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Multiple-buffer-additive strategies for enhanced capillary electrophoretic separation of peptides

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Abstract

A dodeca-peptide from the β-subunit of thyroid stimulating hormone (TSH) was used as a model system for developing "multiple-buffer-additive" strategies to effect the separation of structurally-similar peptides. A series of synthetic peptides included six peptides with identical amino acid composition and two with multiple alanine substitutions at selected positions. Those with identical amino acid composition included the native and reverse sequences of residues 101–112 of the β-TSH and four "computer-shuffled" amino acid sequences. Buffer additives such as acetonitrile (ACN), hexane sulfonic acid (HSA), and hexamethonium bromide (HxMBr), were shown to alter selectivity dramatically. HSA, an ion-pairing agent, and ACN, known to alter the hydrophobic environment of the solute, and HxMBr, which is thought to negate solute–wall interactions, are shown to independently effect only partial resolution of the mixture. It is shown that only with the proper combination of HSA and ACN are all mixture components resolved. These results re-affirm that CE selectivity may be altered by changes in buffer ionic strength or with the addition of HSA, but also show that further changes in selectivity can be achieved through alteration of buffer hydrophobicity. The observed changes in selectivity accompanying the addition of HSA and ACN may be due to differing electrophoretic mobilities resulting from nearest-neighbor effects or subtle differences in peptide secondary structure or solvation. This emphasizes the importance of employing multiple-buffer-additive strategies for effecting the resolution of peptide mixtures that are difficult to separate.

1. Introduction

High-performance capillary electrophoresis (HPCE) is an analytical technique applicable to both small (drug-like) and large (macromolecular) components of biological interest. The capability of attaining separation selectivities complementary to those with high-performance liquids chromatography (HPLC) in a rapid, automated and reproducible manner has set the stage for HPCE to become a premier method for the separation of molecules of a diverse size and nature in complex matrices [1–4]. The use of polyimide-coated fused-silica capillaries allows for the efficient dissipation of Joule heat and, hence, for electrophoretic separations to be carried out in free-solution under high fields (up to 30 000 V).

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The identification of conditions for the separation of synthetic dipeptides was one of the first successes for HPCE [5,6]. In the time since these initial reports, the high separation efficiencies inherent with HPCE have been shown to be useful for the analysis of peptides which have undergone subtle structural changes including deamidation [1], single amino acid substitution [7,8], geometrical isomers which vary only in the surface of the peptide exposed to the matrix [9] and oxidation [10] as well as glycosylation, sulfonation, phosphorylation or covalent bond formation (e.g. disulfide) [11]. Key to achieving high-efficiency separations is the use of low-pH buffer systems which leads to maximal protonation of silanol groups on the inner capillary wall (i.e. reduction of the negative character) and the peptides (making them highly positively charged). Under these conditions, high separation efficiencies are attainable as a result of minimal peptide–wall interactions).

The tremendous selectivity of CE is clearly defined through the ability to resolve peptides with the small structural differences described above. The selectivity is further emphasized in a study by Frenz et al. [7] who used strategically located histidine and arginine residues in "shuffled" peptide sequences (same total composition, shuffled amino acid order) to evaluate the parameters governing selectivity. These peptides, all theoretically possessing the same charge-to-mass (q/m) ratio were shown to have different mobilities upon modification of the pH. This demonstrated that separation was not simply based on a simple mass-to-charge ratio, but that other parameters, such as the variable nature of the surface presented to the matrix or "nearest-neighbor" effects, may play a role.

With the series of experiments described in this study, we demonstrate the utility of various buffer additives to effect the separation of structurally similar peptides. The remarkable separations obtained by appropriate combinations of additives demonstrates the sensitivity of CE to subtle differences in the charge-to-mass ratio, peptide secondary structure, and "nearest-neighbor" effects.

2. Experimental

2.1. Materials

Sodium hydroxide, phosphoric acid (85%), hydrochloric acid and acetonitrile (ACN; HPLC–UV spectral grade) were purchased from Fisher Scientific. All chemicals for peptide synthesis were purchased from Applied Biosystems (Foster City, CA, USA). Borax (sodium tetraborate), boric acid, hexamethonium bromide (HxMBr), hexamethonium chloride (HxMCl), decamethonium bromide (DcMBr), and hexanesulfonic acid (HSA) were purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL, USA).

2.2. Peptide synthesis

The thyroid-stimulating hormone (TSH) peptide corresponding to the native sequence (residues 101–112) and its analogues were synthesized by 9-fluorenylmethoxycarbonyl (FMOC) amino acid strategy on an ABI 431A peptide synthesizer (Applied Biosystems) using the protocols and reagents provided by the manufacturer. Each peptide was purified by RP-HPLC using a Vydac C_{18} column (25 × 2.2 cm) using a trifluoroacetic acid (TFA)–acetonitrile buffer system. Peptide integrity was monitored by either amino acid analysis or plasma desorption mass spectrometry.

2.3. Buffer and sample preparation

Phosphate buffer was made by diluting a 1.0 *M* stock solution and adjusting the pH with NaOH. All buffers were made with Milli-Q (Millipore) water, and filtered through an 0.2-μm filter (Gelman) before use. Additives were made as 1 *M* stock solutions, and added to the appropriately pH'ed buffer to the final concentration. Stock solutions of peptides were solubilized in Milli-Q purified water at a final concentration of 1 mg/ml. The mixture of peptides was made by taking an equal volume of each

peptide resulting in a concentration of 125 μg/ml for each peptide. Samples were frozen until used. Peptide solutions were filtered (0.2 μm, Millipore) before use. Confirmation of peptide peak identity was made by double injection of the mixture and a solution containing a single peptide.

2.4. High-performance liquid chromatography

HPLC separation was carried out on an ABI 130A separation system (Applied Biosystems) using an ABI Aquapore OD-300 (C_{18}) (100 × 2.1 mm) column. Running conditions included a flow-rate of 0.2 ml/min with a gradient of 0 to 25% B over the course of 30 min starting at 6 min (buffer A: 25 m*M* sodium phosphate, pH 2.5; buffer B: acetonitrile–water (40:60) with 25 m*M* sodium phosphate, pH 2.5). Detection was at 215 nm.

2.5. CE Instrumentation

HPCE separation was carried out on a Beckman P/ACE System 2050 or 2100 interfaced with an IBM 55SX computer utilizing System Gold software (V. 7.1) for instrument control and data collection. All peak information (migration time) was obtained through the System Gold software.

2.6. CE separation conditions

Separations were carried out in a phosphate running buffer, ranging in concentration from 50 to 150 m*M*, pH 2.0 with or without additives, such as HSA, ACN and HxMBr. The standard method used was as follows: a three-column volume rinse with running buffer, 5 s pressure injection (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) of peptide mixture (125 μg/ml of each peptide), separation at constant voltage (with the inlet as the anode and the outlet as the cathode), a five-column volume wash with 0.1 *M* NaOH followed by a five-column volume rinse with running buffer. Capillaries were polyimide-coated fused silica, 57 cm (50 cm to the detector) × 50 μm I.D. Capillary temperature was maintained at 28°C. Detection was by absorbance at 200 nm.

3. Results and discussion

One of the clear advantages of CE over other complimentary techniques is the ease with which the selectivity can be changed. This can be achieved through a number of approaches including the use of buffer additives which add a chromatographic component to the separation (e.g. micelles with detergents) or those which dynamically coat the inner surface of the capillary (alkylamines).

The vast literature on peptide analysis by CE clearly defines the utility of this technique for resolving peptides (see [11] and references cited therein). Among these are numerous examples describing the usefulness of single-component, low-pH buffer for resolving peptides with struc-

Table 1
Amino acid sequence of the peptide containing residues 101 to 112 from the β-subunit of TSH and its analogues used in this study

Peptide	Description	Amino acid sequence
1	Native	Lys–Thr–Asn–Tyr–Cys–Thr–Lys–Pro–Gln–Lys–Ser–Tyr
2	Ala_8-Substituted	Lys–Ala–Ala–Ala–Cys–Ala–Lys–Ala–Ala–Lys–Ala–Ala
3	Ala_7-Substituted	Lys–Ala–Ala–Tyr–Cys–Ala–Lys–Ala–Ala–Lys–Ala–Ala
4	Reverse	Tyr–Ser–Lys–Gln–Pro–Lys–Thr–Cys–Tyr–Asn–Thr–Lys
5	Shuffle 1	Lys–Pro–Asn–Lys–Ser–Tyr–Cys–Tyr–Thr–Gln–Thr–Lys
6	Shuffle 2	Gln–Pro–Ser–Lys–Lys–Thr–Tyr–Cys–Lys–Thr–Tyr–Asn
7	Shuffle 3	Lys–Pro–Thr–Gln–Tyr–Asn–Lys–Ser–Thr–Tyr–Lys–Cys
8	Shuffle 4	Lys–Lys–Asn–Lys–Pro–Tyr–Cys–Thr–Gln–Thr–Ser–Tyr

tural differences as minimal as a single amino acid change. For the purpose of defining subtle selectivity changes in low pH buffer systems as a function of "multiple-buffer additives", a series of peptides were used where the peptides involved differed only in amino acid sequence. Using a dodeca-peptide (residues 101–112) from the β-subunit of TSH as a model peptide, a series of synthetic dodeca-peptides containing variations on the "native" primary sequence (Table 1) was synthesized. The synthetic peptides included the "native" and "reverse" sequences, as well as four peptides with "shuffled" amino acid sequences of the native TSH peptide. Two additional peptides containing multiple alanine substitutions at selected (neutral) positions in the sequence were also synthesized. These eight peptides provide an excellent model mixture for evaluation of buffer additives for enhancing selectivity for the CE separation of structurally similar peptides.

The RP-HPLC separation of the peptide mixture is given in Fig. 1. Four peptides are completely resolved, while the remaining four are only partially resolved. The fact that the native (peptide 1) and reverse (peptide 4) sequences are well resolved highlights the importance of nearest-neighbor effects or slight differences in secondary structure resulting from the reversed N- and C-terminal amino acids.

Fig. 2 shows the separation of the model peptides in a phosphate buffer, pH 2.05, where the concentration of phosphate is varied from 50 to 150 m*M*. As the phosphate concentration is increased the expected increase in migration time for all peaks as a result of decreased electroosmotic flow (EOF) is observed. The resolution of the structurally similar peptides, 1, 4, 5 and 6, is enhanced slightly at higher phosphate concentrations, despite the increase in system current accompanying these higher concentration buffers (37, 52 and 75 μA for 50, 100 and 150 m*M*, respectively). The fact that peptide 6 is only barely resolved as a shoulder in 50 m*M*, lost into the peak containing peptides 1 and 4 (as a back shoulder) in 100 m*M*, and optimally

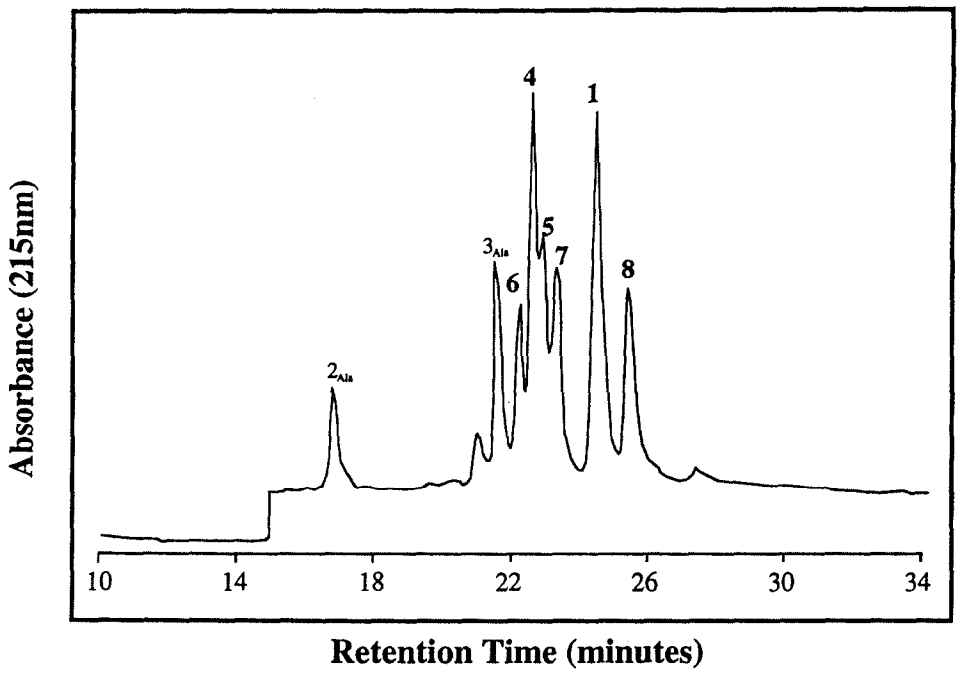

Fig. 1. HPLC separation of the components of the peptide mixture. An ABI Aquapore OD-300 (100 × 2.1 mm) column was equilibrated with buffer A (25 m*M* sodium phosphate, pH 2.5) and a gradient of 0 to 25% B [buffer B: acetonitrile–water (40:60) with 25 m*M* sodium phosphate, pH 2.5] applied over the course of 30 min starting at 6 min.

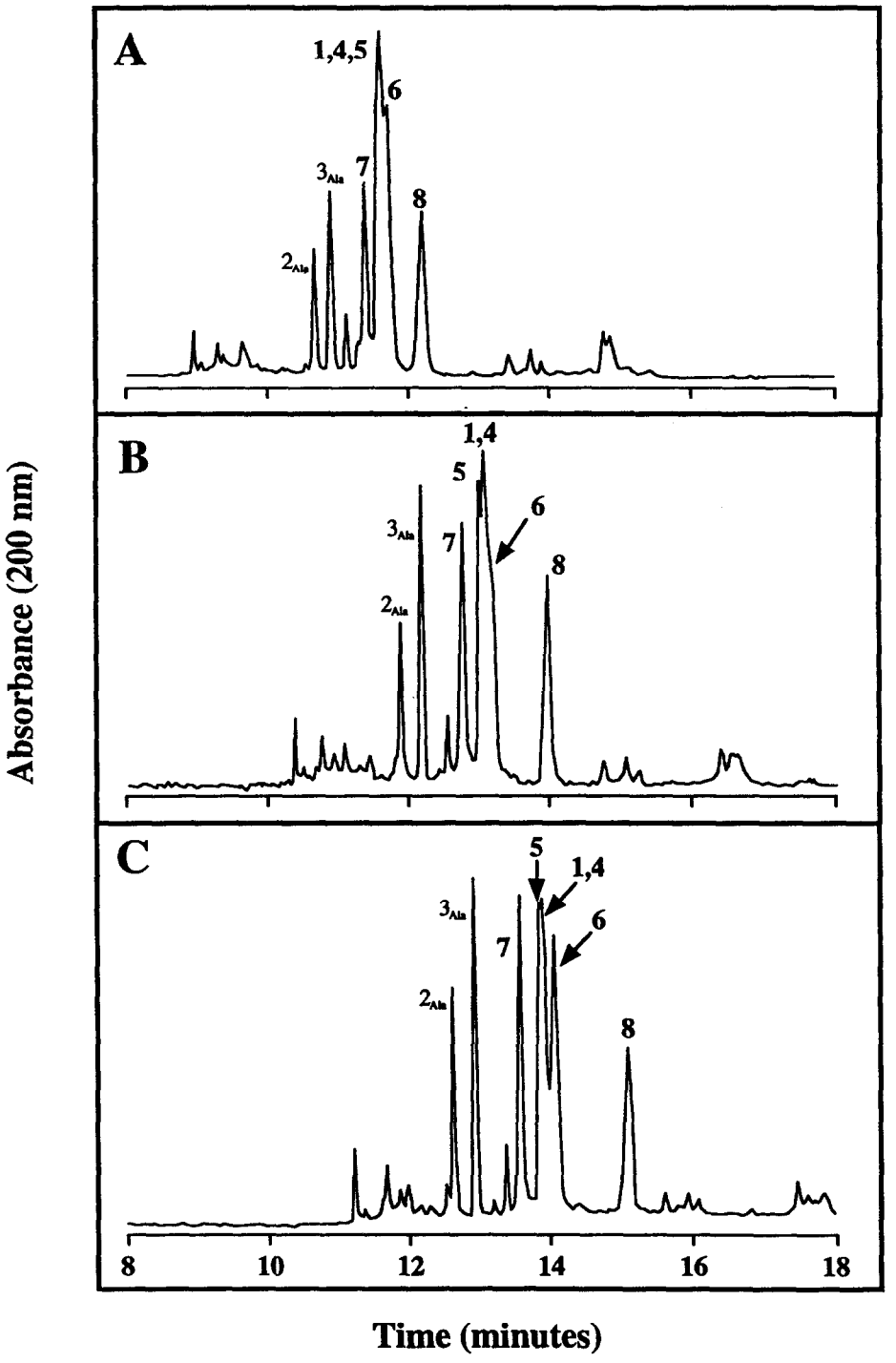

Fig. 2. Separation of the shuffled peptides in varied concentrations of phosphoric acid, pH 2.0. (A) 50 m*M* phosphoric acid, (B) 100 m*M* phosphoric acid, (C) 150 m*M* phosphoric acid. Bare fused-silica capillary, 57 cm (50 cm to the detector) × 50 μm I.D. Separation conditions: 15 kV, 28°C.

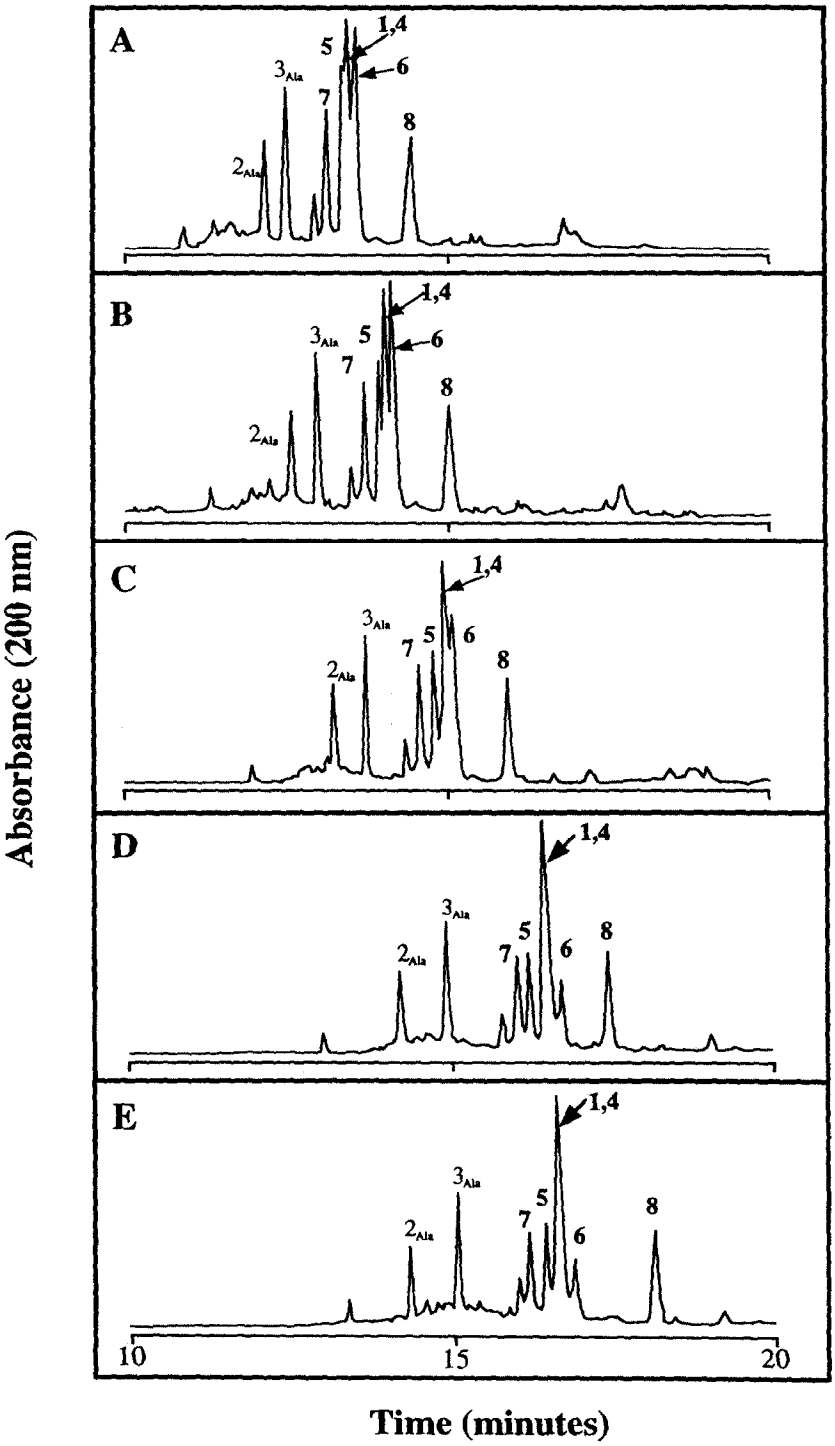

Fig. 3. Dependence of the separation of the peptides on the concentration of hexanesulfonic acid. The separation buffer contained: (A) 50 m*M* phosphoric acid, pH 2.0, 10 m*M* HSA; (B) 50 m*M* phosphoric acid, pH 2.0, 25 m*M* HSA; (C) 50 m*M* phosphoric acid, pH 2.0, 50 m*M* HSA; (D) 50 m*M* phosphoric acid, pH 2.0, 100 m*M* HSA; (E) 100 m*M* HSA, in distilled water, titrated to pH 2.0 with sulfuric acid. Bare fused-silica capillary, 57 cm (50 cm to the detector) × 50 μm I.D. Separation conditions: 15 kV, 28°C.

resolved in 150 m*M* phosphate indicates that slight changes in selectivity are occurring with the increases in ionic strength and decreases in EOF.

The addition of HSA to the separation buffer has been shown to be useful for the separation of peptides [12]. Its effect on the separation of the model peptide mixture as a result of the addition of HSA to 50 m*M* phosphate buffer (pH 2.05) is given in Fig. 3. The most obvious effect resulting from the presence of HSA is the increased migration time for all peaks as a result of the increased ionic strength and subsequent decrease in EOF. When HSA is present at concentrations below the critical micellar concentration (Fig. 3A; HSA = 10 m*M*), the resolution of peptide 6 is very similar to that observed with no HSA and 150 m*M* phosphate (Fig. 2C). The resolution is significantly improved with the addition of HSA at the critical micellar concentration (CMC = 460 m*M*) where peptide 6 is clearly resolved and peptide 5 begins to resolve from the peak containing peptides 1 and 4 (Fig. 3B). Further increase in the HSA concentration led to better resolution of peptide 5 from the peak containing peptides 1 and 4 and appeared to be optimal at 100 m*M* (Fig. 3C and D). Analysis of the same sample mixture in 100 m*M* HSA without 50 m*M* phosphate (pH 2.05 attained by titration with sulfuric acid) showed that the observed resolution was not dependent on the presence of phosphate in the separation buffer (Fig. 3E).

Acetonitrile has been shown to be a useful additive for peptide analysis by CE [13]. This additive has been postulated to enhance CE separation through either differential solvation of the peptides or solvent-induced differences in the ionization of the peptide termini and/or charged amino acid side chains. The effect of acetonitrile (in 50 m*M* phosphoric acid, pH 2.0) on the resolution of the model peptides is given in Fig. 4. The presence of this organic additive at a concentration of 10% (v/v) led to the separation of peptides 4 and 5 from 1 and 6. Doubling the acetonitrile concentration resulted in better separation of peptide 6 from peptide 1 which was now a shoulder on the peak containing peptides 1, 4 and 5.

When none of the individual components (described in the previous figures) allowed for resolution of all eight peptides, combinations of these additives were tested. The separation conditions that proved to be optimal for separation of these peptides, including the partial resolution of the native (1) and reverse (4) peptides, was a buffer containing 50 m*M* phosphate (pH 2.05), 100 m*M* HSA and 10% ACN. Fig. 5 shows a comparison of the separation obtained with phosphate alone (A), phosphate and HSA (B) and phosphate, HSA and ACN (C and D). Resolution of the native and reverse peptides appears to be optimal in the presence of 10% ACN. This separation was observed under no other conditions. Increasing the ACN concentration to 20%, changes the selectivity to a significant extent leading to the single peak resolution of peptide 4, but the loss of resolution between peptides 1 and 6.

A buffer additive shown to be useful for resolving glycoproteins with small structural differences [14] was also tested (Fig. 6). The separation of the peptide mixture in the presence of hexamethonium bromide, a bis-quaternary ammonium compound, in the presence and absence of acetonitrile was tested. The addition of HxMBr (final concentration, 5 m*M*) to 50 m*M* phosphate buffer, pH 2.05, resulted in a slight increase in migration time, but not near the magnitude observed at more neutral pH values [14]. The general migration order of the peaks was similar to that observed in phosphate buffer alone, i.e. peak 2, peak 3, peak 7, peaks 1, 4, 5, 6 and peak 8. Partial resolution of peptides 4 and 5 from the peak containing 1 and 6 was observed and could be selectively changed by the addition of 10% acetonitrile. Under these conditions, peptides 1, 4, 5 and 6 are not resolved as single entities but instead as two peaks, one containing peptides 4 and 5 the other containing peptides 1 and 6.

4. Conclusions

The results of this study demonstrate that the presence of multiple additives in the separation

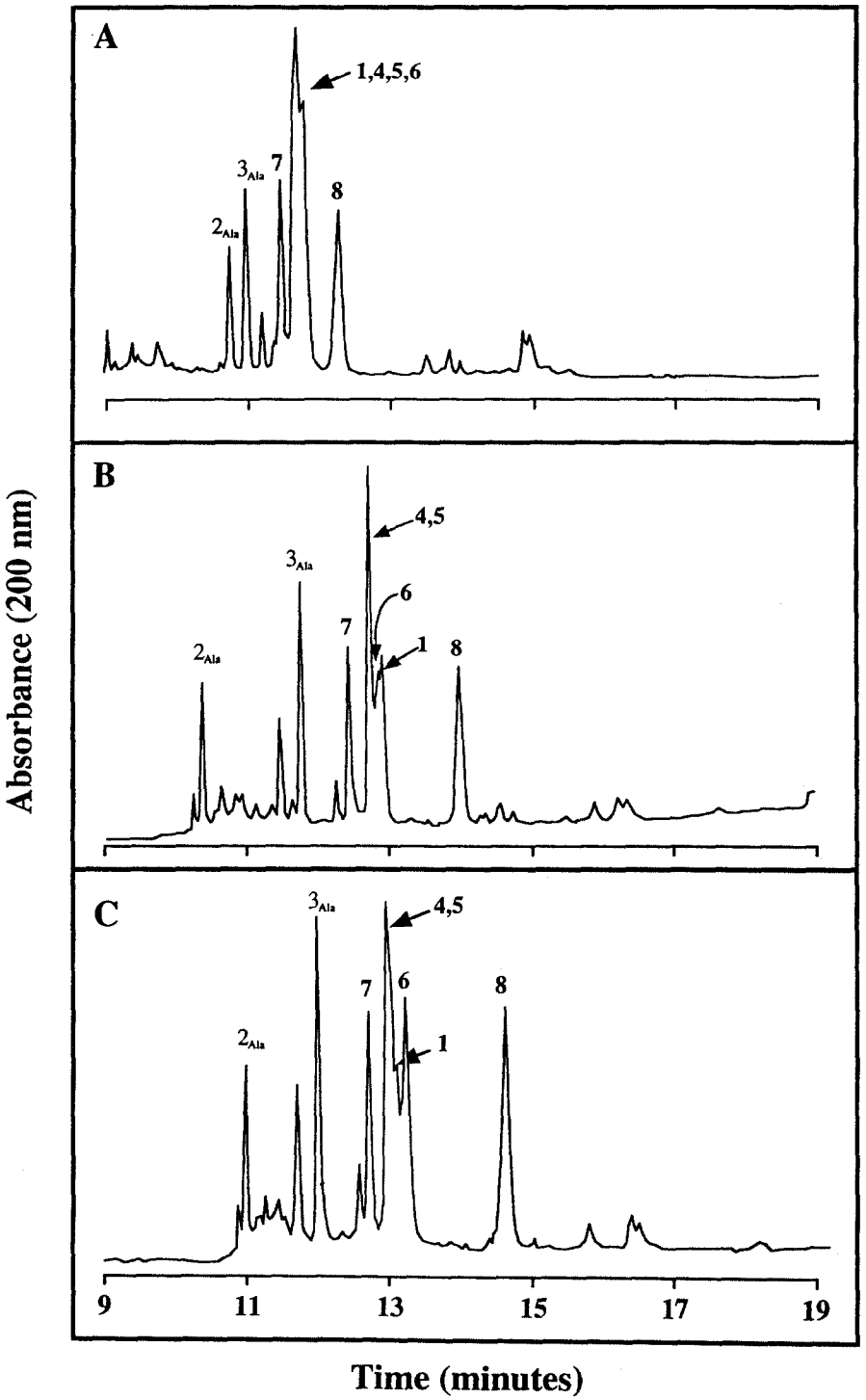

Fig. 4. The effect of acetonitrile on resolution of the peptides in 50 m*M* phosphoric acid, pH 2.0. The separation buffer contained: (A) 50 m*M* phosphoric acid, pH 2.0, no additive; (B) 50 m*M* phosphoric acid, pH 2.0, 10% (v/v) acetonitrile; (C) 50 m*M* phosphoric acid, pH 2.0, 20% (v/v) acetonitrile. Bare fused-silica capillary, 57 cm (50 cm to the detector) × 50 μm I.D. Separation conditions: 15 kV, 28°C.

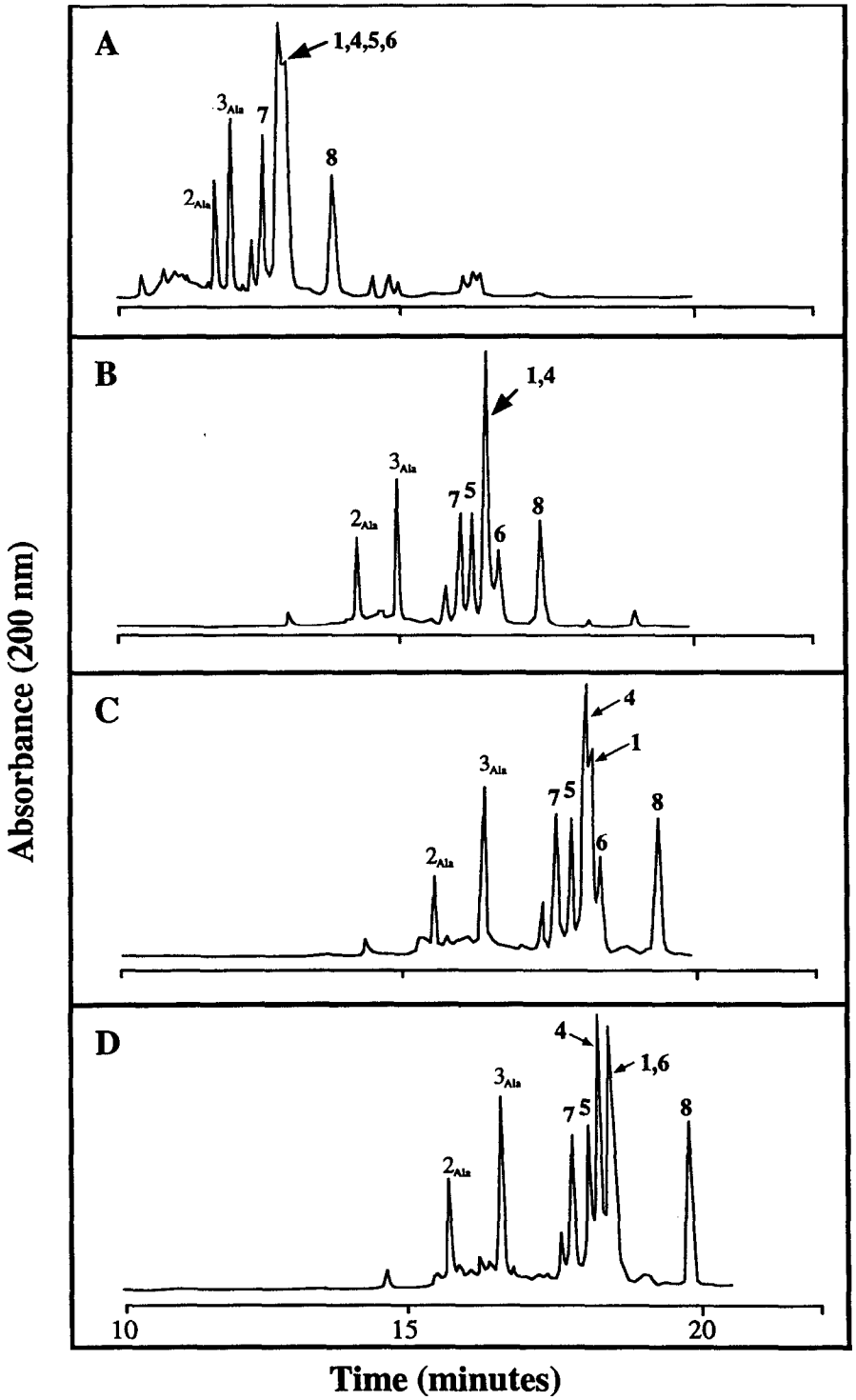

Fig. 5. The combined effect of acetonitrile and hexanesulfonic acid on the separation of the peptides in 50 m*M* phosphoric acid, pH 2.0. The separation buffers contained: (A) 50 m*M* phosphoric acid, pH 2.0; (B) 50 m*M* phosphoric acid, pH 2.0, 100 m*M* HSA, 0% (v/v) acetonitrile; (C) 50 m*M* phosphoric acid, pH 2.0, 100 m*M* HSA, 10% (v/v) acetonitrile; (D) 50 m*M* phosphoric acid, pH 2.0, 100 m*M* HSA, 20% acetonitrile. Bare fused-silica capillary, 57 cm (50 cm to the detector) × 50 μm I.D. Separation conditions: 15 kV, 28°C.

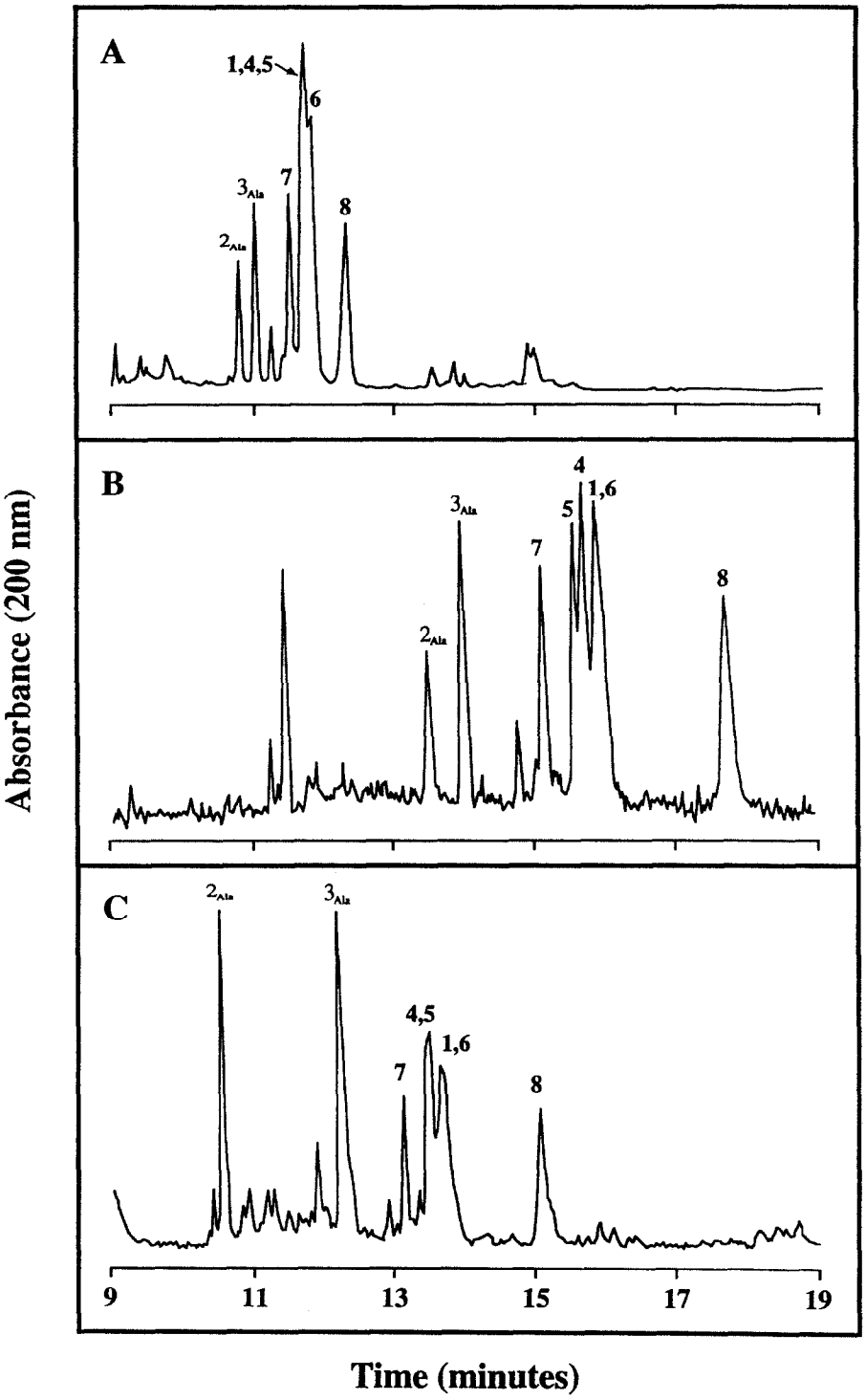

Fig. 6. The separation of the peptides in the presence of hexamethonium bromide and acetonitrile. The separation buffer contained: (A) 50 m*M* phosphoric acid, pH 2.0; (B) 50 m*M* phosphoric acid, pH 2.0, 5 m*M* hexamethonium bromide; (C) 50 m*M* phosphoric acid, pH 2.0, 5 m*M* hexamethonium bromide, 10% (v/v) acetonitrile. Bare fused-silica capillary, 57 cm (50 cm to the detector) × 50 μm I.D. Separation conditions: 15 kV, 28°C.

buffer can lead to subtle but important changes in CE selectivity. This has been shown through the successful separation of peptides with identical composition but different amino acid sequence. In free solution CE or CZE, charge and mass are thought to be the main parameters determining the electrophoretic mobility of analytes. The partial separation of the model peptides effected with simple changes in buffer ionic strength highlight the sensitivity of CE to subtle changes in mass-to-charge ratio, perhaps through nearest-neighbor effects on the pK_a value of the protonatable residues. The addition of the ion-pairing agent, HSA, at concentrations considerably less than the CMC has dramatic effects on the resolution of the peptides. There may be sequence-specific differences in the ability of the negatively-charged HSA to ion-pair with the positively-charged peptides, perhaps a result of subtle charge differences due to nearest-neighbor effects. The addition of ACN changes the hydrophobicity of the solvent and affects separation probably by changing the solvation state of the peptide and/or alterating the interaction of the peptides with HSA. None of the individual manipulations in buffer composition (ionic strength, ion pairing or hydrophobicity) resulted in resolution of all mixture components; it was only through modification of all of these parameters that resolution of the individual components was successful. This study indicates that by altering the ionic environment, the solvation state of the peptide and the hydrophobicity of the buffer, one may effect the separation of structurally-similar molecules. Multiple-buffer-additive strategies should therefore be explored to exploit the combinatorial selectivities when individual additives do not effect adequate separation.

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