Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1009 (2003) 3–14
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Separation of structurally related synthetic peptides by capillary zone electrophoresis

Yuanzhong Yang, Reinhard I. Boysen, Jenny I-Chen Chen, Hooi Hong Keah, Milton T. W. Hearn*

Australian Research Council Special Research Centre for Green Chemistry, Centre for Bioprocess Technology and Australian Centre for Research on Separation Science, Monash University, P.O. Box 23, Clayton, Victoria 3800, Australia

Abstract

The separation of two different sets of synthetic peptides has been investigated by high-performance capillary zone electrophoresis utilising naked, fused silica capillaries. The effects of electrolyte pH, buffer concentration, capillary length and electric field strength on the separation efficiency and selectivity were systematically varied, with the highest resolution achieved with buffer electrolytes of low pH and relatively high ionic strength. Under optimised separation conditions utilising the ''short end injection'' separation approach with negative electric field polarity, a series of eight structurally-related synthetic peptides were baseline resolved within 4 min without addition of any modifier of the background electrolyte with separation efficiencies in the vicinity of 600 000 theoretical plates/m. Further significant enhancement of separation efficiencies could be achieved by taking advantage of the ''long end injection'' approach with positive electric field polarity. The outcome of these experimental variations parallels the ''sweeping'' effect that has been observed in the capillary electrochromatographic and micellar electrokinetic separations of polar molecules and permits rapid resolution of peptides with focusing effects. In addition, small changes in the electrolyte buffer pH and concentration were found to have a significant impact on the selectivity of synthetic peptides of similar intrinsic charge. These observations indicate that multi-modal separation mechanisms operated under these conditions with the unmodified fused silica capillaries. This study, moreover, documents additional examples of peptide-specific multi-zoning behaviour in the high-performance capillary zone electrophoretic separation of synthetic peptides.

Keywords: Efficiency; Selectivity; Separation mechanism; Peptides

1. Introduction

During the past decade, high-performance capillary zone electrophoresis (HPCZE) has become an attractive and powerful technique for the analytical separation of peptide [1–3], protein [4–6], glycoprotein [7], oligonucleotide [8] and other biomolecule mixtures [9]. These applications reflect the simplicity of the technique, its high separation efficiency, short analysis time and facility to be coupled with other high sensitivity detection methods such as electrospray ionisation mass spectrometry (ESI-MS) [10,11]. However, binding of positively charged analytes to the wall of uncoated capillaries can be problematic with HPCZE separations [12,13] particularly when the buffer electrolyte composition has not been selected with care. It is often assumed that

*Corresponding author. Tel.: +61-3-9905-3720; fax: +61-3-9905-5882.
E-mail address: milton.hearn@med.monash.edu.au (M.T.W. Hearn).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00446-1

there are two kinds of interactions established between these (bio)analytes and the capillary wall: electrostatic and hydrophobic interactions [14–18]. At pH values above pH 3.0, the naked fused silica capillary wall has a negative charge due to ionisation of the silanol groups, a process that is essential for the generation of the electroosmotic flow. At pH values below their p*I* values, proteins or peptides will increasingly carry a net positive charge, and may then interact electrostatically with the negatively charged inner wall of a fused silica capillary. Hydrophobic interactions can also occur between the siloxane backbone network of fused silica and hydrophobic moieties accessible at the surface of these biosolutes [17]. Since most HPCZE separations employ aqueous media, hydrophobic regions of peptides will not be well solvated, further enhancing the potential involvement of other self-association binding processes [17,19].

These wall effects at the surface of the bare silica capillary may degrade the separation performance, and can lead to loss of sample, peak tailing, unstable electroosmotic flow and poor reproducibility [12,17]. Several solutions have been proposed to address these problems with polar, particularly basic, analytes including the use of: (a) high concentrations of electrolyte buffer [20,21]; (b) extreme pH values of the electrolyte buffer [22]; (c) dynamic coating of the surface of the capillaries [15,23]; or (d) chemical derivatisation of the capillary surface with ligands of different functionality [24,25]. The first approach can be easily employed although it is limited by the fact that buffers of high ionic strength tend to generate excessive Joule heating in the capillary with deterioration of separation efficiency. The use of high ionic strength buffers also leads to a reduction in the electroosmotic flow and makes the selection of low field strengths necessary. The combination of low field strength and low electroosmotic flow (EOF) then leads to prolonged separation times with peptides. However, if the Joule heating can be effectively controlled and the other separation parameters are carefully selected, this approach permits the separation of peptides and proteins within a reasonable range of separation times. With naked fused silica capillaries, buffers of extreme pH value can also be chosen to minimise adsorption of peptides onto the capillary wall. At pH values $\geq$9, when both the capillary wall and most peptides are anionic, electrical repulsion between the negatively charged double layer at the wall surface and peptides occurs; whilst at pH$\leq$3.0 with the silanol groups of the capillary wall fully protonated the interaction of most peptides in their cationic state can be significantly suppressed. Limitations of this latter approach include: (i) the generation at pH extremes of high concentrations of hydroxonium or hydroxide ions, which are very conductive and can easily cause Joule heating problems; (ii) extreme pH values may not be optimal for the separation since under such conditions the charge to mass ratios for peptides of similar sequence length can be nearly identical; and (iii) hydrophobic interactions are not eliminated. Although chemical derivatisation of the inner wall of fused silica capillaries with ligands of different functionality is an effective method to eliminate some of these wall effects, this approach generally leads to a significant reduction in electroosmotic flow and electromigration velocity of peptides and often leads to enhanced hydrophobic interactions.

The phenomenological occurrence of multi-modal separation processes reflecting the interaction of positively charged bioanalytes with silanol groups in capillary electrophoresis is well documented [18,19,26]. However, to our knowledge no comprehensive study has been carried out that has: (a) investigated the dependence of these secondary retention behaviours in relationship to the amino acid sequence utilising a comprehensive set of overlapping peptide analogues; (b) systematically explored the influence of experimental conditions that promote selectivity differences; or (c) examined an experimental framework as part of an optimisation strategy, to allow these selectivity differences to be efficiently exploited in capillary zone electrophoresis with structurally related peptides of similar charge-to-size relationships. By combining the strategies described above, namely the use of buffer electrolytes of high ionic strength and pH variation in combination with ‘‘short end injection’’ and ‘‘long end injection’’ separation techniques, rapid and highly efficient separations of structurally-related synthetic peptides can be achieved by capillary zone electrophoresis in the absence of any background electrolyte modifier, such as an alkylamine or organic solvent. Moreover, these results demonstrate

that: (a) experimental conditions can be selected to suppress the influence of wall effects with naked fused silica capillaries; (b) the occurrence of sequence-dependent secondary retention processes with structurally related peptides can be efficiently evaluated, and (c) these effects can be exploited to optimise the separation conditions.

2. Experimental

2.1. Chemicals and reagents

2.1.1. Chemicals and reagents for solid-phase peptide synthesis

Trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), piperidine, 1-hydroxybenzo-triazole (HOBt), *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU), Wang-resin with the first amino acid attached, Glu [rink amide 4-methyl benzhydrylamine (MBHA)] resin and the 9-fluorenylmethyloxycarbonyl (Fmoc) protected l-α-amino acids were purchased from Auspep (Melbourne, Australia). Phenol was obtained from Merck (Kilsyth, Australia). Thioanisole, acetic anhydride and 1,3-diiso-propylethylamine (DIEA) were obtained from Aldrich (Milwaukee, WI, USA). All solvents were of analytical grade.

2.1.2. Chemicals and reagents for reversed-phase HPLC

Acetonitrile (HPLC grade) was obtained from Biolab Scientific (Sydney, Australia), TFA was obtained from Auspep. Water was distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA).

2.1.3. Chemicals and reagents for capillary electrophoresis

Tris(hydroxymethyl)aminomethane (Tris) and 2,4-dihydroxypyrimidine (uracil) were purchased from Sigma (St. Louis, USA); sodium dihydrogenphosphate, disodium hydrogenphosphate and ammonium acetate were purchased from BDH (Kilsyth, Australia); NaOH, hydrochloride acid 36% (v/v), orthophosphoric acid 85% (v/v) and acetic acid were obtained from Ajax (Sydney, Australia); methanol, ethanol and iso-propanol (HPLC grade) were obtained from Biolab Scientific. Unless otherwise stated, all reagents were of analytical grade.

2.2. Instruments

2.2.1. Capillary electrophoresis instrumentation

Studies were performed with a HP^{3D}CE capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) at a temperature maintained at 22±1 °C. The temperature of the cassette containing the capillary was controlled by a circulating air fan, and registered by a temperature monitor (U-lab, Melbourne, Australia) and read between 21 and 23 °C during the period over which the experiments were carried out. The detection of peptides was by diode-array detection (DAD) at 214 and 254 nm.

2.3. Experimental procedures

2.3.1. Synthesis and purification of peptides 1–17

Peptides 1–17 were synthesised by solid-phase peptide synthesis (SPPS), either manually or with a PS3 Protein Technologies Automated Peptide synthesiser (Rainin, Woburn, MA, USA) using Fmoc synthesis protocols [27,28] with double-coupling where necessary, according to the amino acid sequence. The Fmoc group was cleaved with piperidine/DMF (1:4, v/v) and the reaction cycles for each additional Fmoc-amino acid using HBTU/HOBT activation were performed using DIEA as the base and a coupling time of 1 h. Peptides were cleaved from the resin and deprotected using a cleavage cocktail consisting of phenol (0.75 g), thioanisole (0.5 ml), ethandithol (0.25 ml), water (0.5 ml) and TFA (10 ml). All the peptides were lyophilised and subsequently purified by RP-HPLC on a semi-preparative TSK ODS-120T column (300×21.5 mm I.D., Tosoh, Yamaguchi, Japan) packed with 10-μm particles using a linear gradient of 0–100% B in 60 min at a flow-rate of 6 ml/min. Eluent A was water with 0.1% TFA and eluent B was 0.1% TFA in water/acetonitrile (40:60 v/v). The purified peptides were analysed on an TSK C_{18} column (150×4.6 mm I.D.) with a linear gradient of 0–85% B in 25 min at a flow-rate of 1 ml/min, and the peptides were detected at 214 nm. Peptide identity and molecular mass were confirmed by

electrospray mass spectrometry (ESI-MS) and matrix assisted laser desorption ionisation (MALDI) time of flight (TOF) MS. All peptide samples used for the HPCZE experiments were dissolved as stock solutions in water (1 mg/ml) and peptide mixtures were prepared by mixing aliquots of each peptide.

2.3.2. Capillary electrophoresis

Stock solutions of 100 m*M* phosphate buffers, pH 2.0–3.0, were prepared by titrating 100 m*M* sodium dihydrogenphosphate with phosphoric acid; whilst the 100 m*M* phosphate buffer, pH 7.0, was prepared by titrating 100 m*M* disodium hydrogenphosphate with 100 m*M* sodium dihydrogenphosphate; The stock solution of 100 m*M* ammonia acetate buffers, pH 4.0 and pH 5.0, were made by titrating 100 m*M* ammonia acetate with acetic acid; the 100 m*M* Tris–HCl buffer was made by titrating 100 m*M* Tris solution with hydrochloric acid. All the separation buffers were prepared, firstly by diluting the stock solutions to the proper concentration with Milli-Q water, then filtered through a 0.22-μm pore sized filter, and finally degassed by ultrasonicating for 10 min prior to use.

Bare fused silica capillaries with an internal diameter of 100 μm, outer diameter of 360 μm and a total length of 34.0 cm obtained from Chromatographie Service (Langerwehe, Germany) were used. For voltages between 6 and 10 kV, depending on the buffer conditions, typical currents in the range of 10–60 μA were obtained. The length from inlet to the detection window was 25.5 cm, and from the outlet to the detection end was 8.5 cm. In this study, analyses carried out with injection of the sample at the inlet end of the capillary under pressure-injection conditions (+5 mbar, 2 s) and applying positive voltage to achieve separation are termed ''long end injection'' separations. Similarly, when the sample was injected into the outlet end of the capillary under pressure-injection conditions (−5 mbar, 2 s) and a negative voltage was applied, these analyses are termed ''short end injection'' separations.

For all analytical separations the following methods were routinely employed using these capillaries: (i) −5 mbar (or +5 mbar), 2-s pressure injection of the sample from the outlet (or inlet) end of the capillary; (ii) constant voltage separation for 5–15 min; (iii) a 1-min wash with water; (iv) a 3-min wash with 0.1 *M* NaOH; (v) a 2-min wash with water; (vi) a 10-min wash with separation buffer. A 50-mbar internal pressure was applied to the inlet to perform the above wash steps. All electrophoretic separations were carried out at least in duplicate. Peptide identification was performed by ESI-MS, sample spiking and by utilising the specific DAD UV absorbance characteristics of the individual peptides. Uracil (1 mg/ml) was used as the EOF marker in all the experiments.

2.3.3. Computational procedures

The physical characteristics of peptides 1–17, their molecular masses, M_r, net charge values, q, associated with each peptide sequence, assuming an average random coil conformation for the species under the different pH conditions of the separation, were calculated using the Henderson–Hasselbach equation [29], and their charge-to-size-ratio parameters, ξ_{fric} ($\xi_{fric}=q/M_r^{2/3}$), at pH 2.0 and 3.0, were calculated utilising the pK_a values of the side chains according Dawson et al. [30] and for the C- and N-terminus according to Rickard et al. [31], implemented in the *Charge* software developed in this laboratory, formatted as Microsoft Excel version 5.0 files, whilst the statistical analysis employed the Sigmaplot 4.01 program (Jandel Scientific) linear and non-linear regression procedures.

3. Results and discussion

All peptides used in this study were synthesised on either the Wang-resin with the first amino acid attached or the Glu-(rink amide MBHA) resin by Fmoc-based solid-phase synthesis protocols, according to their specific amino acid sequence. The peptides have free N- and C-termini, and are positively charged below pH 5.0. For the first group, peptides 1–6 and peptide 8 are fragments of parent peptide 7 and involve truncations from either the N- or the C-terminus. Similarly, peptides 9–15 and peptide 17 are fragments of peptide 16 truncated from either the N- or the C-terminus. The amino acid sequences, molecular masses, calculated effective charges, q, at different pH values and charge-size ratio parameters, ξ_{fric}, of these structurally related synthetic peptides are presented in Table 1.

Table 1
Amino acid sequence, molecular mass M_r, net-charge values q, and charge-mass-ratio parameter $\zeta_{fric}=q/M_r^{2/3}$, of peptide 1–17

Peptide code	Sequence	M_r	q					ζ_{fric}	
			pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 8.0	pH 2.0	pH 3.0
1	DHDINR	768.78	2.92	2.39	1.01	0.08	−1.19	0.0347	0.0285
2	WDHDINR	955.00	2.92	2.39	1.01	0.08	−1.38	0.0301	0.0246
3	SWDHDINR	1042.08	2.92	2.39	1.01	0.07	−1.82	0.0284	0.0232
4	NSWDHDINR	1156.18	2.92	2.39	1.01	0.07	−1.82	0.0265	0.0217
5	HNSWDHDINR	1293.32	3.92	3.39	2.00	0.99	−1.37	0.0330	0.0285
6	HHNSWDHDINR	1430.46	4.92	4.39	3.00	1.91	−1.35	0.0387	0.0346
7	HHHNSWDHDINR	1567.60	5.92	5.39	3.99	2.83	−1.34	0.0438	0.0399
8	HHHNSW	816.83	3.94	3.61	3.11	2.76	−0.35	0.0451	0.0413
9	QHNHFHR	975.04	4.94	4.61	4.11	3.76	0.37	0.0502	0.0469
10	DQHNHFHR	1090.13	4.93	4.50	3.55	2.84	−0.17	0.0465	0.0425
11	QDQHNHFHR	1218.26	4.93	4.50	3.55	2.84	−0.63	0.0432	0.0394
12	HQDQHNHFHR	1355.40	5.93	5.50	4.54	3.75	−0.34	0.0484	0.0449
13	IHQDQHNHFHR	1468.56	5.93	5.50	4.54	3.75	−0.34	0.0459	0.0426
14	NIHQDQHNHFHR	1582.66	5.93	5.50	4.54	3.75	−0.79	0.0436	0.0405
15	TNIHQDQHNHFHR	1683.77	5.93	5.50	4.54	3.75	−0.34	0.0419	0.0388
16	HTNIHQDQHNHFHR	1820.91	6.93	6.50	5.53	4.67	−0.33	0.0465	0.0436
17	HTNIHQD	863.89	2.84	2.25	1.48	0.91	−1.37	0.0313	0.0248

For peptides separated in HPCZE, the apparent electrophoretic mobility, μ_{app} is the summation of the contributions from the electroosmotic flow mobility, μ_{EOF}, and the effective electrophoretic mobility, μ_e, therefore:

$$\mu_e = \mu_{app} - \mu_{EOF} \quad (1)$$

Changes in the composition and concentration of the buffer electrolyte will directly impact on the electrophoretic mobility of a charged peptide through the dependency:

$$\mu_e = \frac{e}{6\pi\eta} \sum_{k=j}^{k=-i} \frac{k}{r_{s,k}} \chi_k \quad (2)$$

where χ_k is the molar fraction of any ionised species, j; e is the electron charge (1.60×10^{-19} coulomb), η is the solution viscosity (water: 8.95×10^{-4} N s/m^2 at 298 K), $r_{s,k}$ is the Stokes radius (i.e. the radius of the sphere equivalent to the hydrated peptide) of any species j; and k the dimensionless value of the species integer charge. As the buffer electrolyte composition changes, the Stokes radius, r_s, of the peptide will also change and this will have an additional impact on the electrophoretic mobility. Similar considerations also apply when the temperature of the buffer electrolyte is varied, particularly for polypeptides with secondary structures.

For the first group of peptides, peptides 1−4 contain the same polar, ionisable amino acids and only differ in their sequences by the progressive extension with neutral or hydrophobic amino acid residues. According to Eqs. (1) and (2), these peptides were expected to exhibit similar intrinsic charge characteristics and electromobilities over the investigated pH range although the shapes of their charge titration curves as a function of pH may not necessarily be superimposable [32]. Since capillary zone electrophoresis is a charge/size-based separation technique, resolution of such peptides with very similar charge characteristics has often proved challenging. On the other hand, for peptides 5–7, there is an additional sequence extension of one positively charged amino acid residue—a histidine—from peptide 5 to peptide 7. As a consequence, separation of these three peptides and their resolution from peptides 1 to 4 at defined μ_{EOF} values was anticipated to be more straightforward. Fig. 1 illustrates the separation of peptides 1–8 at an electrolyte concentration of 10 mM for different buffer conditions corresponding to the specific pH values of pH 2.0, 4.0, 5.0 and 8.0, utilising the ''short-end injection'' approach with negative polarity of the

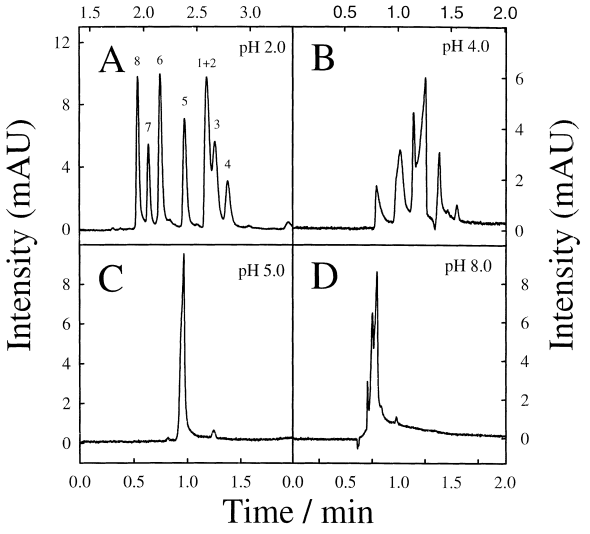

Fig. 1. Separation of peptides 1–8 with different pH buffers at a concentration of 10 m*M*: (A) phosphate buffer, pH 2.0; (B) ammonium acetate buffer, pH 4.0; (C) ammonium acetate buffer, pH 5.0; (D) Tris–HCl buffer, pH 8.0. Conditions: naked fuse silica capillary, 34.0 cm (8.5 cm effective length), 100 μm I.D; separation voltage: −6.8 kV; injection: −5 mbar, 2 s; UV detection at 214 nm.

electric field. When 10 m*M* Tris–HCl at pH 8.0 was used as the electrolyte buffer, as shown in Fig. 1D, a high EOF was achieved but the eight peptides were only partially resolved into three peaks. Under such conditions all of the peptides and the capillary wall are negatively charged. At pH 5.0, which is close to the isoelectric points (p*I*) values of peptides 1–4, the eight peptides migrated together as a single fronting peak, as depicted in Fig. 1C. Since in this case the charge on peptides 1–4 is nearly zero, their co-migration is not unexpected; however, the ξ_{fric} differences for peptides 5–8 should have facilitated their separation from peptides 1–4. At pH 4.0, the eight peptides were resolved into five fronting and tailing peaks as shown in Fig. 1B, consistent with some interaction between positively charged peptides and the negatively charged capillary wall. As can be seen from Fig. 1A, the best resolution was achieved at pH 2.0, even though the effective capillary length was only 8.5 cm and the electroosmotic flow under such conditions was small because the silanol groups of the capillary wall were essentially fully protonated. Under these conditions, these peptides migrated through the capillary essentially according to their effective electrophoretic mobilities, e.g with a negligible electroosmotic flow μ_{EOF}, and with fully protonated peptides not interacting electrostatically with the nearly fully protonated silanol groups accessible at the capillary wall. When no interaction occurs between the peptide and the capillary wall, a linear dependency is expected between the magnitude of the electric field strength and the migrational velocity of a charged peptide in a capillary electrophoretic experiment. Fig. 2 shows the results of field strength studies at 10 m*M* phosphate buffer, pH 2.0 for peptides 4–8. It can be seen that for these peptides the migration velocities followed a linear correlation with the applied field, manifestly indicating no wall interaction effects under these conditions.

The distinguishing advantage of conducting these experiments as ‘‘short end injection’’ separations is that very short analysis times can be achieved. However, under these conditions the separation of complex peptide mixtures may not necessarily be complete due to the short capillary length. When such circumstances arise, a ‘‘long end injection’’

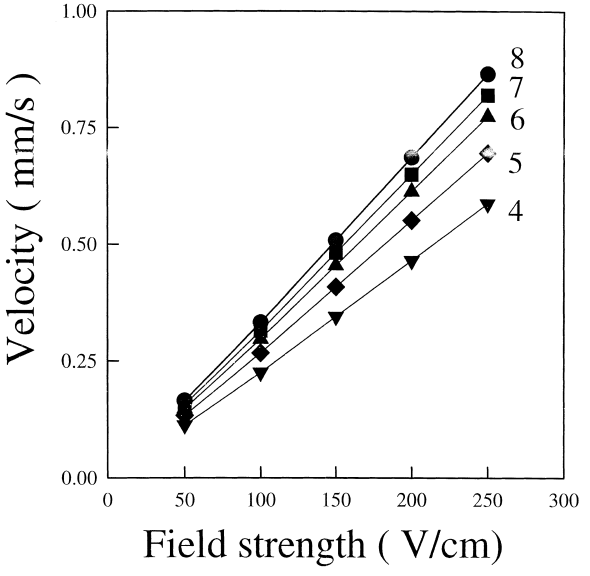

Fig. 2. The influence of field strength of 50–250 V cm^{-1} corresponding 1.7–8.5 kV on the migration velocities of peptides 4–8. Conditions: 10 m*M*, pH 2.0 phosphate buffer; naked fused silica capillary: $L=34.0$ cm, $l=8.5$ cm, 100 μm I.D; injection: −5 mbar, 2 s; UV detection at 214 nm.

separation with the polarity of the electric field reversed can be conducted in order to obtain better resolution. Thus, even though the separation efficiency was high ($N > 300\,000$ plates/m for peptides 7 and 8) for the ''short end injection'' experiments, peptides 1 and 2 could not be separated under these conditions. When a ''long end injection'' separation was performed utilising a 25.5-cm capillary under the same buffer conditions, peptides 1 and 2 still co-migrated. However, when compared to the ''short end injection'' separation, higher resolution factors and peak efficiencies were obtained for all of the other peptides, as illustrated in Fig. 3. In fact, the separation efficiency was 7.5-fold higher with the ''long end injection'' separation than obtained for the ''short end injection'' separation for peptide 7 and about sixfold higher for the remaining peptides in this set. Theoretically, if the column length increases by threefold, which was the case in these experiments, an increase in separation efficiency of ≤3-fold, rather than six or 7.5-fold, would be expected.

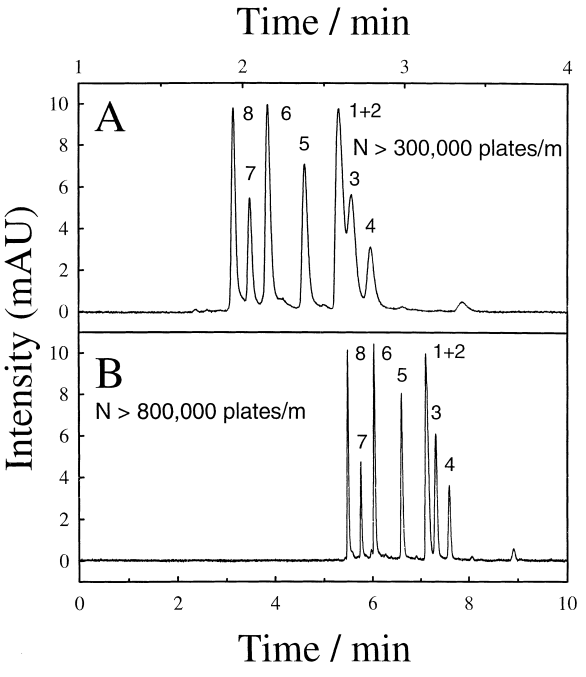

Fig. 3. Comparison between ''short end injection'' separation (A, effective capillary length: 8.5 cm, −6.8 kV) and ''long end injection'' separation (B, effective capillary length 25.5 cm, +6.8 kV) for peptides 1–8. Conditions: 10 m*M*, pH 2.0 phosphate buffer; injection: 5 mbar, 2 s; UV detection at 214 nm.

Such enhancement in peak efficiency is indicative of a ''sweeping'' phenomenon that results in the charged analyte being focused due to polyion stacking. Towns et al. [12] and Hamrnikova et al. [13] have reported on the impact of polycation adsorption on the separation efficiency in capillary electrophoresis. These workers found that the adsorption of positively charged species occurred preferentially at the capillary inlet and this produced non-uniform axial distribution of the zeta potential within the capillary, whereby leading to a reduction in the separation efficiency by up to 50% presumably due to adverse wall effects. Avoidance of such double layer/wall effects may be the reason for the unusual enhancement of separation efficiencies observed with the ''long end injection'' separations with these two peptide sets in the present study. In order to test this hypothesis further, a new bare fused silica capillary of the same size was utilised, and the separation of peptides 1–8 was carried out under the same conditions (data not shown). In this case, the separation efficiency for the first eluted peaks was about fourfold higher than obtained for the ''short end injection'' separation. Therefore, according to the criteria established by Towns et al. [12] it can be concluded that adsorption of peptide samples to the capillary wall causes an axial asymmetry of the zeta potential of the capillary surface and this significantly influences the separation efficiency of these peptides. On the other hand, the fourfold enhancement of the separation efficiency is larger than expected from theory, where peak tailing is the assumed outcome from multimodal interaction processes. According to the Van Deemter equation [33], the contributors to peak dispersion in liquid chromatographic systems can be described in terms of the following model:

$$H = A + \frac{B}{u} + Cu \tag{3}$$

where H is the height equivalent to a theoretical plate (HETP), u is the velocity of a solute through the column, and A, B and C are constants representing contribution to peak dispersion from eddy diffusion, molecular diffusion and resistance to mass transfer between the stationary and mobile phases, respectively. It is obvious that under high-performance capillary zonal electrophoretic conditions, the

contributions from band dispersion caused by eddy diffusion and mass transfer resistance are negligible, and Eq. (3) can be rewritten as:

$$H = \frac{B}{u} \quad (4)$$

$$B = 2D_m \quad (5)$$

where D_m is the diffusion coefficient of the solute. Since the temperature of the system was well controlled, the possible contribution to band dispersion from Joule heating can be excluded. If the main contribution to peak dispersing was from molecular diffusion, then the longer a peptide takes to migrate through a capillary, the more severe will be the band spreading, and consequently lower separation efficiencies would be predicted for the ''long end injection'' separation. The experimental findings indicated that enhanced, rather than reduced, peak efficiencies could be achieved under appropriate ''sweeping'' conditions with these ''long end injection'' separations, provided low pH buffer electrolyte conditions are employed. To elucidate the origin of this process further, we tested the separation efficiency of this group of peptides of different Stoke's radii at different field strengths and derived their corresponding Van Deemter plots (shown in Fig. 4). As can be seen from these plots, the relationship between H and u cannot be represented in terms of the simple dependencies described by Eqs. (4) and (5), with factors other than molecular diffusion per se influencing the separation efficiency.

In capillary electrophoresis, the concentration of the electrolyte buffer can be adjusted to enhance in some cases the separation performance, with high buffer concentrations suppressing analyte and capillary wall interactions whilst facilitating sample-stacking effects [34]. However, with electrolyte buffer of high molality, Joule heating must be carefully controlled to prevent possible peak dispersion. Lowering the field strength is a usual practice, although prolonged analysis time can then be expected. By employing the ''short end injection'' approach, the concomitant problems associated with the use of low field strength can be easily addressed. Fig. 5 shows the separation of peptides 1–8 at pH 2.0 and different concentrations of phosphate buffer. It is obvious that better separation was achieved at

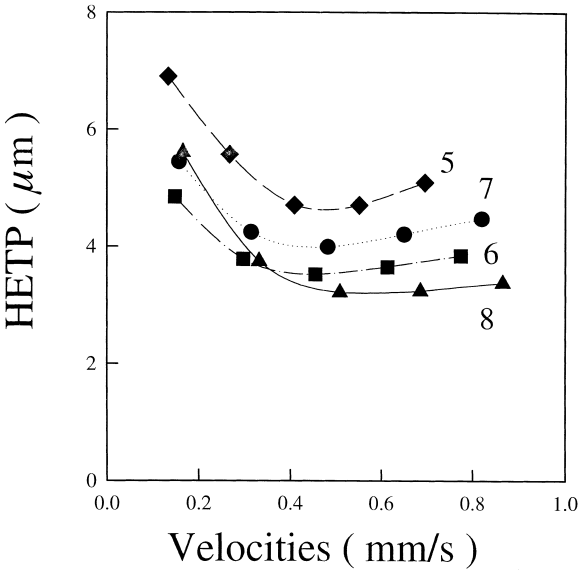

Fig. 4. The influence of migration speed on the separation efficiencies of peptides 5–8. Conditions: 10 m*M*, pH 2.0 phosphate buffer; naked fused silica capillary: $L = 34.0$ cm, $l = 8.5$ cm, 100 μm I.D; injection: −5 mbar, 2 s; UV detection at 214 nm.

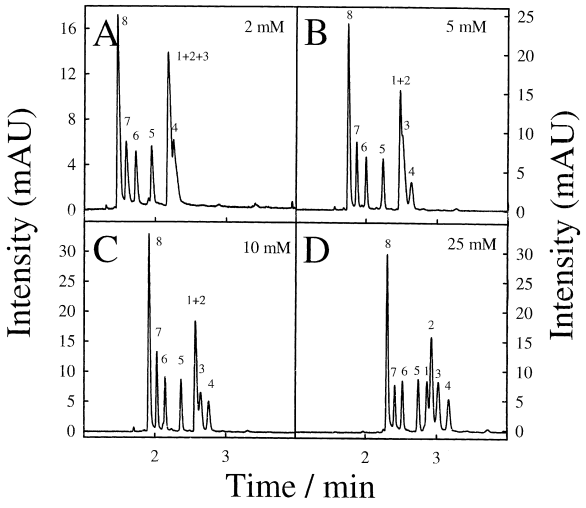

Fig. 5. The influence of buffer concentration on the separation of peptides 1–8: (A) 2 m*M*; (B) 5 m*M*; (C) 10 m*M*; (D) 25 m*M*. Conditions: phosphate buffer, pH 2.0; naked fused silica capillary, 34.0 cm (8.5 cm effective length), 100 mm I.D; separation voltage: −6.8 kV; injection: −5 mbar, 2 s; UV detection at 214 nm.

higher buffer concentrations. When a 25 m*M*, pH 2.0 phosphate buffer, and a field strength of 200 V/cm were applied, as shown in Fig. 5D, the eight peptides could be nearly baseline resolved within 3.5 min with an effective capillary length of only 8.5 cm, and separation efficiencies of about 500 000 plates/m. In order to test whether separation efficiencies could be further improved by sample stacking, the peptide 1–8 sample was diluted onefold by adding either water, methanol, ethanol, or isopropanol and separating these diluted samples under the same condition, e.g. 10 m*M* phosphate buffer, pH 2.0 with a field strength of 200 V/cm. The addition of alcoholic solvents in the sample matrix can greatly reduce the buffer's dielectric constant, viscosity and conductivity, and thus potentially will favour a sample stacking effect [35]. However, the results demonstrated that no substantive changes in separation efficiency were obtained. These observations suggest that sample stacking of the peptide in the monomeric state per se is not the sole cause of enhanced peak efficiencies or the major factor that results in high separation efficiencies under these low pH phosphate buffer conditions.

It was found from the above pH studies that the separation selectivity, especially for peptides 1–4, changed considerably when the pH of the phosphate buffer increased from 2.0 to 3.0, although the silanol groups of the capillary wall will be only partially ionised at pH 3.0. Since these four peptides have the same charge characteristics within this pH range, and peptide–capillary wall interactions were greatly suppressed by the use of high buffer concentrations, significant changes of selectivity between these four peptides should not occur if they were separated solely according to differences in their effective electrophoretic mobilities. However, investigation within this pH range demonstrated a totally different picture, as illustrated in Fig. 6. When 25 m*M* phosphate buffers with a variety of pH values, e.g. pH 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 were used, the relative migration order of peptides 1–4, especially peptide 1 with respect to the other three peptides 2–4, changed dramatically. At pH 2.0, the migration order of peptide 1–4 was 1<2<3<4, perfectly consistent with theoretical calculations [36] based on Eqs. (1) and (2); however, the relative migration order was 2<1<3<4 at pH 2.4, 2<3<1<4 at pH 2.6, and 2~3<4<1 at pH 3.0. The change of migration time of these peptides with pH is shown in Fig. 7. From these observations, we can conclude that a separation mechanism other than that of an electrophoretic process must play a role. Since the migration behaviour of peptide 1, the least hydrophobic peptide within this set of peptides, was distinctly different from peptide 2, which contains an additional tryptophan residue, it is likely that the change of the relative migration order of these peptides as a function of buffer electrolyte pH and concentration is due to secondary retention phenomenon mediated by hydrophobic interactions between these peptides and the capillary wall. Since for a given pH, the field strength plots for peptide 2 was linear, these difference in migration behaviour of peptides 2–8 compared to peptide 1 as the pH was varied (Fig. 7) is thus consistent with a mixed mode separation process occurring, with a significant hydrophobic contribution to the separation mechanism. Other experimental data where the sample concentration was varied demonstrated that at high sample concentrations peptide 1 interacted with peptide 2 with the self-associating species migrating as a single peak zone, whilst at low sample concentration these peptides migrate as discrete one peak zones with

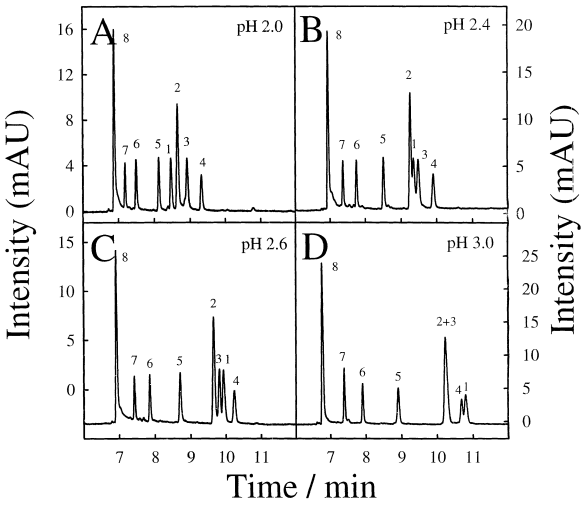

Fig. 6. The influence of buffer pH on the separation of peptides 1–8: (A) pH 2.0; (B) pH 2.4; (C) pH 2.6; (D) pH 3.0. Conditions: phosphate buffer, 25 m*M*; naked fused silica capillary, 34.0 cm (25.5 cm effective length), 100 mm I.D; separation voltage: +6.8 kV; injection: +5 mbar, 2 s; UV detection at 214 nm.

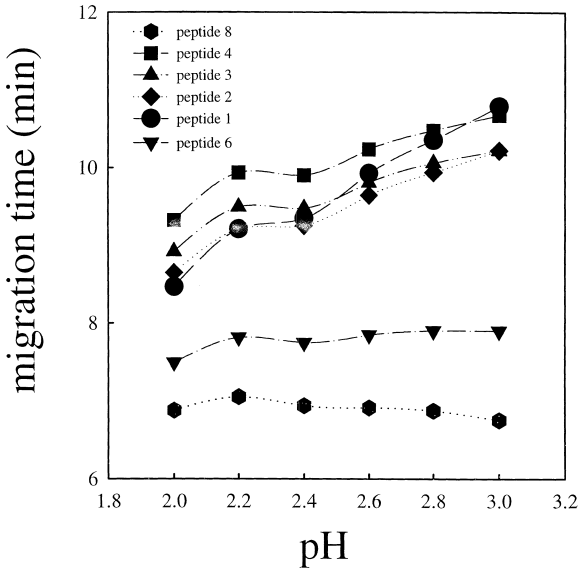

Fig. 7. The influence of buffer pH between pH 2.0 and 3.0 on the migration time of peptides 1–8. Condition: 25 m*M* phosphate buffer; naked fused silica capillary: $L = 34.0$ cm, $l = 25.5$ cm, 100 mm I.D; voltage: +6.8 kV; injection: 5 mbar, 2 s; UV detection at 214 nm.

different, intermediate migration times. This behaviour is consistent with the hypothesis that at high sample concentrations peptide 1 (and 2) can form the respective homodimers and oligomers in these HPCZE systems. This finding highlights the significant dependency of the electromigration time of peptides on sample loading conditions, and suggests that overloading effects may be a common cause for the multi-zoning behaviour observed with these systems.

The separation of the second group of peptides 9–17, which were designed to investigate not only the possibility of sequence-dependent side chain reactions, such as the intramolecular formation of N-terminal pyroglutamate residues during peptide synthesis as well as during storage [27], illustrated an analogous scenario. Most of the peptides in this group demonstrated multiple peaks over the pH ranges investigated in this study as shown in Fig. 8. Because the presence of contaminating compounds in the samples of peptides 9–17 samples can be excluded on the basis of their mass spectroscopic and chromatographic data, other explanations need to be sought for the origin of the multizoning behaviour observed in the electropherograms of these peptides. The occurrence of a pyroglutamate side chain reaction for peptide 9 and 11, as documented by MALDI-TOF-MS and ESI-MS, resulted in the presence of the second peak, which could be easily resolved in HPCZE because of the charge loss of the N-termini of these peptides. Such side chain reactions, cannot however, be the explanation for the multi-peak phenomenon of the other peptides in this second group, which lack an N-terminal glutamine residue. On the other hand, multizoning interconversion of polypeptides and proteins has often been observed in RP-HPLC leading to significantly broadened peaks, whilst Matyska et al. [37] have also observed a similar multizoning effect during the characterisation of open tubular capillary electrochromatography columns for the analysis of synthetic peptides, whereby several possible mechanisms were put forward, including conformational re-organisation. In the case of peptides 12, 14, 15 and 16 ESI-MS evaluation confirmed that the two major peaks shown in each panel of Fig. 8 had identical molecular masses, corresponding to the correct peptide sequence and thus must be a consequence of one or more effects associated with

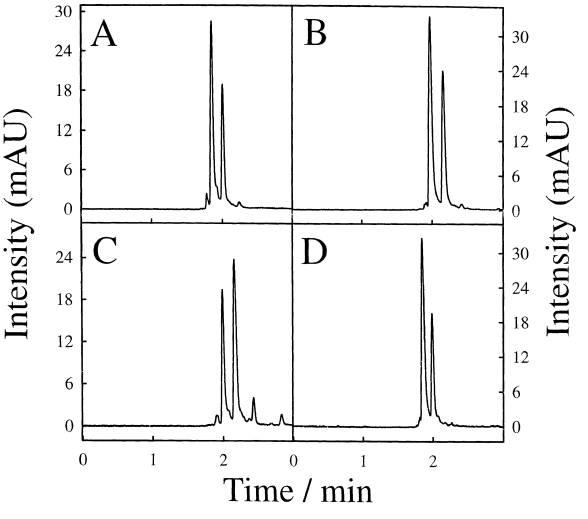

Fig. 8. The multizoning behaviour of several synthetic peptides: (A) peptide 12; (B) peptide 14; (C) peptide 15; (D) peptide 16. Conditions: 10 m*M*, pH 2.0, phosphate buffer; naked fused-silica capillary, 34.0 cm (8.5 cm effective length), 100 mm I.D; separation voltage: −6.8 kV; injection: −5 mbar, 2 s; UV detection at 214 nm.

peptide self-association, wall adsorption effects, racemisation or conformational interconversions. Our findings lead to the conclusion that this multizoning behaviour is highly peptide-specific since this phenomenon was not evident for all of the peptides used in these investigations.

4. Conclusions

As demonstrated in this study, the use of electrolyte buffers of very low pH and relatively high ionic strengths in combination with a ''short end injection'' separation approach enables peptide analogues that have very similar charge characteristics and structures to be efficiently and very rapidly separated by capillary electrophoresis with untreated bare fused-silica capillaries. Using this method the interaction of peptide analytes with the capillary wall can be suppressed; with subtle differences resulting from multimodal interactions can still be exploited to permit the resolution of peptides with very similar physico-chemical properties. The method described herein represents a fast and efficient approach for the separation of peptides with closely related physico-chemical properties. Such high-resolution HPCZE methods provide an alternative and in the case of peptides 1–17 more accurate assessment than RP-HPLC of the presence of deletion peptide impurities in samples of synthetic peptides derived from solid-phase peptide synthetic procedures. Application of such capillary electrophoretic procedures for the characterisation of the more abundant as well as lower abundant components in the crude reaction mixture of synthetic peptides can be further strengthened [38] through the application of principal component analysis and other methods of multivariate mathematical–statistical analysis, thus revealing the nature and extent of side reactions that can occur during solid-phase peptide synthesis protocols. Moreover, by exploiting the ''short end injection'' separation approach high peak efficiencies and shorter electromigration times can be achieved compared to those obtained with the conventional ''long end injection'' separation methods without sacrificing loadability or operational flexibility, thus permitting an optimum separation length for the capillary to be chosen for a given analytical task.

Acknowledgements

The financial support of the Australian Research Council is gratefully acknowledged.

References

[1] V. Kasicka, Electrophoresis 20 (1999) 3084.
[2] M.T.W. Hearn, Biologicals 29 (2001) 159.
[3] K.M. Deantonis, P.R. Brown, Y.F. Cheng, S.A. Cohen, J. Chromatogr. A 661 (1994) 279.
[4] P.G. Righetti, Biopharm. Drug Dispos. 22 (2001) 337.
[5] M.X. Huang, D. Mitchell, M. Bigelow, J. Chromatogr. B. 677 (1996) 77.
[6] F.E. Regnier, S. Lin, Chem. Anal. (NY) 146 (1998) 683.
[7] K. Kakehi, S. Honda, J. Chromatogr. A 720 (1996) 377.
[8] P.G. Righetti, C. Gelfi, M.R. D'Acunto, Electrophoresis 23 (2002) 1361.
[9] S. Hu, N.J. Dovichi, Anal. Chem. 74 (2002) 2833.
[10] B.L. Zhang, F. Foret, B.L. Karger, Anal. Chem. 72 (2000) 1015.
[11] G.A. Valaskovic, N.L. Kelleher, F.W. McLafferty, Science 273 (1996) 1199.
[12] J.K. Towns, F.E. Regnier, Anal. Chem. 64 (1992) 2473.
[13] I. Hamrnikova, I. Miksik, Z. Deyl, V. Kasicka, J. Chromatogr. A 838 (1999) 167.
[14] Z.X. Zhao, A. Malik, M.L. Lee, Anal. Chem. 65 (1993) 2747.
[15] M.J. Thornton, J.S. Fritz, C.W. Klampfl, J. High Resolut. Chromatogr. 20 (1997) 647.
[16] J. Novotna, Z. Deyl, I. Miksik, J. Chromatogr. B 681 (1996) 77.
[17] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, 2000.
[18] Z. Zhao, A. Malik, M.L. Lee, Anal. Chem. 65 (1993) 2747.
[19] W. Nashabeh, K.F. Greve, D. Kirby, F. Foret, B.L. Karger, D.H. Reifsnyder et al., Anal. Chem. 66 (1994) 2148.
[20] J.S. Green, J.W. Jorgenson, J. Chromatogr. 478 (1989) 63.
[21] M.M. Bushey, J.W. Jorgenson, J. Chromatogr. 480 (1989) 301.
[22] H.H. Lauer, D. McManigill, Anal. Chem. 58 (1986) 166.
[23] B.Y. Gong, J.W. Ho, Electrophoresis 18 (1997) 732.
[24] J.K. Towns, J. Bao, F.E. Regnier, J. Chromatogr. 599 (1992) 227.
[25] D. Figeys, R. Aebersold, J. Chromatogr. B 695 (1997) 163.
[26] I. Miksik, J. Novotna, M. Uhrova, D. Jelinkova, Z. Deyl, J. Chromatogr. A 772 (1997) 213.
[27] G.B. Fields, R.L. Noble, Int. J. Pept. Protein Res. 35 (1990) 161.
[28] R.I. Boysen, A.J.O. Jong, M.T.W. Hearn, Biophys. J. 82 (2002) 2279.
[29] B. Skoog, A. Wickman, Trend Anal. Chem., 5 (1986) 82.
[30] R.M.C. Dawson, D.C. Elliot, W.H. Elliot, K.M. Jones, Data for Biomedical Research, Clarendon Press, Oxford, 1986.

[31] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[32] M.T.W. Hearn, H.H. Keah, R.I. Boysen, I. Messana, F. Misiti, D.V. Rossetti et al., Anal. Chem. 72 (2000) 1964.
[33] J.C. Giddings, Dynamics of Chromatography, M. Dekker, New York, 1965.
[34] T. Stroink, E. Paarlberg, J.C.M. Waterval, A. Bult, W.J.M. Underberg, Electrophoresis 22 (2001) 2375.
[35] M.L. Riekkola, M. Jussila, S.P. Porras, I.E. Valko, J. Chromatogr. A 892 (2000) 155.
[36] B.R. Sitaram, H.H. Keah, M.T.W. Hearn, J. Chromatogr. A 857 (1999) 263.
[37] M.T. Matyska, J.J. Pesek, R.I. Boysen, M.T.W. Hearn, Anal. Chem. 73 (2001) 5116.
[38] I. Miksik, A. Eckhardt, T. Cserhati, E. Forgacs, J. Zicha, Z. Deyl, J. Chromatogr. A 921 (2002) 81.