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Capillary zone electrophoresis of pharmaceutical peptides

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ABSTRACT

In peptide analysis, capillary zone electrophoresis (CZE) gives complementary information to that obtained using high-performance liquid chromatography and capillary isotachophoresis. However, a prerequisite for the implementation of CZE for routine quality control purposes is a simple strategy quickly leading to an adequate separation system. A general approach towards the set-up of such systems is presented. A broad range of peptides were used as representative models, *viz.*, adrenocorticotropic hormone (ACTH), the modified ACTH fragment Org 2766, endorphins, cholecystokinin and fragments thereof. In general, the pH and the concentration of the applied electrophoresis buffers are the most important parameters to be considered in the CZE of pharmaceutical peptides.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) is a separation technique with a rapidly growing number of applications in the field of protein and peptide analysis. Several groups have reported on the complementary information obtained from reversed-phase high-performance liquid chromatography (RP-HPLC) on the one hand and the HPCE modes [capillary isotachophoresis (CITP) and capillary zone electrophoresis (CZE)] on the other [1–4]. The combination of these techniques proved to be most valuable in the support of the preparation, purification and characterization of natural and synthetic pharmaceutical peptides. However, a prerequisite for the implementation of CZE for routine quality control purposes for pharmaceutical peptides is the availability of rapid and simple optimization procedures. Considering the amphoteric character of peptides, the most important parameter to be manipulated in method optimization is the pH of the buffer system [5,6]. Another important factor that can easily be varied and that may have a beneficial effect on the separation performance is the concentration of the buffer [7]. Moreover, buffer additives such as methanol or acetonitrile or metal salts such as $ZnSO_4$ may also improve the separation [6,7]. Using a broad range of peptides we studied the influence of the above parameters. The intention of this work was to set up a general strategy for the development of CZE procedures to be applied in routine peptide analyses.

EXPERIMENTAL

Instrumentation

All CZE experiments were performed on a P/ACE System 2100 capillary electrophoresis equipment (Beckman, Palo Alto, CA, USA) equipped with an untreated fused-silica tube (57 cm × 75 μm I.D.; 50 cm from inlet to detector), an autosampler, a temperature-controlled fluid-cooled capillary cartridge, an automatic injector

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TABLE I

SEQUENCES OF PARENT PEPTIDES

ACTH	H–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Ala–Glu–Asp–Glu–Leu–Ala–Glu–Ala–Phe–Pro–Leu–Glu–Phe–OH
Org 2766	H–MetO_2–Glu–His–Phe–D–Lys–Phe–OH
β-Endorphin	H–Tyr–Gly–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser–Gln–Thr–Pro–Leu–Val–Thr–Leu–Phe–Lys–Asn–Ala–Ile–Ile–Lys–Asn–Ala–Tyr–Lys–Lys–Gly–Glu–OH
CCK-8(S)	H–Asp(–SO_3H)–Tyr–Met–Gly–Trp–Met–Asp–Phe–NH_2

and a UV source with a set of selectable wavelength filters.

Calculation of the relative mobilities of the peptides was performed using CAS, a peptide mobility software program developed at Eindhoven University of Technology [4].

Peptide characteristics

To obtain a good overall insight into the influence of the various buffer parameters on HPCE peptide separations in general, four characteristic peptide groups varying widely in isoelectric point (p*I*), molecular mass and chain

TABLE II

PHYSICO-CHEMICAL CHARACTERISTICS OF THE PEPTIDES

Peptide group	Sequence[a]	p*I*	Molecular mass	Chain length	Buffer pH	Mobility[b]	Migration order
1 ACTH	11–24	11.69	1652	14	3.8	0.0431	1
	1–24	10.05	2934	24		0.0379	2
	1–39	9.24	4567	39		0.0216	3
	1–10	7.55	1299	10		0.0151	4
2 Org 2766	3–6	9.74	578	4	3.8	0.0289	1
	5–6	9.70	294	2		0.0229	2
	2–6	7.81	707	5		0.0222	3
	1–6	7.60	870	6		0.0194	4
	4–6	9.82	441	3		0.0175	5
	2–6 (pyroGlu)	8.25	689	5		0.0130	6
	1–3	5.15	447	3		0.0130	7
3 Endorphins	7–15	6.70	998	9	2.2	0.0157	1
	6–15	6.70	1089	10		0.0147	2
	6–16	6.70	1190	11		0.0130	3
4 CCK	2–8 (non-S)	6.80	948	7	7.5	−0.0001	1
	1–8 (cyclic S)	–	1125	8		–	2
	1–8 (non-S)	3.90	1063	8		−0.0097	3
	2–8 (S)	~3.90	1028	7		−0.0099	4
	1–8 (S)	~3.90	1143	8		−0.0184	5

[a] S = Sulphated.

[b] Mobility = calculated electrophoretic mobility.

length were chosen: (1) adrenocorticotropic hormone (ACTH) and fragments; (2) Org 2766 and fragments; (3) endorphins and fragments; and (4) cholecystokinin (CCK) fragments. For each of the four groups, structurally closely related peptide preparations, giving representative information in critical separations, were selected.

The sequences of the parent peptides are given in Table I and the physico-chemical characteristics of the individual peptides in Table II. The porcine ACTH preparation was obtained from Diosynth (Oss, Netherlands). All other peptide preparations were synthesized by the peptide chemistry group of Organon.

ACTH and Org 2766. The principal activity of ACTH is the stimulation of the adrenal cortex to produce and release steroid hormones. The N-terminal part ACTH-(1–24) possesses full biological activity [8]. The (1–10) and (11–24) fragments are the building blocks in the synthesis of ACTH-(1–24). Recently, several studies have shown that ACTH and related peptides also stimulate the recovery of sensorimotor function after nerve damage. The modified ACTH-(4–9) fragment Org 2766 was found to prevent neurophathies induced by cytostatic drugs in both animals and man [9]. In the framework of metabolism studies the separation of Org 2766 and fragments has been investigated [4].

Endorphins and fragments. β-Endorphin, the C-terminal 31-peptide of β-lipotropin, was originally isolated from pituitaries of several species. The peptide is a potent opiate-like compound and displays behavioural properties [10]. Metabolic processing of β-endorphin by enzymes generates the N-terminal 16- and 17-peptides called α- and γ-endorphin, respectively. Removal of the N-terminal tyrosine residue of the endorphins, giving the (2–31), (2–17) and (2–16) fragments, results in preparations that have lost their opiate-like activity but have retained their behavioural properties. In order to facilitate metabolism studies of the proposed antipsychotic compound β-endorphin-(6–17) (Org 5878), the separation of the fragments (6–15), (6–16) and (7–15) has been investigated [11].

Cholecystokinin. The 58-peptide CCK is important in the control of gastrointestinal function. In addition, high concentrations of a sulphated octapeptide sequence (CCK-8S; the sulphated C-terminal fragment) were demonstrated to be present in several brain regions [12]. CCK-8S and (modified) fragments have been investigated for their separatibility.

Operational buffer systems

Five buffer systems, covering the pH range 2.20–8.30, were selected: (1) 25 m*M* phosphoric acid, adjusted to pH 2.20 with 1 *M* NaOH; (2) 20 m*M* formic acid, adjusted to pH 3.80 with β-alanine; (3) 20 m*M* L-histidine, adjusted to pH 6.20 with 2-(N-morpholino)ethanesulphonic acid; (4) 50 m*M* tris(hydroxymethyl)aminomethane (Tris), adjusted to pH 7.50 with acetic acid; and (5) 100 m*M* boric acid, adjusted to pH 8.30 with 1 *M* NaOH.

In addition, the separation performance of the buffers 1, 2 and 4 was studied at higher concentrations, *i.e.*, 50 and 100 m*M* for buffers 1 and 2 and 100 and 200 m*M* for buffer 4. Moreover, the effects of $ZnSO_4$ (50 m*M*), acetonitrile (10%) and methanol (10%), added to the original buffers 1, 2 and 4, was tested.

All chemicals used in the preparation of the buffers were of analytical-reagent grade (J.T. Baker).

Procedure

The peptide mixtures 1–4 (see Table II) were prepared in ultrapure water (Milli-Q) at a concentration of *ca.* 0.2 mg/ml of each component.

At the beginning of each day the capillary was rinsed successively with 1 *M* NaOH and ultrapure water for 45 min each. Prior to each analysis the capillary was rinsed for 3 min with the separation buffer. Samples of the peptide mixtures were injected using pressure for 5 s (corresponding to an injection volume of *ca.* 25 nl). Peak assignment was done by single component injection.

The analyses were performed at a voltage of 25 kV, unless stated otherwise. The fused-silica tube was maintained at 25°C. The components were detected by UV absorbance at 214 nm. After each run the capillary was rinsed successively with 0.1 *M* NaOH and ultrapure water for 3 min each.

RESULTS AND DISCUSSION

ACTH peptides (group 1, Table II)

On applying the operational buffer systems having a neutral or basic pH (3, 4 and 5), the four peptides co-migrated. However, using the acidic buffers (1 and 2), three clearly distinguishable peaks were monitored. The peak shape in the pH 3.8 buffer was slightly better than that in the buffer of pH 2.2. Hence further experiments were performed at pH 3.8. Raising the concentration of this buffer from 20 to 50 mmol/l resulted in a baseline separation of the four peptides in 8 min. Execution of a run at an even higher buffer concentration of 100 mmol/l made it necessary to lower the applied voltage from 25 to 20 kV (prevention of excessive Joule heating: current limit 100 μA). As a result, the separation time was then 12 min. The electrophoresis patterns obtained with the pH 3.8 buffer at concentrations of 20, 50 and 100 mmol/l are shown in Fig. 1.

An even more pronounced increase in the separation time was found when $ZnSO_4$ was added to the buffer. The high ionic strength of this medium made it necessary to lower the applied voltage to 15 kV. As a result, the separation time was 24 min without an improvement in separation. The organic additives acetonitrile and methanol also proved to be ineffective; in fact, the separation decreased. Whereas in the 10% acetonitrile-containing buffer no significant increase in separation time was found, in the buffer containing 10% methanol the separation lasted 30 min. This observation is in line with the results reported by McLaughlin *et al.* [7].

In summary, a buffer of pH 3.8 with a "medium" concentration of 50 mmol/l performed best in the separation of the four ACTH peptides.

On the basis of the amino acid composition of the peptides, one can calculate the approximate charge of the peptides at a particular pH, using the pK_a values of the amino acid residues [4,13]. Dividing the charge by the two-thirds power of the peptide molecular mass, one obtains an estimate of the relative electrophoretic mobility [4,14–16]. For the four ACTH peptides under investigation these relative mobilities, calculated at pH 3.8, are given in Table II, group 1.

The actual migration order of the peptides is in complete accordance with the order predicted from the estimated mobilities: in order of decreasing mobility, (11–24), (1–24), ACTH itself and finally the (1–10) peptide (Table II, group 1).

Org 2766 group (group 2, Table II)

For the optimum pH of the buffers tested, similar results as for the ACTH group were obtained, *i.e.*, the best separation was achieved in the system of pH 3.8. Four of the seven peptides are clearly separated whereas the separation of the other three peptides is critical (Fig. 2A). Raising the buffer concentration from 20 to

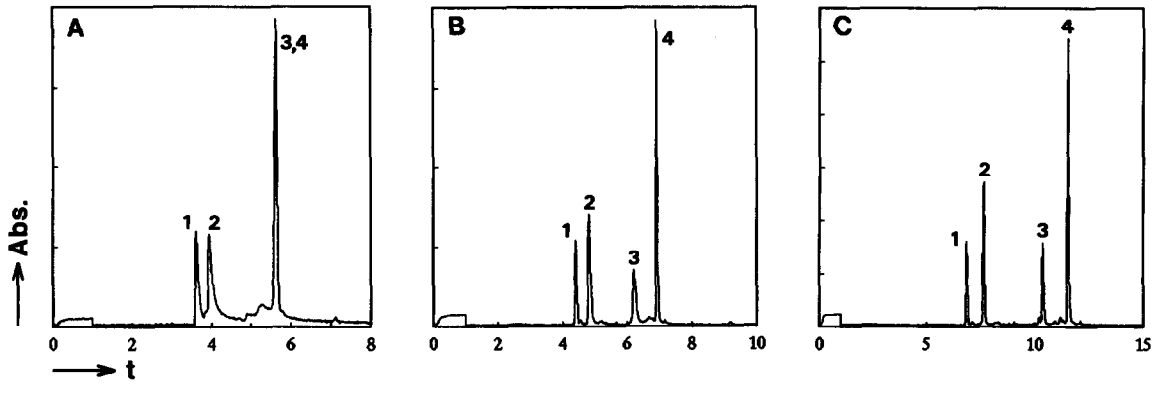

Fig. 1. CZE separation of ACTH peptides in buffer of pH 3.80. Concentration: (A) 20; (B) 50; (C) 100 m*M*. Peaks: 1 = (11–24); 2 = (1–24); 3 = (1–39); 4 = (1–10). *t* = Migration time (min); Abs. = absorbance at 214 nm.

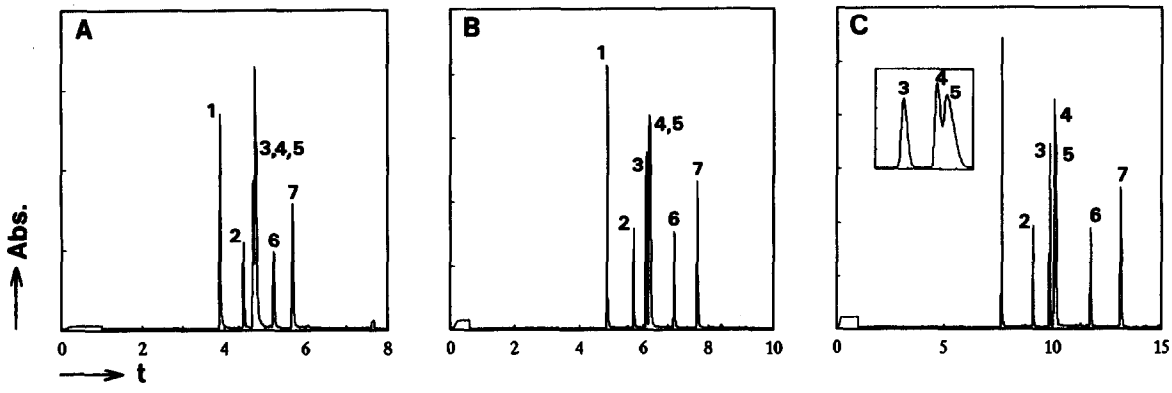

Fig. 2. CZE separation of Org 2766 peptides in buffer of pH 3.80. Concentration: (A) 20; (B) 50; (C) 100 m*M*. Peaks: 1 = (3–6); 2 = (5–6); 3 = (2–6); 4 = (1–6); 5 = (4–6); 6 = (2–6)pyroGlu; 7 = (1–3). The inset in (C) shows the actual separation of peptides 3, 4 and 5. Axes as in Fig. 1.

50 mmol/l improved the resolution: the (2–6) peptide is partly separated from the (1–6) peptide (Org 2766 itself) and the (4–6) peptide. However, the last two peptides still co-migrate (Fig. 2B). Further elevation of the buffer concentration to 100 mmol/l gave the best result, with complete separation of five of the seven peptides and partial separation of the (1–6) and (4–6) peptides (Fig. 2C). Owing to the lower voltage that had to be applied with this buffer, the separation time was 14 min. As with the ACTH peptides, the addition of $ZnSO_4$ gave no improvement in the separation (in this high ionic strength buffer, the voltage had to be lowered to 15 kV, resulting in an analysis time of 24 min). Also, the addition of 10% acetonitrile or 10% methanol was ineffective.

Summarizing these results, the buffer of pH 3.8 at a high concentration of 100 mmol/l gave the best separation.

At the applied pH of 3.8 the actual migration order of the seven peptides was again completely in line with the predicted order derived from the calculated relative electrophoretic mobilities (Table II, group 2).

Endorphin peptides (group 3, Table II)

We have previously reported on the separation of α-, β- and γ-endorphin and their N-terminally shortened des-Tyr fragments [17]. With a system that was not further optimized, *i.e.*, 20 m*M* phosphate buffer (pH 2.80) at 25 kV, a clear separation of the six peptides was obtained (Fig. 3).

As a sequel to that study on closely related endorphin fragments, we investigated the separation of the neutral peptide triad β-endorphin-(6–16), -(6–15) and -(7–15). This is considered to be a severe test for the current strategy as these peptides have similar molecular masses and an identical p*I* value of 6.70. Again, the acidic buffers performed best. In this instance the most acidic buffer of pH 2.2 gave the best result, with three partially separated peaks, whereas at pH 3.8 two components co-migrated. Raising the concentration of the pH 2.2 buffer from 25 to 50

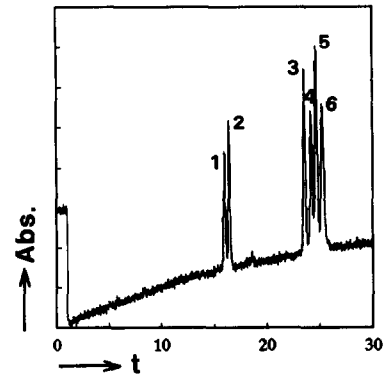

Fig. 3. CZE separation of α-, β- and γ-endorphin and their N-terminally shortened des-Tyr fragments in buffer of pH 2.80. Peaks: 1 = des-Tyr β-endorphin, (2–31); 2 = β-endorphin, (1–31); 3 = des-Tyr γ-endorphin, (2–17); 4 = des-Tyr α-endorphin, (2–16); 5 = γ-endorphin, (1–17); 6 = α-endorphin, (1–16). Axes as in Fig. 1.

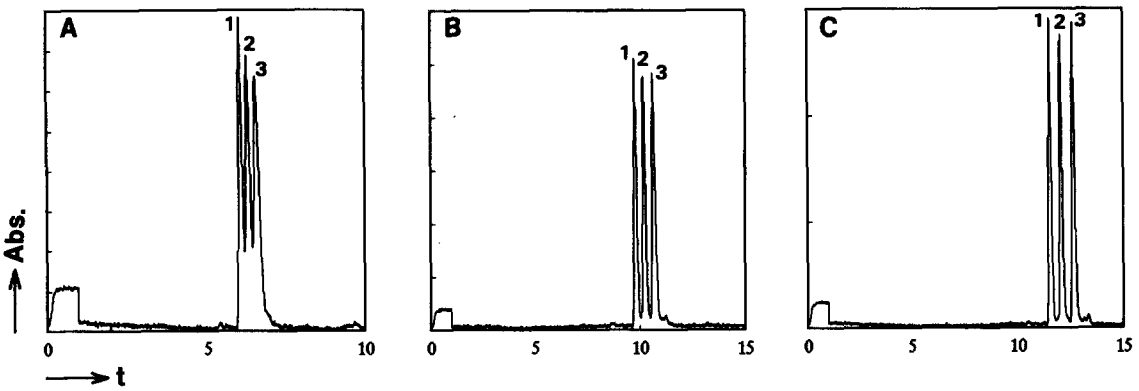

Fig. 4. CZE separation of β-endorphin fragment peptides in buffer of pH 2.20. Concentration: (A) 25; (B) 50; (C) 100 m*M*. Peaks: 1 = (7–15); 2 = (6–15); 3 = (6–16). Axes as in Fig. 1.

mmol/l resulted in a nearly baseline separation of the three peptides. As the applied voltage had to be lowered from 25 to 20 kV with the 50 mmol/l buffer, the separation time was increased by *ca.* 5 min (Fig. 4A and B). A further increase in the buffer concentration to 100 mmol/l (applied voltage again 20 kV) gave a further minor improvement of the separation (Fig. 4C). Addition of $ZnSO_4$ was ineffective, giving long migration time of 25 min (applied voltage 15 kV) and no improvement in separation. Addition of acetonitrile or methanol decreased the resolution.

In summary, for these neutral peptides the buffer of pH 2.2 at a molarity of 100 m*M* gave the best separation.

Also for these peptides the migration order found corresponded with the predicted order (Table II, group 3).

CCK fragments (group 4, Table II)

For the acidic CCK peptide fragments a completely different electrophoretic behaviour to that with the basic and neutral peptides was found. The low-pH buffers gave inadequate separation. However, using the neutral or basic buffers the separation improved considerably. The best result was obtained with the pH 7.50 buffer in which a reasonable separation of the five peptides was obtained (Fig. 5A). Raising the buffer concentration to 100 mmol/l further improved the peak shape, although the separation

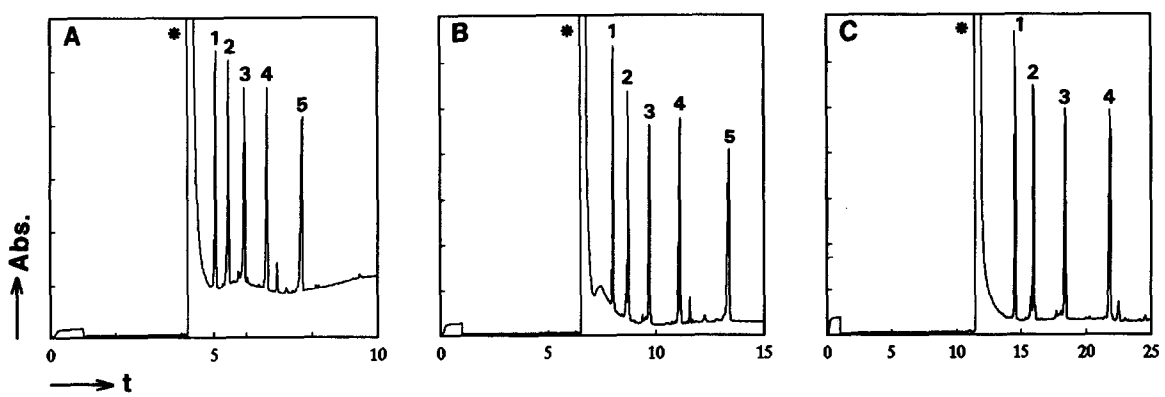

Fig. 5. CZE separation of CCK peptides in buffer of pH 7.50. Concentration: (A) 50; (B) 100; (C) 200 m*M*. Peaks: 1 = (2–8)non-S; 2 = (1–8)cyclic-S; 3 = (1–8)non-S; 4 = (2–8)S; 5 (1–8)S (S = sulphated); * = dimethylformamide (solvent constituent). Axes as in Fig. 1.

time was doubled owing to the lower voltage of 20 kV that had to be applied (Fig. 5B). At a concentration of 200 mmol/l at 15 kV, only four of the five components migrated within 25 min (Fig. 5C). Addition of $ZnSO_4$, acetonitrile or methanol to the buffer gave no improvement in separation.

Hence for these acidic peptides the neutral buffer of pH 7.5 at a "medium" concentration of 100 mmol/l performed best.

Again, the experimentally found order of migration corresponded with the calculated migration order (Table II, group 4).

CONCLUSIONS

In the set-up of standard CZE procedures to be used in the quality control of closely related peptides, a simple strategy for obtaining adequate information is needed. In this work we investigated the parameters that may have a major impact on the separation. In line with earlier observations [5,6], the buffer pH is the most important parameter to be considered. However, it turned out that the optimum buffer pH depends strongly on the p*I* values of the peptides. Using five "standard" buffers, we found that for peptides with a basic and neutral character, the best separation is achieved in the low-pH region. Additional advantages of the low-pH buffers are the substantial reduction in the electroosmotic flow and the elimination of solute–wall interactions, resulting in a better reproducibility of the separation [18]. For peptides with an acidic character, the neutral pH region is preferred for a good separation.

After careful adjustment of the buffer pH, the second parameter to be considered for an optimum separation is the buffer concentration. In general we found that "medium" and "high" concentration buffers, *i.e.*, 50–100 mmol/l, performed best. For the peptide groups investigated, no beneficial effect on the separation was found with the buffer additives $ZnSO_4$, 10% acetonitrile or 10% methanol.

An additional guide in setting up an optimum separation is the calculation of the expected relative mobilities at a particular pH. For all the peptide groups investigated, the experimentally found migration order corresponded with the order estimated from the calculated relative mobilities. In this respect, plotting the calculated relative mobilities against the pH, as proposed by Van de Goor *et al.* [4], is a further extension of this approach. Taking the three endorphins as a representative example, such a plot is shown in Fig. 6.

In conclusion, we propose the following strategy for the rapid optimization of an adequate routine CZE analysis of closely related peptides: for basic and neutral peptides low-pH standard buffers should be tested first, and for acidic peptides the optimization should be started with buffers of neutral pH. If necessary, further improvement in the separation can be obtained by careful fine tuning of the buffer pH and/or using a higher buffer concentration. Using this strategy, CZE can be applied to the analysis of pharmaceutical peptides on a routine basis. Together with the complementary information from RP-HPLC and CITP, a valuable quality control system for these peptides may be established.

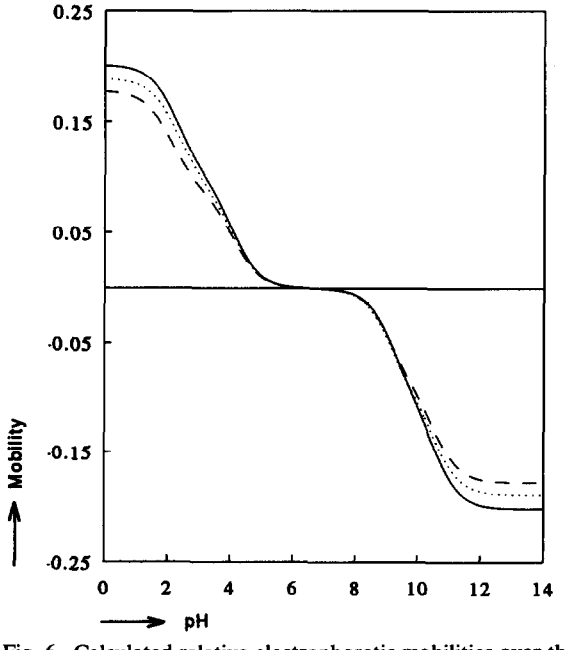

Fig. 6. Calculated relative electrophoretic mobilities over the pH range 0–14 for the three endorphin fragment peptides. Solid line, (7–15); dotted line, (6–15); dashed line, (6–16).

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