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Capillary zone electrophoresis of peptides: prediction of the electrophoretic mobility and resolution

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Abstract

The determination of the pK_a values of some selected peptides of similar size was performed by microtitration, which makes possible an accurate determination of the peptide charge as a function of the solution pH. Capillary zone electrophoresis separation of these peptides on modified capillaries at acidic pH showed that the electrophoretic mobility correlates with the peptide charge. This observation suggests that when an appropriate charge value is used, the basic electrophoretic equation is respected and, at least at a peptide charge value less than 1, the utilization of alternative semi-empirical predictions is not necessary. As a general rule, a peptide separation at acidic pH values is to be preferred to that at basic pH values. In fact, at basic pH a separation in the absence of both electroosmotic flow and of spurious interactions between the peptides and the inner wall of the capillary is difficult, owing to the instability of capillary modification. Further, from the differences in the peptide charge, a prediction of the best resolution as a function of the pH could be obtained; in fact, the resolution, for peptides of similar size and in the absence of electroosmotic flow, is connected to a simple equation, where the principal term depends on the effective charge of the peptides, which is a function of the pH of the solution and the pK_a values of the peptides. The predictions of resolution at acidic pH agreed well with the experimental results; the spatial resolution measured in the separation of met- and leu-enkephalin was virtually coincident with the predicted resolution; in the case of a mixture of four model tetrapeptides of sequence GGNA, GGQA, GGDA and GGEA some anomalous results with respect to the predicted resolutions were observed. Nevertheless, an acceptable prediction can also be made in this case.

1. Introduction

Capillary zone electrophoresis (CZE) with modified or bare capillaries offers new opportunities in the analysis of polar biomolecules such as small peptides. The performance of a

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CZE peptide separation can be approximated from correlations between physical peptide attributes and the observed electrophoretic mobility μ, which is defined as the steady-state velocity under unit field strength.

In this respect, several models have been proposed; Grossman *et al.* [1] suggested that the electrophoretic mobility can be related to the logarithm of the peptide charge and is inversely proportional to approximately the square root of the number of the amino acids (AA) of the peptide; this semi-empirical correlation was partly confirmed by our result on the tryptic map of horse myoglobin [2]. Rickard *et al.* [3] observed that the electrophoretic mobility is approximately proportional to the peptide charge and inversely proportional to its molecular mass to the 2/3 power. Hilser *et al.* [4] concluded that the correlation proposed by Grossman *et al.* displays a slightly better linearity than that proposed by Rickard *et al.*; Hilser *et al.* also suggested that the observed deviations in both models derive from an inadequate treatment of peptide size. A more accurate theoretical approach was offered by Compton and O'Grady [5,6], who correlated the peptide mobility with various physico-chemical properties of the molecule and the electrophoretic solution, on the basis of the Debye–Hückel–Henry theory. This last model might explain why different semi-empirical approaches display similar results reconciling the different models.

The aim of this work was to investigate the correlation between peptide properties and electrophoretic mobility; further, on the basis of simple theoretical considerations, a prediction of the optimum resolution for the separation of two sets of model peptides as a function of the pH of the electrophoretic buffer was attempted.

For the achievement of this objective several experimental simplifications were adopted, as follows. The two sets of model peptides used for the optimization were the couple met- and leu-enkephalin and, separately, four tetrapeptides of GGXA sequence. This choice was made to avoid appreciable differences in peptide size and to focus on peptide charge, which, in our opinion, is the major factor responsible for the deviation observed between predicted and experimental mobilities. The couple met- and leu-enkephalin should provide information about the mobility and the separation of two peptides differing in the carboxy terminus, while the four tetrapeptides GGNA, GGQA, GGDA and GGEA should provide information about slight structural modifications at the level of side-chains. All the predictions made before and mentioned above use a peptide charge computation based on mean pK_a values derived from values reported in the literature. As a consequence, a sensitive predictive error might derive from too inaccurate peptide pK_a values. Hence the pK_a values of the peptides under study were determined by microtitration. In order to eliminate the contribution of the electroosmotic flow (f_{eo}) at the observed mobility, the separations at acidic pH were performed using a capillary modified by a monolayer of polyacrylamide [7]. As this modification is unstable at basic pH, the separations in this pH range were performed with a bare capillary and in the presence of f_{eo}.

2. Experimental

2.1. Instrumentation

The capillary electrophoretic separations were performed on a Beckman (Palo Alto, CA, USA) PACE system 2100, equipped with a Beckman Gold 711 system for automated apparatus control and data acquisition. The peptide microtitrations were performed on a Radiometer (Copenhagen, Denmark) automated titrator composed of a ABU93 triburette dispenser, a stirred and thermostated SAM90 sample station equipped with a G-222 C glass microelectrode and a K422 reference electrode, a VIT90 videotitrator and Tetrafile software for automated data treatment.

2.2. Chemicals and reagents

All common reagents were of analytical-reagent grade purchased from Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany). The

peptides under study [YGGFM (met-enkephalin), YGGFL (leu-enkephalin), GGNA, GGQA, GGDA and GGEA] were purchased from Bachem (Bubendorf, Switzerland). Standard buffer solutions were obtained from Carlo Erba. Acetanilide (analytical-reagent grade), HCl and NaOH (Titrisol Normex solutions) were supplied by Merck. The electrophoretic solutions were prepared with the use of ultra-pure water.

2.3. Procedures

The peptide microtitrations were performed on peptide solutions in the concentration range 5–70 mg ml^{-1} by the use of Titrisol Normex HCl or NaOH solutions. The data treatments for the peptide pK_a value determinations were performed following the mathematical procedure described under Results and Discussion, using our own software.

The fused-silica capillaries utilized were provided by Beckman and included on the Beckman electrophoretic cartridge. The peptide capillary electrophoretic separations at acidic pH were performed on capillaries coated with linear polyacrylamide directly on the cartridge and on the capillary electrophoretic apparatus using the procedure described by Hjertén [7]; the separations at basic pH were performed on uncoated capillaries. The capillary dimensions were 56.5 cm (50.0 cm to the detection window) × 75 μm I.D. Sodium phosphate (80 mmol l^{-1}) was used to prepare the electrophoretic buffers at acidic pH, whereas sodium phosphate (64 mmol l^{-1}) and sodium tetraborate (16 mmol l^{-1}) were used at basic pH. Standard sample solutions were prepared by dissolving each peptide in water at a concentration of 2.5 mg/ml and by diluting different volumes of the standard peptide solutions 1:1 with the separation buffer, in order to recognize the peptide identity during the electrophoretic separation. Separations at acidic pH were performed, unless mentioned otherwise, at a constant voltage of 25 kV and at 20°C; those at basic pH were performed at a constant voltage of 12 kV and at 20°C. In both instances the anode electrode was coincident with the capillary injection terminal. Injection was performed by pressure for 1 s, corresponding to an injection volume of *ca.* 4.5 nl (measured using injection markers). At basic pH with uncoated capillaries a solution of acetanilide at a concentration of 2 mg ml^{-1} was used as the electroosmotic flow marker.

3. Results and discussion

3.1. Electrophoretic mobility

The peptides submitted to CZE analysis were the couple met-enkephalin (YGGFM) and leu-enkephalin (YGGFL) and the four tetrapeptides GGDA, GGEA, GGNA and GGQA. The separation of the two enkephalins was chosen to study the properties of two peptides differing at the carboxy terminus. The set of the four tetrapeptides of sequence GGXA was chosen to study the effect of the side-chains on the separation, *i.e.*, the difference in charge observable on replacing aspartic with glutamic acid and the difference in charge observable with very small modifications of the side-chain on the terminals (such as in the couple GGNA–GGQA); further, the optimization of a system of four peptides needs a more complex predictive approach.

The peptide charge fraction Z at any pH can be obtained from the algebraic sum of the molar fractions of the negative groups (N_i) and positive groups (P_i):

$$Z = \sum_i N_i + \sum_i P_i \quad (1)$$

The groups which in a peptide can assume a negative charge are the carboxy terminus and the lateral groups of aspartic and glutamic acid, cysteine and tyrosine; for each of these groups, having a dissociation constant K_a, the molar fraction of the charge is equal to

$$N_i = [A^-]/([A^-] + [AH]) = \varphi/(1 + \varphi) \quad (2)$$

where $\varphi = K_a/[H^+] = 10^{pH - pK_a}$. The groups which can assume a positive charge are the amino terminus and the lateral groups of lysine,

arginine and hystidine; for each of these groups the fraction of the charge is given by

$$P_i = [BH^+]/([BH^+] + [B]) = 1/(1 + \varphi) \quad (3)$$

The peptide charge can be thus calculated at any pH using the peptide pK_a values; they can be assumed from theoretical considerations of the pK_a values of the dissociable groups, but an important error may be introduced; for this reason, the pK_a values of the model peptides were measured by titration. As some dissociable groups have very close pK_a values, as in the case of GGDA and GGEA peptides, the titration curve was converted into a curve expressing the buffering capacity of the peptide as a function of the pH [$\partial(\text{mmol})/\partial(\text{pH}) = f(\text{pH})$] and this curve was fitted according to the following equation:

$$\partial(\text{mmol})/\partial(\text{pH}) = aC \cdot 10^{-\text{pH}}/(C - 10^{-\text{pH}})^2 + b \sum_i (10^{\text{pH}-\text{p}K_i})/(10^{\text{pH}-\text{p}K_i} + 1)^2 \quad (4)$$

where a and b are free parameters, C is the concentration of the titrant (HCl), which can be obtained by the fit, and the first term of the equation takes into account the buffering capacity of the titrant.

In Table 1 the pK_a values obtained by this method for the two sets of model peptides are reported; the pK_a values utilized by Grossman *et al.* [1] and Rickard *et al.* [3] are also reported for comparison. The experimental values are similar to the values reported by Rickard *et al.* [3]. Nevertheless, the latter are identical and, on the basis of their values, the separation of the enkephalins or of the couple GGNA–GGQA should be impossible. The pK_a values used by Grossman *et al.* [1] derive from the pK_a of free amino acids and they are too different from the experimental values; with their use a good mobility prediction is not possible and, as in the case of the other theoretical values, the separation of the couple GGNA–GGQA should be impossible.

Table 1
Peptide pK_a values obtained by titration measurements

Peptide	Parameter	Experimental	Ref. 1	Ref. 3
YGGFM	pK_{a1}	3.45 ± 0.01	2.28	3.20
	pK_{a2}	7.36 ± 0.01	9.11	7.70
	pK_{a3}	10.36 ± 0.02	10.11	10.30
YGGFL	pK_{a1}	3.69 ± 0.01	2.17	3.20
	pK_{a2}	7.40 ± 0.01	9.11	7.70
	pK_{a3}	10.34 ± 0.02	10.11	10.30
GGNA	pK_{a1}	3.65 ± 0.01	2.34	3.20
	pK_{a2}	8.30 ± 0.02	9.78	8.20
GGQA	pK_{a1}	3.61 ± 0.01	2.34	3.20
	pK_{a2}	8.27 ± 0.02	9.78	8.20
GGDA	pK_{a1}	3.53 ± 0.01	2.34	3.20
	pK_{a2}	4.16 ± 0.01	3.82	3.50
	pK_{a3}	8.37 ± 0.02	9.78	8.20
GGEA	pK_{a1}	3.66 ± 0.01	2.34	3.20
	pK_{a2}	4.47 ± 0.01	4.18	4.50
	pK_{a3}	8.31 ± 0.02	9.78	8.20

The last two columns report theoretical pK_a values from the literature.

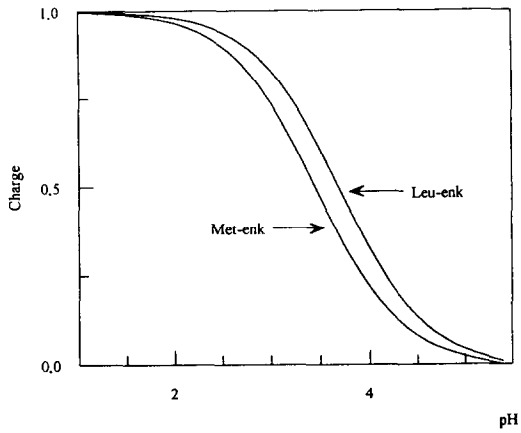

Fig. 1. Charge (Z) values of leu-enkephalin (leu-enk) and met-enkephalin (met-enk) at acidic pH values obtained from the pK_a values measured by peptide microtitration and reported in Table 1.

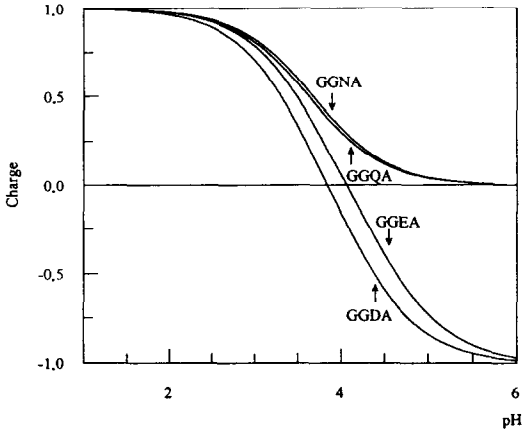

Fig. 3. Charge (Z) values of four tetrapeptides of GGXA sequence at acidic pH values obtained from the pK_a values measured by peptide microtitration and reported in Table 1.

In Figs. 1 and 2 the charges of leu- and met-enkephalin at acidic and basic pH ranges, respectively, are presented; in Figs. 3 and 4 the charges of the four tetrapeptides of the GGXA sequence are shown. The charges are calculated using the Eqs. 1, 2 and 3 and the pK_a values obtained from titration data (Table 1).

The electrophoretic mobility μ should be connected to the charge by the classical electrophoretic relationship based on the Stokes' law of viscosity:

$$\mu = Ze/6\pi\eta r_s \quad (5)$$

where η is the solution viscosity, e is the electronic charge and r_s is the peptide Stokes radius. This relationship could be applied only at zero ionic strength [5].

By considering the mobility of the two en-

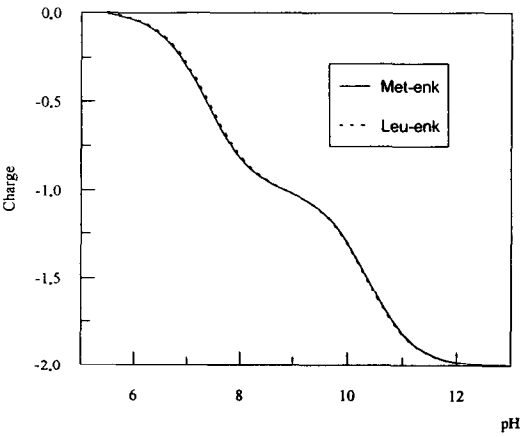

Fig. 2. Charge (Z) values of (dashed line) leu-enkephalin (leu-enk) and (solid line) met-enkephalin (met-enk) at basic pH values obtained from the pK_a values measured by peptide microtitration and reported in Table 1.

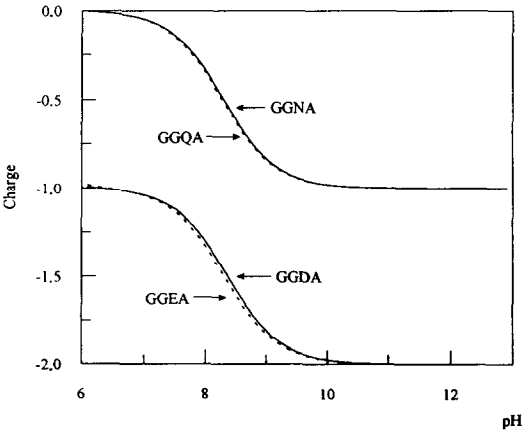

Fig. 4. Charge (Z) values of four tetrapeptides of GGXA sequence at basic pH values obtained from the pK_a values measured by peptide microtitration and reported in Table 1.

kephalins or by separately considering the set of the four tetrapeptides, it can be assumed that the radius does not play a role in the separation, for very similar peptide structures. Further, our electrophoretic separations at acidic pH were performed with the use of a capillary modified by a monolayer of polyacrylamide to avoid any spurious contribution to the mobility due to the electroosmotic flow or to peptide interactions with the capillary inner wall. Under these conditions the differences observed in the electrophoretic mobility at different acidic pH values should only be connected to a different peptide charge; in Fig. 5, the correlations observed between μ and Z for the enkephalins and for the four tetrapeptides with the GGXA sequence are reported. The angular coefficients of the enkephalins are smaller than those of GGXA peptides; this is in agreement with an inverse proportionality between electrophoretic mobility and peptide size.

Grossman *et al.* [1] suggested that Eq. 5 is not properly applicable to peptides and proposed a semi-empirical relationship:

$$\mu = [A \log(Z + 1)/n^B] + C \quad (6)$$

where n represents the number of AA of the peptide and A, B and C are parameters obtained by the best fit; whereas B provides a relationship between n and the peptide radius, A and C are connected with the electrophoretic experimental conditions [1,2].

Compton and O'Grady [5,6] modified Eq. 5 by applying the Debye–Hückel–Henry theory to account for ionic effects:

$$\mu = Ze\Phi(kr)/[6\pi\eta r_s(1 + k)] \quad (7)$$

where $\Phi(kr)$ is the Henry function. By expressing the size dependence of the electrophoretic mobility using molecular mass (M) rather than r_s, they obtained a general equation of the form

$$\mu = K_1 Z/(K_2 M^{1/3} + K_3 M^{2/3}) \quad (8)$$

where K_1, K_2 and K_3 are three terms which include common physical constants, the solution ionic strength and the frictional ratio (f/f_0). Further, they indicated that, after some simplification and transformations of Eq. 7, a relationship similar to Eq. 6 is obtainable. On the other hand, if $K_2 << K_3$, Eq. 8 is equivalent to the correlation observed by Rickard *et al.* [3], where

$$\mu \propto Z/M^{2/3} \quad (9)$$

Hence it seems that the different approaches of Grossman *et al.* and Rickard *et al.* provide similar results because both are particular cases deriving from the more general theory elaborated by Compton and O'Grady.

Our results are in agreement with a linear relationship between Z and μ; in our opinion the use of experimental peptide pK_a values ensures a direct proportionality between peptide charge and mobility, as predicted from the basic electrophoretic Eq. 5 and different semi-empirical models could not be justified. In this respect it is important to note that the use of erroneous pK_a values similar to those proposed by Grossman *et al.* [1] provides a good correlation between μ and $\log(Z + 1)$ [2]; in other words the function between the electrophoretic mobility and $\log(Z + 1)$ is less sensitive to errors in pK_a values than the function between μ and Z. This observation could explain the results of Hilser *et al.* [4], who have found a better correlation between μ and $\log(Z + 1)$ than between μ and Z.

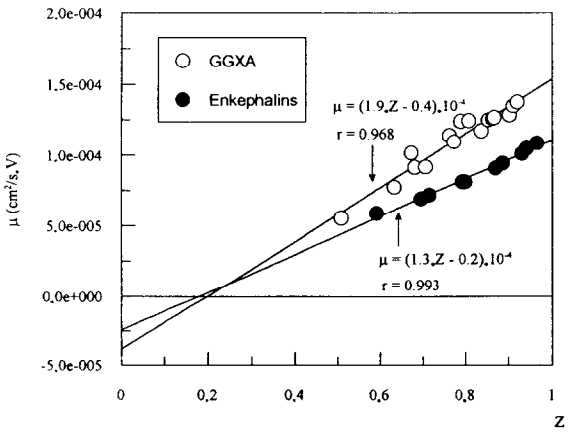

Fig. 5. Correlations between the observed electrophoretic mobility (μ) and the charge (Z) calculated at different acidic pH values for (●) the enkephalins and (○) the four GGXA tetrapeptides.

3.2. Resolution

Jorgenson and Lukacs [8] suggested the following expression for resolution (R_s) in CZE:

$$R_s = (1/4)(\mu_1 - \mu_2)[V/2D(\mu + \mu_{osm})]^{1/2} \quad (10)$$

where μ_1 and μ_2 are the mobilities of two consecutive separands, V is the applied voltage, D is the mean molecular diffusion coefficient, μ is the mean mobility and μ_{osm} is the mobility of the electroosmotic flow. The coefficient of diffusion D is given by

$$D = kT/6\pi\eta r_s \quad (11)$$

where k is the Boltzmann constant and T the absolute temperature. As reported previously, under acidic experimental conditions and with the modified capillary, μ_{osm} can be considered to be equal to zero. In the separation of the enkephalins or in the separation of the four tetrapeptides of GGXA sequence, sensitive differences in the radius are not present. Hence, in the absence of electroosmotic flow, introducing Eqs. 5 and 11 into Eq. 10 can be easily achieved:

$$R_s = (1/4)[\Delta Z/(Z_1 + Z_2)^{1/2}](qV/kT)^{1/2} \quad (12)$$

This equation is similar to that proposed by Kenndler and Friedl [9]. At constant voltage and temperature, the resolution should be directly proportional to $[\Delta Z/(Z_1 + Z_2)^{1/2}]$, which can be called the pH factor of resolution (FR_s).

In Fig. 6 the absolute difference in charge and the theoretical resolution from the measured pK_a values of met- and leu-enkephalin are plotted as a function of pH in the acidic pH range. To obtain the resolution values FR_s was multiplied by $(qV/16kT)^{1/2}$ ($q = 1.602 \cdot 10^{-19}$ C, $k = 1.381 \cdot 10^{-23}$ J K^{-1}, $T = 298$ K, $V = 25\,000$ V). A sensitive shift towards higher pH values between the maximum of the charge difference and the maximum of resolution is observed. In any case, the best resolution in the separation of met- and leu-enkephalin can be predicted to be at pH 3.5–4.0. Unfortunately, in this pH range the observed electrophoretic mobility is too low to obtain the separation in a reasonable time and the analysis of the experimental resolution was performed in the pH range 2.3–3.2.

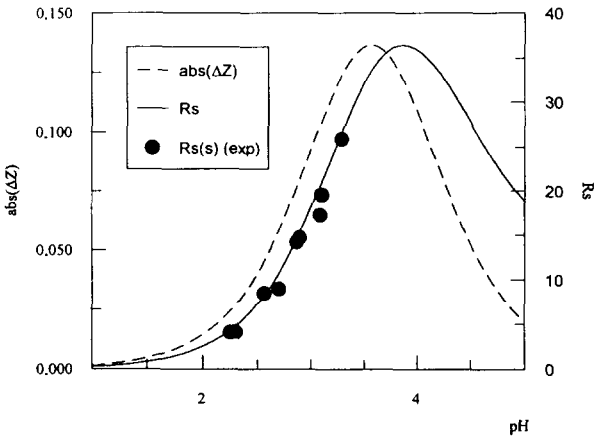

Fig. 6. (Dashed line) charge differnces (ΔZ) and (solid line) theoretical resolution (R_s) calculated from the Eq. 12 as a function of the pH in the acidic pH range for the enkephalins. The closed circles represent experimental values of spatial resolution [$R_{s(s)}$] obtained in CZE separations at different pH.

The experimental (temporal) resolution in a chromatographic separation can be calculated as:

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \quad (13)$$

where t_2 and t_1 are the migration times of two consecutive peaks and w_1 and w_2 are the corresponding peak widths measured in time units at the base of the peak (temporal width). In CZE separations, in the absence of electroosmotic flow, this relationship should be converted into spatial resolution because the electrophoretic zones do not cross the detector window at the same velocity; thus a variation in temporal widths might not reflect real peak enlargement. As pointed out by Huang *et al.* [10], the conversion from temporal (w_t) to spatial width (w_s) is given by

$$w_s = (L/t)w_t - w_d \quad (14)$$

where L is the length of the capillary measured at the detection window and w_d is the vertical spatial width of the detection window. The experimental spatial resolution $R_{s(s)}$ is thus easily obtained by

$$R_{s(s)} = 2L[(t_2 - t_1)/t_1]/[L(w_1/t_1 + w_2/t_2) - 2w_d] \quad (15)$$

where the term at the numerator accounts for the spatial difference between the two peaks at time t_2. Under our conditions the differences between the values of $R_{s(s)}$ and R_s are small and the correction has no practical significance.

In Fig. 6, the values of $R_{s(s)}$ measured at various pH values for the separation of the enkephalins by the use of Eq. 15 are reported in comparison with the values of resolution predicted theoretically with the use of Eq. 12. The agreement between experimental and theoretical resolution is excellent. As an example, in Figs. 7 and 8 the CZE separations of met- and leu-enkephalin at pH 2.20 and at 2.86, respectively, are reported.

In order to study the optimum resolution in the acidic range for the separation of the four tetrapeptides of GGXA sequence, the difference in charge and the predicted resolution of the couples GGNA–GGQA [ΔZ(NQ); R_s(NQ)], GGQA–GGEA [ΔZ(QE); R_s(QE)] and GGEA–GGDA [ΔZ(ED); R_s(ED)] were computed; only these pairs were considered because, on the basis of the charge values presented in Fig. 3, the predicted migration order is GGNA, GGQA, GGEA, GGDA. The charge differences in the acidic range are presented in Fig. 9

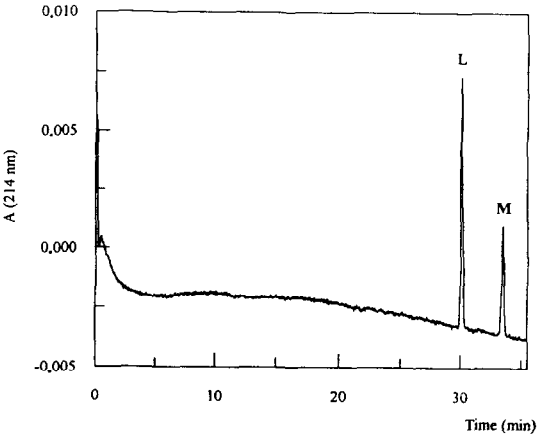

Fig. 8. CZE separation of leu-enkephalin (L) and met-enkephalin (M) at pH 2.86. Applied voltage, 25 kV; temperature, 20°C; other conditions as reported under Experimental.

and the R_s values are shown in Fig. 10. The order of electrophoretic migration of the four peptides is well respected at any pH. A baseline separation is established for two peaks with equal area at a value of approximately 1.5; thus, from Fig. 10, a window of resolution for a complete separation should open at a pH value greater than 2.6. The couples GGQA–GGEA and GGEA–GGDA show experimental $R_{s(s)}$ values which agree with the theoretical prediction. The $R_{s(s)}$ values observed for the couple GGNA–GGQA are puzzling; in fact, the latter

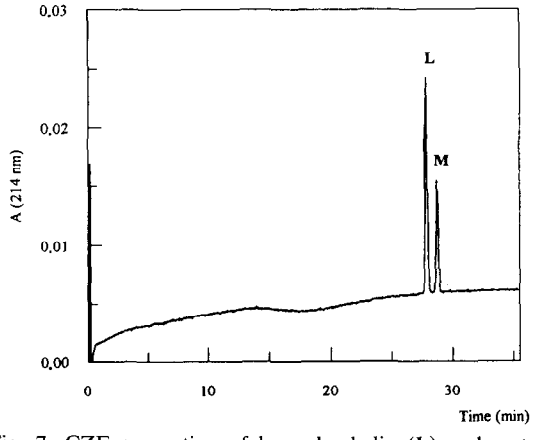

Fig. 7. CZE separation of leu-enkephalin (L) and met-enkephalin (M) at pH 2.20. Applied voltage, 25 kV; temperature, 20°C; other conditions as reported under Experimental.

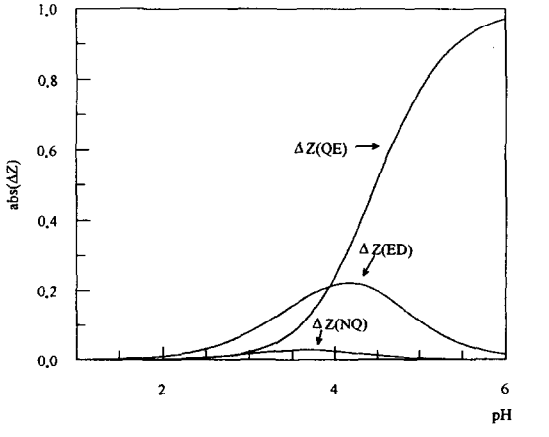

Fig. 9. Calculated peptide charge differences of the couples GGNA–GGQA [ΔZ(NQ)], GGQA–GGEA [ΔZ(QE)] and GGEA–GGDA [ΔZ(ED)].

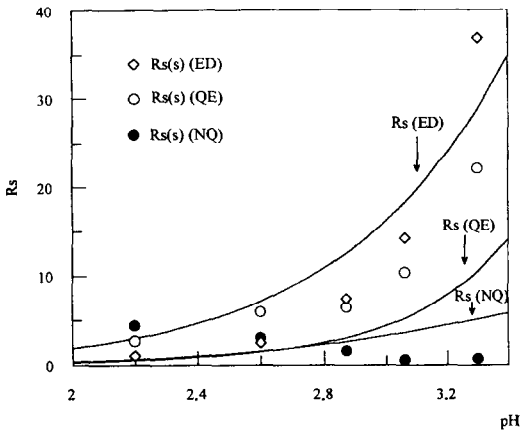

Fig. 10. Theoretical resolution (R_s) calculated for the acidic pH range by the use of Eq. 12 for the CZE separation of the four tetrapeptides of GGXA sequence. The values of the experimental spatial resolution [$R_{s(s)}$] measured in several electrophoretic separations at acidic pH are reported for comparison. ◇ = $R_{s(s)}$(ED); ○ = $R_{s(s)}$(QE); ● = $R_{s(s)}$(NQ).

represents the limiting couple in the separation, because of the very small charge differences; the $R_{s(s)}$ presents a relatively high valuc (*ca.* 5) at pH 2.26 and it decreases as a function of pH, in disagreement with the prediction. As a consequence, whereas with the use of Eq. 12 an increase in total resolution should be expected as a function of pH, compatible with acceptable migration times, from the measured $R_{s(s)}$ values thc best separation of the four tetrapeptides occurs at pH ≈ 2.6.

3.3. CZE separation at basic pH

The analysis of the charge differences calculated in the basic pH range (Figs. 2 and 4) immediately shows that an electrophoretic separation either of the couple met- and leu-enkephalin or of the four tetrapeptides of GGXA sequence is not possible; the small charge difference and the consequent small difference in the electrophoretic mobility, on the basis of Eq. 10, should provide a very low resolution. Thus, as expected, the CZE separation of enkephalins with a bare capillary in the pH range 8.4–10.3 showed no resolution between the two peptides; the same result was obtained in the separation of GGXA peptides for the couples GGNA–GGQA and GGDA–GGEA; an element of the couple GGDA–GGEA has a negative charge which is about one unit of charge greater than the negative charge of an element of the couple GGNA–GGQA at any basic pH. Owing to the high ionic strength, the applied voltage at basic pH was lower than that at acidic pH (12 kV instead of 25 kV); in the hypothetical absence of electroosmotic flow, by the use of Eq. 12, a one unit charge difference should provide a resolution value of *ca.* 80. The resolution measured in the basic pH range for the couple GGNA–GGDA showed a value between 2 and 4. It must be pointed out that for the measurement of the resolution, in the presence of high f_{eo}, Eq. 15 is not applicable; in fact, under these conditions the substance transit at the detection window mainly depends on the constant f_{eo}. Its value within the pH range 8.4–10.3 was found to be constant and equal to about $1.0 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}, by the use of acetanilide as a marker, indicating that above pH 8.4 the silanol dissociation on the inner wall of the capillary is virtually complete. The electrophoretic mobility of the peptides under study, under these conditions is about $0.5 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}; the very low resolution values measured could thus derive from an interaction between the peptide and the inner wall of the capillary.

4. Conclusions

The experimental determination of the peptide pK_a values by microtitration permits an accurate determination of the peptide charge as a function of the pH of the solution used for the CZE separation; this avoids the introduction of a systematic error in the treatment of the data and allows satisfactory relationships to be obtained between the peptide charge and its electrophoretic mobility. To gain further information, the pK_a values of other model peptides can be determined, although their availability and cost might represent serious problems; on the other hand, a prediction from titration results of the optimum pH conditions for a peptide separation

has little practical significance, as in most instances the peptides are not available in adequate amounts. Nevertheless, the results of this study show that the pK_a values of the free amino acid [1,2] are too inaccurate to provide an acceptable prediction, whereas the pK_a values proposed by Rickard *et al.* [3], close to the experimental values, are too similar between themselves to permit a separation prediction in the case of very similar peptide structures. It must be pointed out that the latter are the most difficult separations in biochemical analysis, because other analytical methods such as reversed-phase high-performance liquid chromatography usually fail in the separation of small polar peptides of similar structure. In our opinion, owing to the high values of resolution, the separation of peptides with a similar structure is easily obtainable by CZE.

Eq. 12 represents a simple way to gain information about a CZE separation of weak electrolytes with similar structure and slight charge differences; in fact, even though a precise determination of the pK_a values of similar peptides was not possible, some general suggestions can be provided, as follows.

First, it is advisable to perform the CZE separation at acidic pH with a modified capillary; in fact, whereas at acidic pH a baseline separation of peptides with a difference in charge less than 0.03 was observed, at basic pH a baseline separation was difficult to obtain, also with a peptide charge difference of *ca.* 1.0. In effect, the comparison between acidic and basic pH conditions is not correct, because in the former instance the use of modified capillary allows the separation to be performed in the absence of electroosmotic flow and in the absence of strong interactions between the peptides and the inner wall of the capillary, whereas at basic pH an easy and stable capillary modification is not actually available. Our conclusions concerning the peptide separations at basic pH should be thus reconsidered when the technical problems concerning easy and stable capillary modifications have been definitively solved.

In the acidic pH range it is advisable to try the separation, when possible, towards pH 3.0–3.5 in order to achieve a pH value close to the peptide pK_a values, where the charge differences are maximum. Further, from the FR_s of Eq. 12, at these pH values the sum $Z_1 + Z_2$ is small enough to give an adequate resolution.

On the basis of Eq. 12, it is advisable to perform the separation at low temperature (20°C or less), when the CE apparatus allows its accurate control, and at high separation voltages, consistent with the desired current and the ionic strength of the electrophoretic buffer.

The prediction of the electrophoretic resolution following Eq. 12 does not allow correct results to be obtained in the case of the couple GGNA–GGQA, which, on the basis of the charge difference, should have a better resolution in relation to a pH increase. This is not due to the effect of the slight differences in the ionic strength of the solutions because on performing the separations at constant ionic strength similar results were obtained (data not reported). A possible explanation is that the structural difference of a CH_2 group results in a sensitive difference in the electrophoretic mobility in a small tetrapeptide with a GGXA sequence and this difference is greater at high than at low mobility; this behaviour is not observable with the couple GGDA–GGEA because the charge difference plays the main role in the electrophoretic separation. Also for enkephalins the charge difference plays a major role in the separation, and the structural differences are greatly masked, certainly more than for the GGXA peptides.

The CZE separation of small polar peptides at acidic pH with coated capillaries gives good results in short times with a peptide charge difference of less than 0.03 unit charge; if effectively, such as in the couple GGQA–GGNA, a structural CH_2 difference is sufficient to provide a good separation when the peptide charges are very close, the selectivity is exceptional and CZE can properly be considered the technique of choice for the separation of small polar peptides.

5. References

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