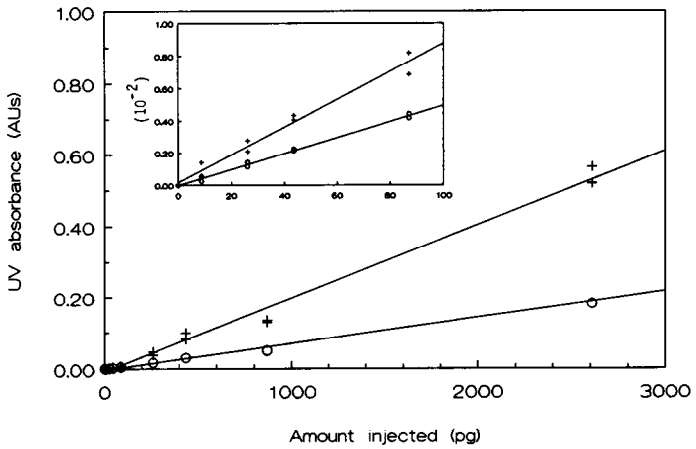

Fig. 6. Calibration graphs for I using buffers (+) 1 and (o) 6. The inset shows the lower region of the curve.

amount of sample used was in the range 8–2610 pg and all measurements were performed in duplicate. A good correlation was found between the amount injected and the UV absorbance, with regression coefficients of 0.995 and 0.999, respectively. The samples were dissolved in water and therefore a non-uniform electric field gradient was present after the injection. Dissolving the sample in the operating buffer improves the linearity of the calibration graph. Detection limits were *ca.* 5 pg injected in both instances. As mentioned earlier, buffer 1 shows poorer reproducibility.

Fig. 7 shows the electropherogram for I in buffer system 6. No significant impurities can be observed. It should be borne in mind, however, that fragments 4–9 and 5–9 elute very closely in this system. A large surplus of 4–9 as compared with 5–9 can easily mask the presence of the latter. However, checking in system 1, where these two components do not elute very closely, did not show any extra peak.

Addition of SDS to the buffer system can influence the selectivity. In Fig. 8 three electropherograms are shown of I and fragments in buffer 6 with different amounts of SDS added. Mainly compounds which are overall zero charged at the pH of buffer 6 are strongly influenced by addition of SDS. Charged compounds (*e.g.*, fragment 4–6), are hardly influenced, especially when one takes into account the decrease in the EOF. Therefore, compounds which are difficult to separate or which migrate very closely can be separated if they have different affinities for the micellar phase. Fragments 4–9 and 5–9 show an improved resolution when the amounts of SDS added is increased. On the other hand, fragments 6–9 and 7–9 continue to migrate with almost similar velocity. Fig. 9 shows the complete behaviour in the region 0–90 m*M* SDS. Plate numbers decrease, however, at high concentrations of SDS because it contributes to the conductivity of the electrophoresis buffer. When we compare the conductivity of buffer 6 (32 μA at 25 kV) with the same buffer containing 90 m*M* SDS (123 μA at 25 kV), we can observe a clearly increased production of Joule heat and a decrease in plate number.

Using HPLC, in the initially performed 60-min run (gradient 1, Table II) all six peptides were completely separated and showed a relatively wide difference in reten-

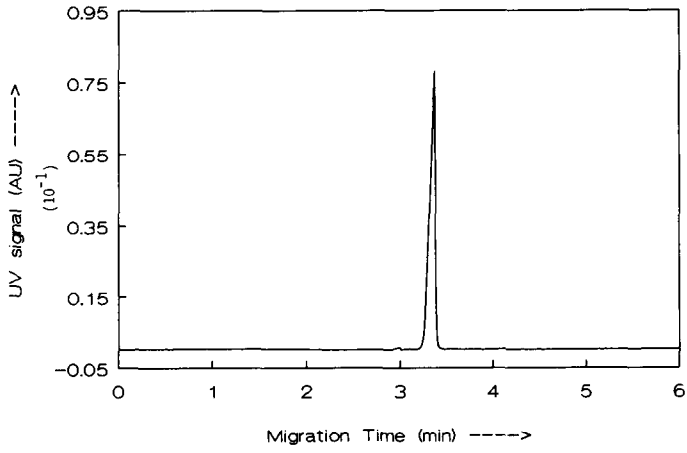

Fig. 7. Electropherogram of 4350 pg of I injected using buffer 6.

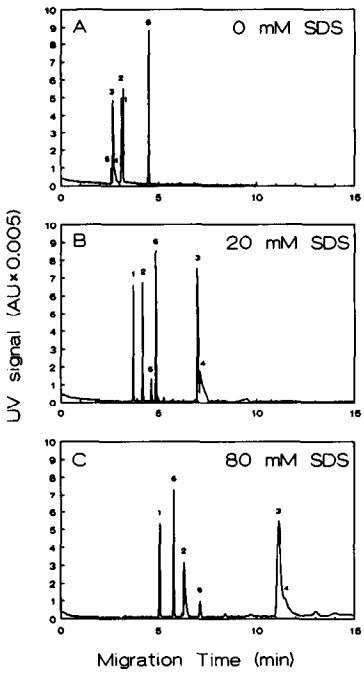

Fig. 8. Electropherograms of I and fragments separated using buffer 6 with 0, 20 and 80 m*M* SDS added.

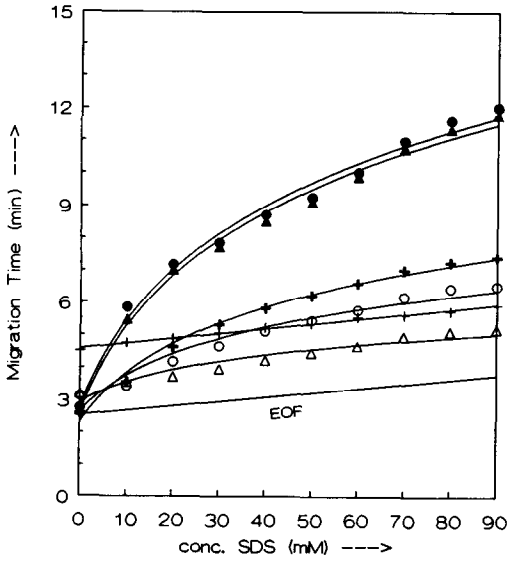

Fig. 9. Influence of addition of SDS to buffer 6 on the separation of I and fragments. (△) 4–9; (○) 5–9; (▲) 6–9; (●) 7–9; (✦) 8–9; (+) 4–6.

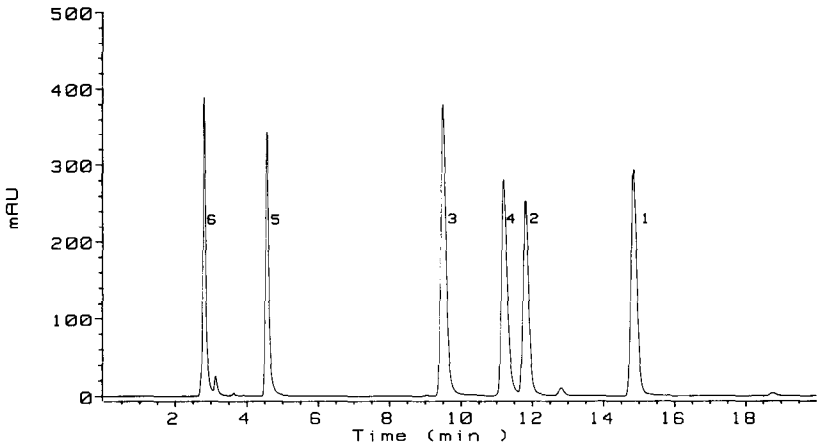

Fig. 10. HPLC elution pattern of I and fragments. (1) 4–9; (2) 5–9; (3) 6–9; (4) 7–9; (5) 8–9; (6) 4–6.

tion times. We therefore tried to perform a faster elution by applying a run with the same solvent composition but over a 30-min period (gradient 2) Again a good separation of the six compounds was obtained. Finally, we further speeded up the separation to 20 min by raising the temperature to 45°C and slightly adapting the solvent composition (gradient 3). Even in this short run a complete separation of the peptides was obtained, as illustrated in Fig. 10. In all three gradients the actual elution order of the peptides was in accordance with the predicted elution order based on the peptide hydrophobicity calculation of Meek and Rosetti [15] for phosphate–acetonitrile gradients. Only the pair 7–9/5–9 eluted in the reverse order.

As expected, no correlation was found between the CZE analysis in the aqueous buffer systems and HPLC. Using two-phase systems in both MECC and HPLC, leads to the question of whether these two methods can give comparable results. In reversed-phase HPLC we have a moving polar mobile phase and an apolar stationary phase, whereas in MECC the 'stationary phase' is also apolar but migrating. However, no similar migration orders were found between the HPLC and MECC.

CONCLUSION

Small peptides are suitable for analysis by capillary electrophoretic methods. The mobility of these compounds can often be predicted fairly well using the pK_a values of the separate amino acids, especially when the investigated peptides are closely related. One should keep in mind, however, that pK_a values of amino acids in peptides can be influenced by neighbouring groups. Both the elution orders and measured migration times, however, were close to the predicted values, although a correction factor had to be used.

Choice of buffer systems can therefore be made based on calculations, although some parameters have to be taken into account. The buffer components should preferably have a high buffering capacity in the given pH region and have a low conduc-

tivity. In this respect, fairly high-concentration buffers can be used without problems of Joule heating. A small UV absorbance of the buffer is recommended because it makes the determination of the EOF possible without addition of a marker. This EOF dip can be used to check the reproducibility of the system and can be used as an internal standard for correction of migration times.

To identify fragments in purity control of peptides, the migration behaviour of standards should be known. If not, one should use several systems to minimize possible overlapping of compounds. For fairly apolar species the addition of SDS can cause extra selectivity which can be used to improve critical separations.

The different separation mechanism in CE, both CZE (based on charge/mass ratio) and MECC (based on the affinity for the micellar phase), and HPLC (mainly based on hydrophobicity), will result in complementary information. Therefore, both techniques are valuable in peptide analysis.

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