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Peptide separations and dissociation constants in nonaqueous capillary electrophoresis: Comparison of methanol and aqueous buffers

Nonaqueous capillary electrophoresis was evaluated for its potential to separate peptides in methanolic background electrolytes in comparison to aqueous-methanol (50% v/v) and water. Isomeric aspartyl dipeptides and Leu- and Met-enkephalin served as model compounds. pK_a values were determined in the three solvent systems based on the apparent pH scale and in the case of methanol additionally based on the conventional pH scale. Changing from water to methanol led to an increase of the ionization constants describing the dissociation equilibria of the carboxyl group and the amino group, respectively. The pK_a shift was more pronounced for the carboxylic acid function leading to a compression of the mobility-pH curve. As reported for aqueous buffers, efficient separations of the peptides were achieved in methanolic background electrolytes including the resolution of the diastereomers of the isomeric α- and β-aspartyl dipeptides. In contrast to aqueous buffers, the separation of Leu- and Met-enkephalin could also be obtained in buffers in methanol at high pH.

Keywords: Dissociation constant / Nonaqueous capillary electrophoresis / Peptide EL 5313

1 Introduction

Synthetic and natural peptides as well as peptidomimetics comprise a significant portion of the currently used pharmaceutical drugs. Highly efficient separation techniques are required for their determination in quality control or bioanalysis. In recent years, capillary electrophoresis (CE) has been recognized as a fast and simple analytical technique for the analysis of peptides including stereoisomers [1, 2]. Traditionally, aqueous buffer solutions have been used as background electrolytes in CE. However, many studies have shown that water may be replaced by organic solvents. Compared to aqueous media, nonaqueous solvents offer a wider range of dielectric constants, polarity, viscosity and autoprotolysis constants. Due to the high efficiency and selectivity, nonaqueous capillary electrophoresis (NACE) has evolved as an interesting alternative to aqueous CE for the separation of closely related compounds [3–5]. Additional advantages of NACE is the better compatibility with mass spectrometry [6, 7] and electrochemical detection [8, 9]. For both detection modes lower detection limits have been reported compared to CE using aqueous buffers [10]. Therefore, the application of nonaqueous solvents in CE represents an attractive approach for pharmaceutical and biomedical analysis [11–14].

Peptides as hydrophilic compounds are primarily analyzed using aqueous buffers with organic solvents serving as run buffer additives [1, 2]. Only few studies of peptide analysis in organic solvents have been described. Hansen and co-workers [15] separated Ala-Phe diastereomers using ammonium acetate-acetic acid in a 1:1 mixture of methanol and acetonitrile. Gramicidin S and bacitracin were analyzed by NACE-MS in a background electrolyte consisting of ammonium acetate and formic acid in mixtures of acetonitrile and methanol [6]. Czerwenka *et al.* [16] reported the enantiomer separation of *N*-protected alanine peptides containing up to six amino acid residues using *tert.*-butylcarbamoylquinine as chiral counterion in mixtures of methanol and ethanol. This system also allowed the separation of all four possible stereoisomers of the *N*-acyl-Ala-Ala.

The electrophoretic migration behavior in CE strongly depends on the protolysis of the analytes in the background electrolyte as described by the pK_a value of the solutes. However, acid-base equilibria of compounds in water and in nonaqueous solvents are very different. Porras *et al.* [17] investigated systematically the pK_a values of different monoacidic bases in methanol as compared to water establishing a conventional pH scale in addition to the frequently used apparent pH values of buffers in organic solvents. Another effect of the organic solvent is the modification of the actual mobility (the

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Abbreviation: NACE, nonaqueous capillary electrophoresis

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mobility of the fully charged species) depending on the different viscosity (η) and dielectric constant (ε) of the solvent [18].

The present study was conducted in order to investigate the dissociation behavior of peptides in nonaqueous solvents as compared to water and a 1:1 mixture of methanol and water. The pK_a values were determined based on the conventional pH scale as established by Porras *et al.* [17] as well as based on the apparent pH of the background electrolytes. The diastereomeric aspartyl peptide derivatives α-DL-Asp-L-PheOMe and β-DL-Asp-L-PheOMe as well as the neuropeptides Met-enkephalin and Leu-enkephalin were used as model compounds.

2 Materials and methods

2.1 Chemicals

Leu-enkephalin, Met-enkephalin, α-L-Asp-L-PheOMe and β-L-Asp-L-PheOMe were purchased from Bachem (Heidelberg, Germany). The diastereomeric pairs α-L-Asp-L-PheOMe/α-D-Asp-L–PheOMe and β-L-Asp-L-PheOMe/β-D-Asp-L-PheOMe were prepared from Z-D/L-Asp and L-PheOMe according to Yang *et al.* [19]. Methanol (HPLC grade), acetic acid, chloroacetic acid, dichloroacetic acid, sodium chloroacetate, sodium acetate, ammonium acetate, boric acid, tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (36%), sodium hydroxide and 30% sodium methylate in methanol were from VWR (Darmstadt, Germany). Sodium trichloroacetate and potassium dichloroacetate were from Sigma-Aldrich (Steinheim, Germany). All buffer reagents were of the highest grade of purity commercially available.

2.2 Electrolyte solutions

Buffers were prepared by mixing the appropriate amounts of the acid and the corresponding sodium salt in the respective solvents as summarized for methanol in Table 1. Conventional pH was calculated according to the Henderson-Hasselbalch equation as described by Porras *et al.* [17]. Electrolyte solutions in the acidic pH range in methanol-water and water were prepared as outlined for pure methanol in Table 1. Note that the two highest conventional pH values are out of the buffering range of the acetic acid (conventional pK_a = 9.7). Alkaline buffers in methanol-water and water were prepared by adjusting 25 mM Tris to the appropriate pH by addition of 6 M hydrochloric acid or by adjusting 25 mM boric acid solution by the addition of 1 M sodium hydroxide. The buffer solutions were filtered through a 0.47 μm filter and degassed by sonication prior to use. All solutions were prepared freshly on a daily basis. All freshly prepared buffers contained less than 0.025% w/w of water determined by Karl Fischer titration. The water content of the BGEs determined after the electrophoretic measurements did not exceed 0.05% w/w. pH measurements were performed with a inoLab Cond Level 1 potentiometer (WTW, Weilheim, Germany), equipped with a Schott electrode

Table 1. Electrolyte composition of the background electrolytes in methanol

Buffer component 1	Buffer component 2	pH_{app}[a)]	pH_{conv}[b)]
25.0 mM Trichloroacetic acid	25.0 mM Sodium trichloroacetate	2.2	4.9
2.5 mM Trichloroacetic acid	25.0 mM Sodium trichloroacetate	3.2	5.9
125.3 mM Chloroacetic acid	25.0 mM Sodium chloroacetate	4.4	7.1
25.0 mM Chloroacetic acid	25.0 mM Sodium chloroacetate	5.1	7.8
6.3 mM Chloroacetic acid	25.0 mM Sodium chloroacetate	5.7	8.4
125.0 mM Acetic acid	25.0 mM Sodium acetate	6.3	9.0
25.0 mM Acetic acid	25.0 mM Sodium acetate	7.0	9.7
7.9 mM Acetic acid	25.0 mM Sodium acetate	7.5	10.2
5.0 mM Acetic acid	25.0 mM Sodium acetate	7.6	10.4
3.1 mM Acetic acid	25.0 mM Sodium acetate	7.9	10.6
0.8 mM Acetic acid	25.0 mM Sodium acetate	8.4	11.2
0.1 mM Acetic acid	25.0 mM Sodium acetate	9.0	12.0
25.0 mM Boric acid	adjusted to pH_{app} with 1 M NaOH in MeOH	9.9	–
25.0 mM Boric acid	adjusted to pH_{app} with 1 M NaOH in MeOH	10.3	–
25.0 mM Ammonium acetate	25.0 mM Sodium methylate	11.3	–
15.0 mM Ammonium acetate	35.0 mM Sodium methylate	12.2	–
5.0 mM Ammonium acetate	45.0 mM Sodium methylate	12.5	–

a) Apparent pH scale, see Section 3.2
b) Conventional pH scale, see Section 3.2

N6180. The electrode was calibrated using aqueous standard pH solutions. The water content of the buffers was measured by Karl Fischer titration using 701 KF Titrino (Metrohm, Herisau, Switzerland).

2.3 Capillary electrophoresis

All experiments were performed on a Beckman P/ACE 5510 instrument equipped with a diode-array detector (Beckman Coulter, Unterschleißheim, Germany) at 25°C using 50 μm ID fused-silica capillaries with an effective length of 30 cm and a total length of 37 cm. UV detection was carried out at 215 nm. Sample solutions (100 μg/mL peptide dissolved in the respective solvent) were introduced at a pressure of 3447.4 Pa for 3 s (0.5 psi). Experiments were conducted under normal polarity, applying a voltage of 25 kV (detection at the cathodic end) or under reversed polarity, applying a voltage of −25 kV (detection at the anodic end) when the electroosmotic flow was too weak. New capillaries were rinsed for 5 min with 1 M aqueous sodium hydroxide, 10 min with water and 5 min with methanol followed by the separation medium for 10 min. Between the analyses the capillary was rinsed for 2 min with methanol and 3 min with the running buffer. Analyte mobility was calculated as the difference between the apparent mobility of each peptide and the mobility of mesityl oxide used as neutral marker. Calculation of the dissociation constants was performed by nonlinear fitting of the experimental data using the program Origin 5.0 (OriginLab, Northampton, MA, USA).

3 Results and discussion

3.1 Determination of the pK_a values of the peptides

CE is a precise and convenient technique for the determination of pK_a values [17, 20–27]. The constants are determined by simply measuring the dependence of the effective mobility of an analyte on the pH of the background electrolyte. In contrast to potentiometric measurements, the compounds do not have to be pure and mixtures of compounds and diastereomers can be analyzed simultaneously.

Figure 1 illustrates the dissociation behavior of a dipeptide. In general, we can consider a protonated species (H_2Z^+) with cathodic mobility, a zwitterionic species (HZ) which has no charge and migrates with the electroosmotic flow, and a dissociated anionic species (Z^-) that migrates towards the anode. An additional dissociation equilibrium will be observed if the side chain of amino acids such as Tyr, Asp, Cys, Glu, Arg, Lys or His contains ionizable groups.

The effective mobility μ_e of the neutral acid HA is described by Eq. (1):

$$\mu_e = \mu_a \, \alpha \tag{1}$$

where μ_a is the actual mobility, *i.e.*, the mobility of the fully ionized analyte, and α is the degree of ionization. For a monovalent acid (HA) α is given by:

$$\alpha = \frac{1}{1 + 10^{pK_a - pH}} \tag{2}$$

Equations (1) and (2) describe the second dissociation equilibrium (HZ/Z^-) of the peptide. The effective mobility of a cationic acid BH^-, *i.e.*, a protonated base, is given by:

$$\mu_e = \mu_b \, (1-\alpha) \tag{3}$$

corresponding to the first ionization step (H_2Z^+/HZ). Substitution of Eq. (2) into Eqs. (1) and (3) results in Eq. (4) describing the dissociation equilibria of the peptide as shown in Fig. 1:

$$\mu_e = \frac{\mu_a}{1 + 10^{pH - pK_{a1}}} + \frac{\mu_b}{1 + 10^{pK_{a2} - pH}} \tag{4}$$

The dependence of the effective mobility of the peptides on the pH of the background electrolyte in water, water-methanol and methanol is summarized in Fig. 2.

Organic solvents also influence the mobility of the fully charged species due to changes of the viscosity (η) and the dielectric constant (ε). A linear increase of the ion mobility (μ_{ion}) with increasing ε/η ratio can be deducted from the well-known relationship [4]:

$$\mu_{ion} = \frac{\varepsilon_0 \varepsilon \xi_{ion}}{\eta} \tag{5}$$

where ε_0 is the permittivity in vacuum and ξ_{ion} is the zeta potential of the analyte ion. The viscosity of water-methanol mixtures display a maximum at about 50% methanol

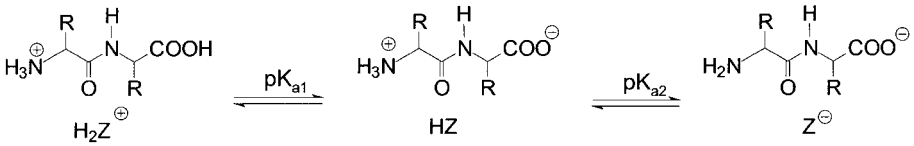

Figure 1. Dissociation behavior of a dipeptide. Residue R without further ionizable groups.

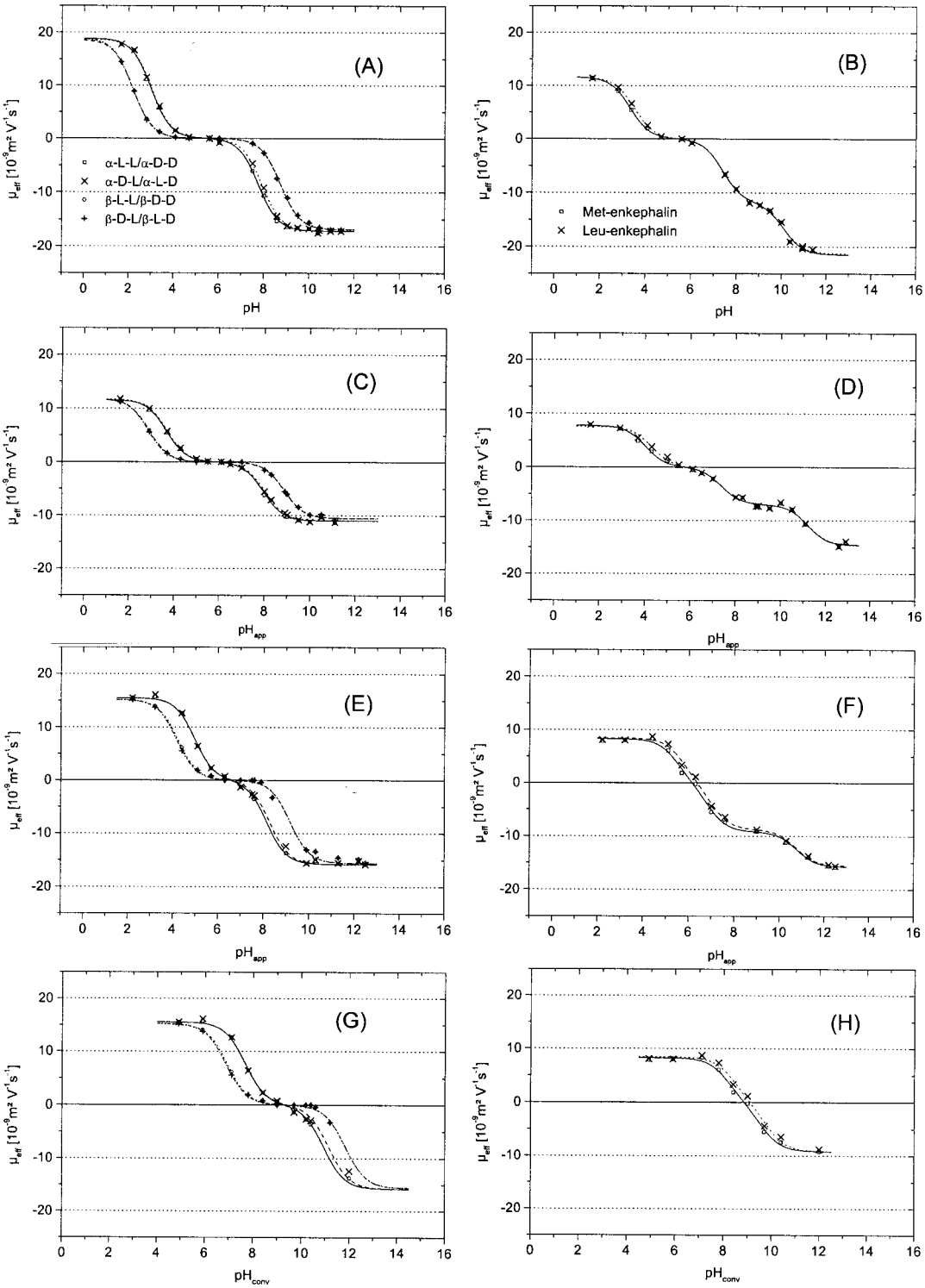

Figure 2. Effective mobility of the investigated peptides as a function of the pH. α/β-DL-Asp-L-PheOMe in (A) water, in (C) methanol-water, in (E) methanol apparent pH scale and in (G) methanol conventional pH scale; (B) Met-enkephalin and Leu-enkephalin in water, (D) in methanol-water, (F) in methanol apparent pH scale and (H) in methanol conventional pH scale. In (G) and (H) the actual mobilities of the third ionization equilibrium (Z^-) were taken from the apparent scale pH-mobility plot (E, F). Note that for the determination of pK_{a3} (in case of the enkephalins) no conventional pH values are available. The experimental data were fitted according to Eq. (5).

resulting in a minimum of the ε/η ratio [28]. This reduces the analyte mobility for the water-methanol mixture resulting in a "flatter" curve as shown in Figs. 2C and D.

The ionization constants of the peptides were calculated by a nonlinear fitting of the experimental data according to Eq. (4) because this approach has been verified to give the least biased determination in comparison with other approaches [21]. For the third dissociation equilibrium of the phenolic group of tyrosine of the neuropeptides an additional term was added for the calculation of pK_{a3} similar to the second term [24]. The pK_a values obtained by curve fitting of the experimental values are summarized in Table 2. The data in methanol and methanol-water are discussed in detail below. The pK_a values of the peptides in water are in good agreement with reported data (Table 3). The present data for aqueous buffers are apparent constants because the ionic strength of the aqueous buffer solutions was not considered.

3.2 Dependence of pK_a values on acidity scales in nonaqueous solvents

In a nonaqueous solvent the pK_a values are only valid with quantitative correlation to a suitable pH scale, because the commonly used (aqueous) pH scale has only a very limited applicability in organic solvents. Different acidity scales are classified in the literature [17, 35]. The quality of the various scales that may be defined depends on the chosen standard state and the experimental conditions of the (potentiometric) measurement of the activities of the solvated protons [35]. In general, a theoretical acidity scale for nonaqueous solvents can be easily defined, but it is rather difficult to approximate the practically measured values to the specifications of the definition. In the present study, the conventional pH scale was used for analyses in methanol and the apparent pH scale has been applied to background electrolyte solutions in water, water methanol mixtures, and methanol.

The conventional scale can be defined by potentiometrical measurements in cells without a liquid junction potential [35]. This scale is derived from the standard scale. The problem concerning single ion activity coefficients applied to standard scale definition are circumvented by defining the activity coefficient of the chloride ion derived from the Debye-Hückel equation as standard ion in the conventional scale. Conventional pH measurements in a cell without liquid junctions are cumbersome for practical purposes. Therefore, Porras *et al.* [17] eluded problems concerning electromotive force (EMF) measurements without liquid junction by the use of solutions based on a weak acid with a defined conventional pK_a in methanol and the corresponding salt. This way, nonaqueous buffer solutions with a well-defined conventional pH were obtained, without further calibration of the pH electrodes and unknown liquid junction potentials. The resulting conventional pH values were calculated according to the Henderson-Hasselbalch equation, which is valid for the weak acids used in methanol.

Standard reference buffers with defined pH values in aqueous organic media have been described [36, 37]. However, the reported values for 50% methanol only comprise pH 3.1–8.7. Therefore, only the apparent pH

Table 2. Ionization constants of the investigated peptides obtained in methanol, 50% v/v methanol-water mixture and water by CE

Analyte	MeOH						MeOH : water 1 : 1			Water		
	Apparent pH scale			Conventional pH scale			Apparent pH scale					
	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a1}	pK_{a2}	pK_{a3}
α-L-Asp-L-PheOMe	4.98 ±0.04	8.15 ±0.05	–	7.68 ±0.04	10.94 ±0.06	–	3.67 ±0.04	7.96 ±0.03	–	3.00 ±0.03	7.76 ±0.02	–
α-D-Asp-L-PheOMe	4.98 ±0.04	8.28 ±0.06	–	7.67 ±0.04	11.15 ±0.07	–	3.70 ±0.04	8.02 ±0.03	–	3.04 ±0.03	7.92 ±0.02	–
β-L-Asp-L-PheGMe	4.24 ±0.05	9.18 ±0.07	–	6.94 ±0.05	11.83 ±0.09	–	2.90 ±0.04	8.85 ±0.03	–	2.20 ±0.04	8.73 ±0.02	–
β-D-Asp-L-PheOMe	4.18 ±0.05	9.18 ±0.07	–	6.88 ±0.05	11.83 ±0.09	–	2.86 ±0.04	8.87 ±0.03	–	2.18 ±0.04	8.73 ±0.02	–
Met-enkephalin	5.49 ±0.11	6.86 ±0.12	10.77 ±0.22	8.19 ±0.13	9.57 ±0.11	–	4.03 ±0.10	7.36 ±0.12	11.19 ±0.13	3.33 ±0.06	7.40 ±0.07	10.10 ±0.09
Leu-enkephalin	5.76 ±0.11	7.01 ±0.13	10.74 ±0.22	8.43 ±0.12	9.80 ±0.11	–	4.30 ±0.10	7.35 ±0.12	11.20 ±0.13	3.51 ±0.06	7.40 ±0.07	10.10 ±0.09

Calculation of the pK_a values according to Eq. (4)

Table 3. Aqueous pK_a values for the investigated peptides obtained by the present CZE method compared with dissociation constants reported in the literature obtained by potentiometry and CE

Analyte	pK_{a1}			pK_{a2}			pK_{a3}		
	Experimental	References		Experimental	References		Experimental	References	
		Potentiometry	CE		Potentiometry	CE		Potentiometry	CE
α-L-Asp-L-PheOMe	3.00	3.19 [29]	3.04 [30]	7.76	7.87 [30]	7.84 [30]	–	–	–
β-L-Asp-L-PheOMe	2.20	2.36 [30]	2.22 [30]	8.73	8.62 [30]	8.73 [30]	–	–	–
Met-enkephalin	3.33	2.28 [31] 3.20 [32] 3.45 [33]	3.22 [24] 3.52 [34]	7.40	9.11 [31] 7.70 [32] 7.36 [33]	7.26 [24]	10.10	10.11 [31] 10.30 [32] 10.36 [33]	10.05 [24]
Leu-enkephalin	3.51	2.17 [31] 3.20 [32] 3.69 [33]	3.36 [24] 3.69 [34]	7.40	9.11 [31] 7.70 [32] 7.40 [33]	7.25 [24]	10.10	10.11 [31] 10.30 [32] 10.34 [33]	10.09 [24]

scale was applied to methanol-water mixtures. For mixed aqueous-organic media deviations between the apparent pK_a and the conventional pK_a values are much lower [20], the apparent pK_a values may somewhat agree with thermodynamic pK_a data. For example, the thermodynamic pK_a values of benzoic acid in ethanol-water mixtures up to 80% ethanol show good agreement with the apparent pK_a values [20]. When comparing the pH of the reference buffers in 50% methanol with the apparent pH as measured with a commercial glass electrode calibrated with aqueous buffers, we found deviations of 0.1–0.15 pH units from the values reported in the literature [36, 37] (data now shown).

For most applications in analytical chemistry pH values based on the apparent scale are applied. For the calibration of the glass electrode aqueous standard buffers are commonly used. Compared to the conventional pH scale, the apparent pH scale leads to larger deviations from the thermodynamic pH scale [17, 35]. All scales mentioned above will lead to different pH values of identical electrolyte solutions and, thus, to different pK_a values of the analytes.

Table 2 summarizes the pK_a values obtained by curve fitting of the mobility data in the various solvent systems of Fig. 2. pK_{a3} describing the dissociation equilibrium of the phenolic group of tyrosine could not be determined in methanol using the conventional scale as this equilibrium fell outside of the pH region defined by the available standard electrolytes [17]. Changing from water to the methanol-water mixture and further to pure methanol as solvent led to an decrease of the acidity of the peptides. In accordance to studies on basic compounds [17], the apparent pK_a values in methanol were about 2.7 units lower than the data referring to the conventional pH scale (Table 2). In addition, small differences in the pK_a values of the diastereomers of the isomeric aspartyl peptides were detected. Such minute differences cannot be easily observed by potentiometry [38] demonstrating the usefulness of CE for pK_a determinations.

3.3 pK_a shift in methanol

As shown in Table 2, both pK_a values describing the dissociation equilibria of the carboxylic acid group (pK_{a1}) and of the protonated amino group (pK_{a2}) increase with increasing concentrations of methanol, translating to a decrease of the acidity of the respective groups. This comparison is based on the apparent scale in water and water-methanol and on the conventional scale for the data in methanol. This effect is more pronounced for pK_{a1} compared to pK_{a2}. As a result the difference between both pK_a values becomes smaller leading to a "compression" of the mobility-pH curves when increasing the concentration of methanol as can be seen in Fig. 2. The inflexion points of the curves in methanol are closer to each other compared to water. In the case of Leu- and Met-enkephalin pK_{a1} and pK_{a2} "merge" into one as no difference between the two ionization equilibria can be observed in the curve obtained for methanol (Fig. 2C). This observation is in accordance with data obtained for ampholytic quinolones in acetonitrile-water mixtures [25]. In mixtures containing high concentrations of acetonitrile where pK_{a1} and pK_{a2} values were very close the two inflexion points merged into one.

The theoretical basis for the interpretation of the pK_a shift is the transfer activity coefficient model, which is based on the standard free energy change by transferring one mole of a species from water to an organic solvent. Different $\triangle$pK_a values for neutral acids (HA) and for cationic acids (HB^+) are known from the literature [39–41].

The changes for protonated bases with a maximum increase of 2.7 pK_a units are significantly smaller than for neutral acids which exhibit an increase of approximately 5 pK_a units. This effect can be explained by a stronger stabilization of cations in methanol in contrast to anions [20]. For the investigated peptides a stronger pK_a shift of the dissociation of the carboxylic acid was observed compared to the ionization equilibrium of the protonated base.

Barbosa and co-workers [25] explained pK_a shifts observed for zwitterionic quinolones in acetonitrile-water mixtures by differences of the effect of the change of the permittivity of the medium on the dissociation process. Thus, in the case of uncharged acids which corresponds to the second ionization step of the peptides ($HZ \leftrightarrow Z^- + H^+$) charges are created and electrostatic interactions become important. Consequently, the values of the corresponding dissociation constant decrease upon increasing the proportion of the organic solvent and concomitantly decreasing the polarity of the medium. In contrast, the dissociation of cationic acids ($H_2Z^+ \leftrightarrow HZ + H^+$, *i.e.*, p$K_{a1}$ of the peptides) does not change the number of charges. In this case the dissociation equilibrium is primarily affected by the solvation of the different species by the solvent while the change of the permitivity of the medium is of minor importance.

The pK_a shift for the mixed aqueous organic solvent is significantly smaller than the pK_a shift in pure methanol (Table 4). A steep decrease of the pK_a values when adding a few percent of water to organic solvents has been described previously for various analytes [17, 20]. The changes for pK_{a1} ($\triangle$pK_a 0.7–0.8 pK_a units) are stronger than for pK_{a2} ($\triangle$pK_a 0.1–0.2 pK_a units). For the second ionization step of the enkephalins no significant changes in the pK_a values were observed when changing from water to methanol-water. The observed changes are within experimental error. For pK_{a3} of the two neuropeptides a slight increase is also observed when changing from water to water-methanol. A valid comparison of the pK_{a3} values between water and methanol is impossible due to the lack of suitable conventional buffer references in this pH range.

Table 4. Differences between the obtained pK_a values in the three different solvents (denoted as $\triangle$pK_a)

Analyte	pK_{a1}		pK_{a2}	
	$\triangle$pK_{a1} MeOH/ water	$\triangle$pK_{a1} MeOH-water/ water	$\triangle$pK_{a2} MeOH/ water	$\triangle$pK_{a2} MeOH-water/ water
α-L-Asp-L-PheOMe	4.68	0.67	3.18	0.20
α-D-Asp-L-PheOMe	4.63	0.66	3.23	0.10
β-L-Asp-L-PheOMe	4.74	0.70	3.10	0.12
β-D-Asp-L-PheOMe	4.70	0.68	3.10	0.14
Met-enkephalin	4.86	0.70	2.17	−0.04
Leu-enkephalin	4.92	0.79	2.40	−0.05

The applied methanolic pK_a values based on the conventional pH scale established by Porras *et al.* [17]. $\triangle$pK_{a3} values of the enkephalins are only obtained between water and methanol-water (1:1), Met-enkephalin $\triangle$pK_{a3} = 1.09, Leu-enkephalin $\triangle$pK_{a3} = 1.10.

3.4 Separation of aspartyl peptides and neuropeptides by NACE

NACE can also be successfully applied to the separation of peptides. As for aqueous systems high resolution of analytes is obtained by using buffers with pH values close to the pK_a values of the peptides maximizing differences in the charge-to-mass ratio of the compounds. Figure 3 shows representative electropherograms of the diastereomers of the isomeric aspartyl peptides and the two neuropeptides under pH optimized acidic conditions using background electrolytes based on chloroacetic acid in methanol, methanol-water and water. Resolution between the neuropeptides was higher in methanol compared to the water-methanol or water while the resolution between the diastereomers of isomeric aspartyl peptides was higher when aqueous buffers were used. The best diastereomer separation in methanolic buffers was obtained using a dichloroacetate buffer at apparent pH 3.8 (Fig. 4). This buffer has not been included in the determination of the pK_a values as lower than expected electrophoretic mobility of the analytes was observed in initial experiments. This lower mobility may be due to ion pair formation as observed for basic drugs using an acetate as background electrolyte in methanol [26].

The peptides could also be separated as anionic analytes in methanolic buffers at apparent pH (pH_{app}) 7.0 and 10.3 (Fig. 5). The separation of the diastereomers of α-DL-Asp-L-PheOMe has been previously achieved in aqueous phosphate buffer, pH 8 [30]. However, the separation of Met- and Leu-enkephalin was not possible in basic aqueous buffers as also described in the literature [33] in contrast to the methanolic background electrolytes (Fig. 5B). Figure 6 illustrates the effect of the apparent pH on the resolution of Met- and Leu-enkephalin in methanol between pH_{app} 2.2 and 5.7. The highest resolution R_s = 5.8 was obtained for pH_{app} 5.7, which is close to the apparent pK_a values of the neuropeptides (Table 2).

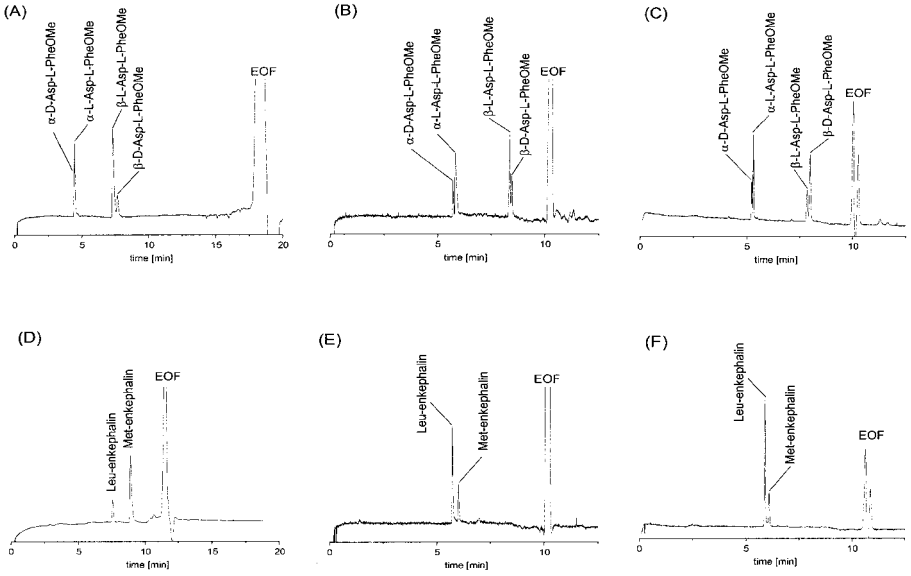

Figure 3. Separation of (A–C) α/β-DL-Asp-L-PheOMe and of (D–F) the enkephalins in the acidic pH range in the three different solvent systems under optimized pH conditions. (A) 25 mM chloroacetate buffer pH_{app} 4.4 in methanol, (D) 25 mM chloroacetate buffer pH_{app} 5.7 in methanol, (B) and (E) 25 mM chloroacetate buffer pH_{app} 3.7 in 50% v/v methanol-water, (C) and (F) 25 mM chloroacetate buffer pH 2.8 in water. Applied voltage, +25 kV.

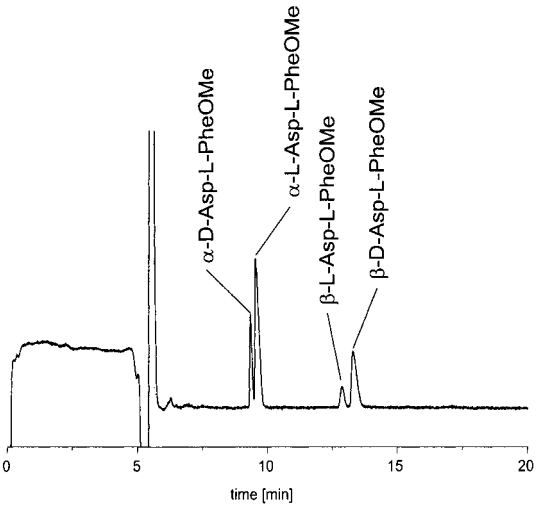

Figure 4. Separation of the α/β-DL-Asp-L-PheOMe diastereomers in methanolic dichloroacetate buffer. 25 mM dichlororacetate buffer pH_{app} 3.8; applied voltage, +25 kV.

4 Concluding remarks

CE has been employed for the determination of the pK_a values of small peptides in methanol, methanol-water and water applying the apparent pH scale as well as the conventional pH scale in methanol. In accordance with literature data on nonpeptide analytes [17, 20, 26] a pK_a shift was observed increasing the concentration of methanol. The shift was more pronounced for pK_{a1} describing the dissociation equilibrium of the carboxyl group compared to pK_{a2} which corresponds to the ioniza-

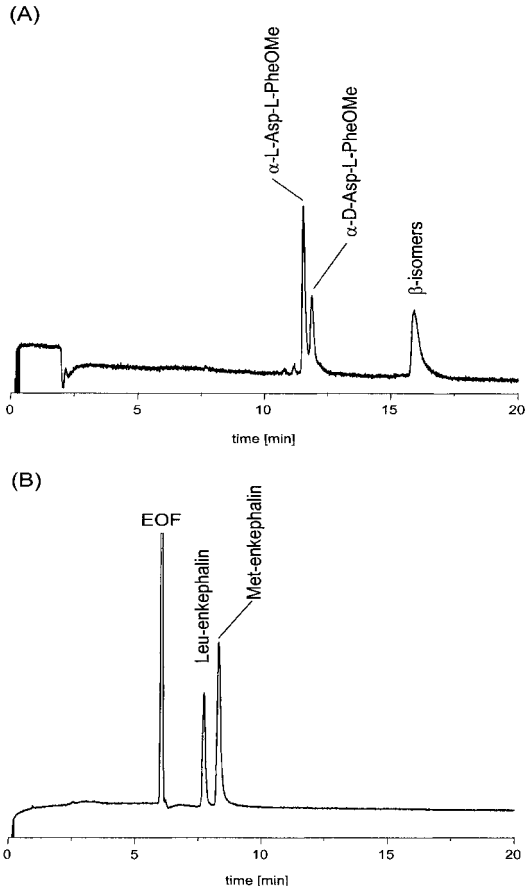

Figure 5. Electropherograms of α/β-DL-Asp-L-PheOMe and of the enkephalins obtained in methanol. (A) α/β-DL-Asp-L-PheOMe in 28 mM borate buffer pH_{app} 10.3; applied voltage, −25 kV; (B) Met- and Leu-enkephalin in 25 mM acetate buffer pH_{app} 7.0; applied voltage, +25 kV.

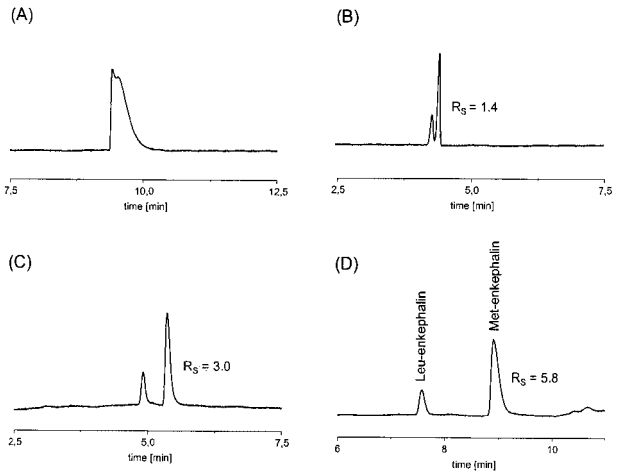

Figure 6. Effect of pH on the resolution of Met- and Leu-enkephalin in methanol. (A) 25 mM trichloroacetate buffer pH_{app} 2.2, (B) 25 mM chloroacetate buffer pH_{app} 4.4, (C) 25 mM chloroacetate buffer pH_{app} 5.1, (D) 25 mM acetate buffer pH_{app} 5.7. Applied voltage, +25 kV.

tion of the amino function. The difference in the pK_a shifts when changing from water to an organic solvent may be explained by differences in the stabilization of anions and cations [20] or by differences in the charge balance during the dissociation process [25].

NACE can be effectively applied to the separation of peptides, a class of compounds that is traditionally analyzed in aqueous buffers. The analytical power of NACE is demonstrated by the separation of the structurally closely related neuropeptides Leu-enkephalin and Met-enkephalin in an alkaline methanolic background electrolyte. This separation is not possible in alkaline aqueous buffers or in methanol-water.

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