Journal of Chromatography, 470 (1989) 241–250
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 223

CAPILLARY ZONE ELECTROPHORESIS OF HISTIDINE-CONTAINING COMPOUNDS

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SUMMARY

Capillary zone electrophoresis has been tested for the separation of angiotensins, cationic heptapeptides and model histidine derivatives. Good separation efficiencies are seen for peptides and model compounds with negative to small positive net charges. For net charge greater than +2, addition of putrescine to pH 6 buffer greatly suppresses ion exchange at anionic sites on fused silica. When operating at pH values where histidine groups are neutral, addition of Zn^{2+} allows separations based on metal, rather than proton, binding. Separation efficiencies and relative migration times are dependent on capillary length when ion-exchange behavior occurs.

INTRODUCTION

Capillary zone electrophoresis (CZE) is a powerful, high-speed separation technique featuring high resolution, flexible operating conditions and on-line detection[1,2]. The method has proved particularly valuable for separating proteins[3–6] and amino acids [7–9]. Relatively little work has been done on peptide separations by CZE. Firestone *et al.*[10] used a commercial isotachophoresis (ITP) analyzer with a 0.5 mm I.D. PTFE capillary to determine Phe–His purity. Use of CZE–mass spectroscopy for the identification of Phe–Phe, Trp–Phe, leucine enkephalin and vasotocin has been reported[11]. Labeled peptides from tryptic digests of chicken egg white have been separated[12], but not identified.

The predominant factor affecting peptide mobility changes over the CZE operating pH range 4–10 is ionization of His moeities. At low pH values, accumulation of positive charge due to protonation of His could lead to interactions with fixed, negative charges on capillary walls, resulting in decreased efficiencies. Previous studies[4] have discussed difficulties in obtaining high efficiency CZE separations of positively charged proteins due to surface interactions. It is not clear how serious this problem is for small peptide separations.

This study reports CZE separations of various His containing compounds: model His derivatives, blocked His–Gly copeptides and human form angiotensins. The net positive charge necessary for significant peak broadening due to surface interactions is assessed, and improvements in efficiencies of cationic peptide separations are explored through use of metal or organic cation additives. Investigations

0021-9673/89/$03.50

are performed using a modified, commercial ITP instrument and a liquid chromatography UV–VIS detector.

EXPERIMENTAL

Apparatus

LKB 2127 Tachophor (Bromma, Sweden) power supply and analyzer units were used to supply high voltage to the CZE buffer reservoirs. The ITP capillary and detector were replaced with two 50-cm 1.5 mm × 0.8 mm I.D. PTFE capillaries. These wide-bore capillaries were filled with working buffer and provided electrical contact to the 25-ml polypropylene beakers used as CZE reservoirs. The terminating reservoir of the Tachophor was fitted with a membrane to prevent drainage to the CZE reservoir. Reservoirs in the Tachophor analyzer unit were filled with deionized water. A schematic diagram of the connections for CZE are shown in Fig. 1. One potential advantage of this arrangement with buffer filled electrical contacts is isolation of the electrolysis products from the CZE reservoirs.

Untreated 75 μm I.D. fused-silica capillaries (Scientific Glass Engineering, Austin, TX, U.S.A.) were used for electrophoresis. Active capillary lengths (anode-to-detector) were 40 or 70 cm with total lengths (anode-to-cathode) of 70 and 100 cm, respectively. Capillaries were mounted through a 1-mm, straight-bore, preparative flow cell cassette of an ISCO V4 UV–VIS detector (Lincoln, NE, U.S.A.), as shown in Fig. 2. A 1-cm section of the outer polymer coating on the capillary at the location of the detector window was removed with a razor blade prior to mounting.

Chemicals

Buffer solutions used for electrophoresis are shown in Table I. CHES, MES and Tricine were obtained from Sigma (St. Louis, MO, U.S.A.). Potassium phosphate

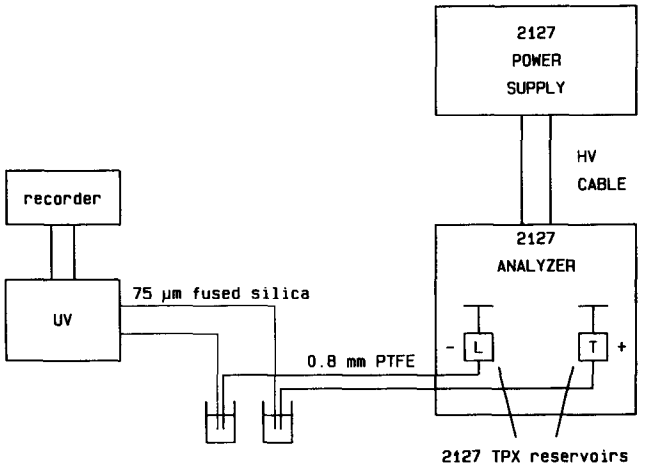

Fig. 1. Schematic diagram of connections from LKB 2127 Tachophor to external buffer compartments and ISCO V4 for CZE.

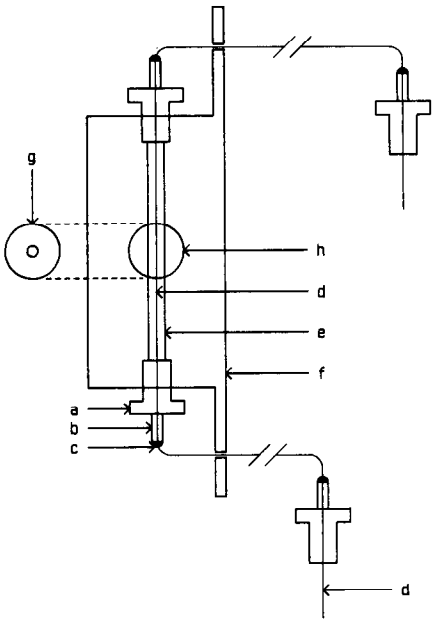

Fig. 2. Detail of connection of fused-silica capillary to ISCO V4 1 mm, preparative flow cell cassette. Side view of cassette. (a) Omnifit 1/4 in. × 28 fitting, (b) 1.5 × 0.3 mm PTFE tubing, (c) epoxy seal, (d) fused-silica capillary, (e) 1-mm flow path, (f) cassette, (g) 0.8-mm incident aperture, (h) flow cell window.

monobasic, potassium hydroxide and potassium chloride were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Zinc perchlorate hexahydrate was obtained from Johnson Matthey (Seabrook, NH, U.S.A.). Buffers were prepared at the molarities listed in Table I, and the pH was adjusted with 1 *M* KOH.

His-containing compounds tested are listed in Table II. All compounds were obtained from Sigma, except for the 4-His, 3,5-diHis and 2,4,6-triHis heptapeptides.

TABLE I

COMPOSITION OF BUFFER SYSTEMS AND RESULTING CURRENTS IN THE 40 cm AND 70 cm × 75 μm CAPILLARIES

pH	*Buffer system*	*μA at 15 kV*	
		40 cm	*70 cm*
6	20 m*M* N-morpholinoethanesulfonic acid (MES) 10 m*M* KCl	17	–
7	10 m*M* KH_2PO_4	14	9.7
7.5	20 m*M* N-tris(hydroxy methyl)methyl glycine (Tricine) 10 m*M* KCl	13	–
8	20 m*M* Tricine 10 m*M* KCl	17	–
9	20 m*M* N-cyclohexylaminoethanesulfonic acid (CHES) 10 m*M* KCl	15	–

TABLE II

COMPOUNDS STUDIED

Histidine derivatives	
(1)	L-Histidine methyl ester (HME)
(2)	Glycyl-L-histidine (Gly-His)
(3)	L-Histidine (His)
(4)	N-Acetyl-L-histidine (NAH)
Model heptapeptides	
(M)	Hca-Gly-Gly-Gly-His-Gly-Gly-Gly-NH-CH_3 (4-His) (Hca = hydrocinnamyl)
(D)	Hca-Gly-Gly-His-Gly-His-Gly-Gly-NH-CH_3 (3,5-diHis)
(T)	PMH-Gly-His-Gly-His-Gly-His-Gly-NH-CH_3 (2,4,6-triHis) (PMH = p-methoxy hydrocinnamyl)
Angiotensins	
(I)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
(II)	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
(III)	Arg-Val-Tyr-Ile-His-Pro-Phe

These peptides were synthesized by standard solid-phase techniques on an automated peptide synthesizer (Biosearch Model 9500, San Rafael, CA, U.S.A.). All His were double coupled using Boc-tosyl His. The resin used was a standard polystyrene 1% cross-linked with divinylbenzene. The peptides were cleaved from the resin using neat methylamine at 4°C (8 p.s.i.g.) for 12 h. Methylamine was removed under vacuum and residual amine neutralized with acetic acid, all at 4°C. The crude mixture was dissolved in water and separated from the spent resin. Purification to homogeneity was accomplished by preparative reversed-phase chromatography using Vydac C_{-18} alumina (The Separations Group, Hesperia, CA, U.S.A.) eluted with a linear acetonitrile–water gradient containing 1% trifluoroacetic acid.

Procedures

UV detection was performed at 220 nm, 0.01 a.u.f.s. for all runs. The LKB power supply was operated at a separation voltage of 15 kV in a constant current mode. Sampling was performed by electromigration at 5kV for 5 s from samples diluted in the appropriate buffer. Electropherograms were recorded on a Kipp and Zonen BD-41 strip chart recorder at 1 cm/min chart speed, or were processed using a data acquisition system described previously[13].

pK_a titrations

Concentration-based pK_a values for the synthetic heptapeptides were determined by potentiometric titration in 0.5 *M* $NaClO_4$. A Metrohm 636 Autotitrator (Herisau, Switzerland) was used with a Metrohm Model 6-0210-100 combination pH electrode. The reference filling solution was 3 *M* NaCl–0.5 *M* $NaClO_4$. The titrant 10 m*M* NaOH–0.5 *M* $NaClO_4$ was standardized *vs.* dried potassium acid phthalate from the National Bureau of Standards (Gaithersburg, MD, U.S.A.). Electrode calibration in pH was performed by titrating 10 ml of 2 m*M* $HClO_4$–0.5 *M* $NaClO_4$. Peptides

were diluted to 1 m*M* in 0.5 *M* $NaClO_4$ and, if needed, excess $HClO_4$ added. All titrations were performed in duplicate and the data analyzed using Motekaitis and Martell's PKAS program[14].

RESULTS

Model compounds

Fig. 3 shows separations obtained for the four model compounds listed in Table I. These compounds were selected to give one anionic species (NAH,4), one cationic species (HME,1) and two nominally neutral species (His,3 and Gly–His,2) in the pH range covered. Good separations are seen with each buffer. Numbers of theoretical plates, *N*, are highest for anionic NAH, 170 000 at pH 9, and are lowest for cationic HME, 11 000 at pH 6. Broader peaks for His are due to a comigrating impurity, which can be seen at pH 7. Peak broadening for the cationic model compounds is measurable; however, reasonable efficiencies for all compounds are seen across this pH range.

Of interest is the reversal of migration order for His and Gly–His between pH 7 and 8. These migration orders are consistent with calculated charges on the molecules at different pH values (see Discussion, Table III).

Cationic heptapeptides

Heptapeptides containing a glycine backbone with one (M), two (D) or three (T) His, as given in Table I, were run at pH 6 – 9. The peptides are blocked at both N- and C-termini, so that only charges on the His govern the total peptide charge.

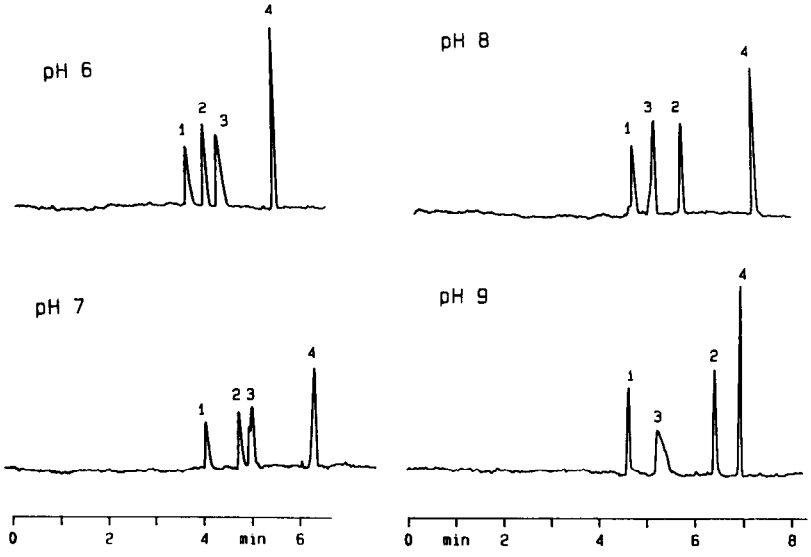

Fig. 3. Separation of small model compounds *vs.* pH. 1 = HME, 2 = Gly–His, 3 = His, 4 = NAH. 0.4 mg/ml each compound; 40-cm capillary; 15 kV.

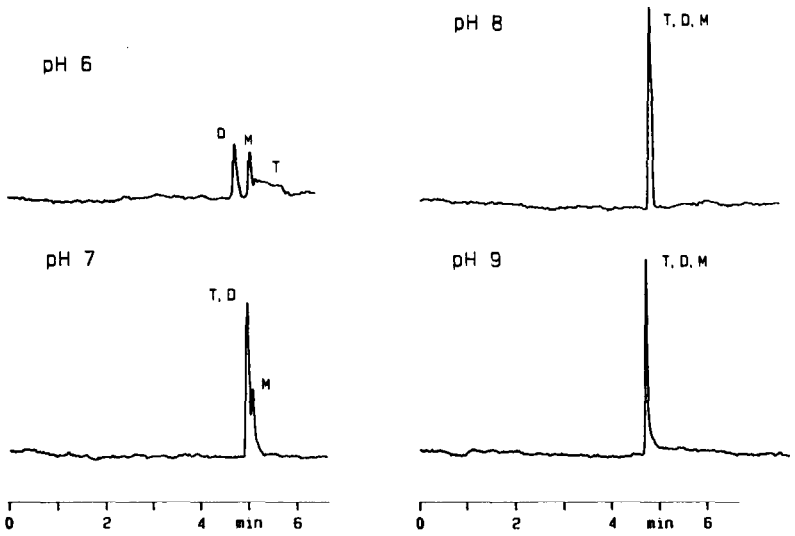

Fig. 4. Separation of cationic heptapeptides *vs.* pH. M = 4-His, D = 3,5-diHis, T = 2,4,6-triHis. 0.5 mg/ml each peptide; 40-cm capillary; 15 kV.

Blocking of the C-terminus also allows relatively high positive charges at neutral-to-basic pH's.

Electropherograms of these peptides are shown in Fig. 4. Separation of the mono- (M) and di-His (D) peptides at pH 6 gives N values of 26 000 and 15 000 and a resolution of 2.8. However, the highly cationic tri-His migrates after mono-His and shows extreme peak broadening. At higher pH values these peptides comigrate as histidines become deprotonated and all net charges approach zero.

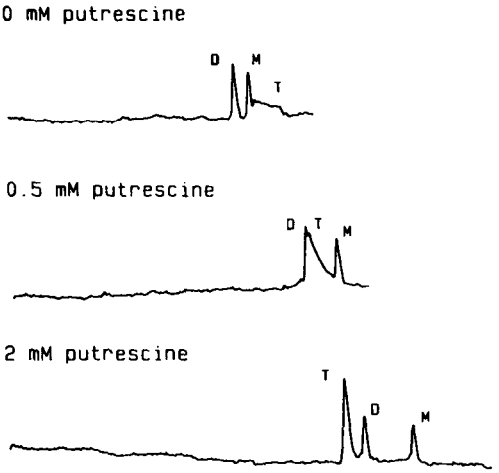

Fig. 5. Separation of cationic heptapeptides in pH 6 buffer with varying amounts of putrescine added. Same conditions as Fig. 4.

Band broadening in CZE will result from any process that is not homogeneously distributed in a radial direction (*e.g.* surface interactions, thermal gradients, laminar flow). While most discussions of adsorptive broadening have focused on proteins, poor efficiencies also have been observed for di- and tri-valent metals[15]. Adsorption of cationic species in CZE can be viewed as simple ion exchange with buffer cations at negative sites on the capillary wall. Ion exchange could be minimized by using buffer cations with strong affinities for surface exchange sites. Lauer and McManigill[4] suggested that polyvalent organic cations can sharpen CZE protein peaks.

Fig. 5 gives the results of adding putrescine to pH 6 buffer to minimize ion exchange of the tri-His peptide. Putrescine (2 m*M*) gives an improved separation of the three His-containing peptides. The peptides now migrate in order of His content with plate numbers of 19 000–62 000. Analysis times are increased only marginally by ζ-potential changes and reductions in electroosmotic flow.

Use of a metal containing buffer also was tested for obtaining improved separation of the peptides. As described above, excessively high charges can occur when protonation of His alone is used to affect a separation. Differential affinities for metals, rather than protons, could be another basis for separation. Fig. 6 shows separations obtained in pH 7.5 tricine buffer with and without added Zn^{2+}. pH 7.5 was chosen so that His protonation would be minimized and Zn–His interactions maximized. Also, operation at a pH substantially less than the buffer pK_a minimizes $Zn(tricine)_2$ formation and possible Zn deactivation.

Improved separation is seen with the Zn-containing buffer. However, the peptides do not migrate in order of expected Zn affinity (tri > di > mono). The tri-His peptide displays a long migration time and relatively low efficiency (N = 7000). Large positive charges are possible for complexes of Zn^{2+}, tricine (HB) and tri-His peptide (H_3T), namely ZnT^{2+}, $ZnHT^{3+}$ and $Zn(B)HT^{2+}$. Thus, late migrating tri-His can be explained by ion exchange of the Zn complexes.

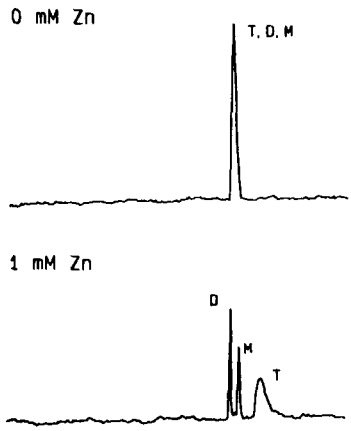

Fig. 6. Separation of cationic heptapeptides in pH 7.5 buffer with and without addition of 1 m*M* Zn $(ClO_4)_2$. Same conditions as Fig. 4.

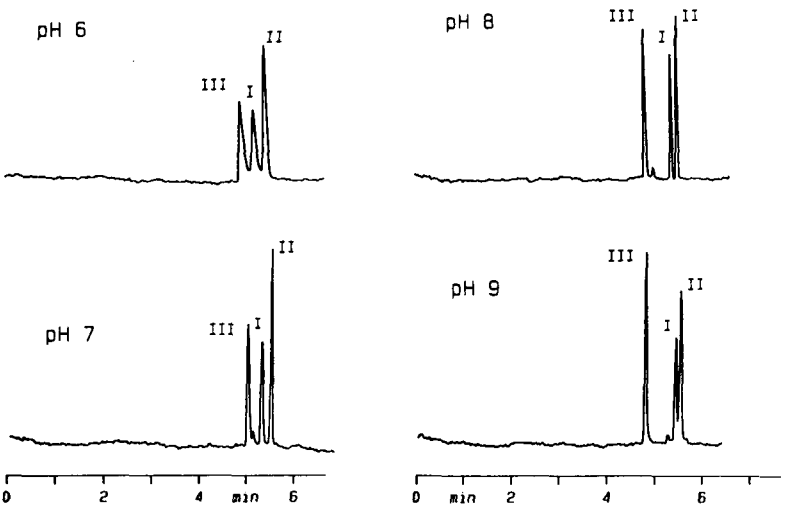

Fig. 7. Separation of human form angiotensins I, II and III *vs.* pH. 0.5 mg/ml each peptide; 40-cm capillary; 15 kV.

Angiotensins

To test CZE for the separation of biologically important cationic peptides, angiotensins I, II and III were run at pH 6–9. Fig. 7 shows that excellent separations are obtained, with the best efficiencies and resolutions occuring at pH 8. *N* values are 64 000 for III, 180 000 for I and 192 000 for II. Resolutions are 3.4 for III/I and 2.5 for I/II. Lower efficiencies are seen as positive charge on the angiotensins increase; however, peak broadening does not rule out the high-resolution separation of these compounds as cations.

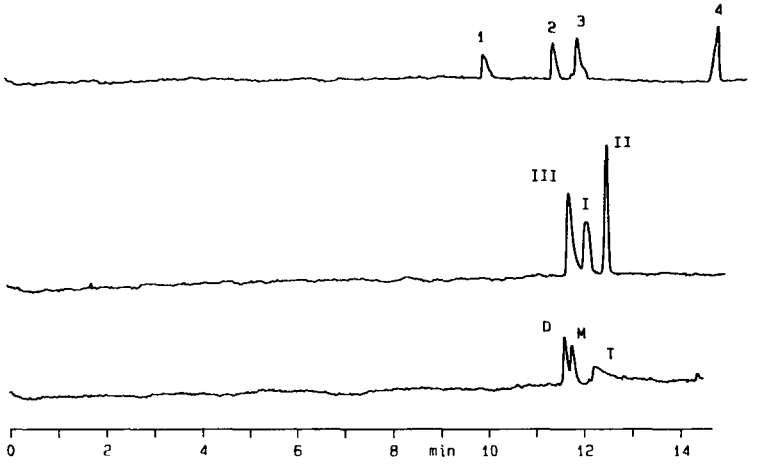

Fig. 8. Separation of His containing compounds in 70-cm long capillary, pH 7 buffer. Top: model compounds, middle: angiotensins, bottom: synthetic peptides. Concentrations same as in Figs. 3, 4 and 7; 15 kV.

The 70-cm capillary separations

Separations are shown in Fig. 8 for each class of compounds using a 70 cm × 75 μm capillary and pH 7 buffer. The longer capillary was used to test for thermal broadening in the previous 40-cm separations. Efficiencies increased by a factor of 2.3 for HME and 3.5 for Gly–His and NAH, indicating that the thermal effect of reducing the current from 14 μA to 9.7 μA on going to the longer capillary is significant.

For cationic peptides, a striking difference in the total separation pattern is seen. All three peptides can be separated at pH 7 in the long capillary, but with tri-His migrating last. Also, N for the tri-His peptide is only 6000 at 70 cm while N = 18 000 for the combined di/tri peak at 40 cm. Finally, the migration time of angiotensin III is longer for the 70-cm capillary relative to I and II, with essentially no increase in plate number.

These results indicate that ion-exchange retardation of highly charged, cationic compounds is dependent on capillary length. Increased capillary length leads to lower efficiencies for ion-exchanging cations, in contrast to diffusion limited separations where N is independent of length[2].

DISCUSSION

While the results presented here show lower separation efficiencies for cations than for neutrals and anions, acceptable peak shapes were obtained for all compounds except tri-His at pH 6. To assess the positive charge necessary for severe peak

TABLE III

CONCENTRATION-BASED pK_a VALUES AND CALCULATED CHARGE *VS.* pH FOR COMPOUNDS STUDIED

pK_a		*Calculated net charge*			
		pH 6	*pH 7*	*pH 8*	*pH 9*
Histidine derivatives[a]					
HME	7.2, 5.3	+1.1	+0.6	+0.1	0.0
Gly–His	8.1, 6.7, 2.5	+0.8	+0.3	−0.4	−0.9
His	9.1, 6.0, 1.7	+0.5	+0.1	−0.1	−0.4
NAH	7.0, 2[b]	−0.1	−0.5	−0.9	−1.0
Heptapeptides[c]					
tri-His	7.3, 6.7, 6.2	+2.4	+1.1	+0.2	0.0
di-His	7.1, 6.3	+1.6	+0.7	+0.1	0.0
mono-His	6.8	+0.9	+0.4	+0.1	0.0
Angiotensins[d]					
III His =	6.7	+1.9			
I His =	7.1, 6.3	+1.6			
II His =	6.7	+0.9			

[a] Ref. 18, I = 0.1 or 0.16 M.
[b] Estimated.
[c] This work, I = 0.5 M.
[d] Estimated, see text.

degradation due to surface ion exchange, net charges for each compound were calculated using cumulative Henderson–Hasselbach relations[16]. Heptapeptide pK_a values were determined as described in the Experimental section, while literature values were used for His derivatives. For angiotensins, N-termini, Tyr and Arg side chains were assumed to be fully protonated and C-termini and Asp side chains were assumed to be fully deprotonated at pH 6. His residues were given values corresponding to those for the mono- and di-His heptapeptides. Charges at pH > 6 were not calculated for the angiotensins due to uncertainties in N-terminus and interior Tyr pK_a values.

Table III gives the calculated charge-pH data for the compounds tested. Inspection of the Table reveals that a net charge of $> +2$ is necessary for the severe ion-exchange retardation seen with tri-His in a 40-cm capillary. Good correlations are seen between charge and migration orders within compound classes. Accurate determination of migration times and electroosmotic flow *vs.* pH should enable CZE to be used for pK_a determinations, similar to procedures developed for ITP[17].

Results presented here indicate that CZE in 40-cm capillaries is capable of high efficiency separations of peptides and model compounds if charges are $< +2$. Use of ion-exchange supressors such as putrescine extends the range of CZE to more positively charged compounds. Alternatively, metal binding at higher pH's can be used to improve separations of His-rich peptides. Separation of the 3,4-, 3,5-, 3,6- and 2,6-positional isomers of the di-His heptapeptide was attempted using Zn^{2+} buffers. pH 7.5 buffer containing 1 m*M* Zn^{2+} is slightly effective in splitting out one isomer, but higher metal concentrations give excessive ion-exchange broadening. Tailoring the pH/metal content of CZE buffers and use of ion-exchange suppressors or deactivated capillaries could yield high-resolution separations of peptides based on both His content and spacing. Also, mobility modifications observed here with Zn^{2+} complexation suggest the potential for chiral separations of underivatized enantiomers using simple UV detection.

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