Ovidius University Annals of Chemistry Volume 20, Number 2, pp.153-158, 2009

Determination of ionization constants of a series of therapeutic peptide hormones by capillary electrophoresis in polybrene-coated capillaries

Ghiulendan APTISA[a,b], Fernando BENAVENTE[*b], Victoria SANZ-NEBOT[b], Elisabeta CHIRILA[a] and José BARBOSA[b]

[a] *Chemistry Department, Ovidius University, 124 Mamaia Blvd, 900527 Constanta, Romania*
[b] *Department of Analytical Chemistry, University of Barcelona, Av Diagonal 647, 08028 Barcelona, Spain*

Abstract In this work, equations describing the effect of pH on the electrophoretic behaviour are used to investigate migration of a series of polyprotic amphoteric peptide hormones between pH 2 and 12 in polybrene-coated capillaries. Polybrene (hexadimethrin bromide) is a polymer composed of quaternary amines that strongly adsorbs to the fused-silica inner surface, preventing undesired interactions between the peptides and the inner capillary wall. The ability of using polybrene-coated capillaries for determination of accurate ionization constant values has been evaluated and the optimum pH values for separation of a mixture of the studied peptide hormones have been selected.

Keywords: CE, coated capillary, dissociation constant, hexadimethrin bromide, peptides, pK, prediction.

1. Introduction

In capillary zone electrophoresis of ionizable compounds, a model relating electrophoretic mobility to pH of the background electrolytes can be easily derived for prediction of separation and determination of ionization constants [1-4]. This model has been proved for a wide range of ionizable compounds [1-4]. However, these investigations have been traditionally perfomed using bare fused-silica capillary columns despite the benefits of using coated capillaries under certain circumstances [5-7].

Coating of bare fused-silica capillaries is the most effective way to reduce analyte adsorption to the inner capillary wall, modify the EOF and improve the separation selectivities in CE [5-7]. Physically adsorbed Polybrene-coated capillaries have demonstrated an excellent performance for the analysis of peptides and proteins [6-7]. Polybrene (i.e. hexadimetrhin bromide, PB) is a polycationic polymer composed of quaternary amines that strongly adsorbs to the fused-silica inner surface and enables the EOF to be reversed toward the anode. The net positively charged amine layer formed on the capillary wall reverses the EOF, and the field polarity used for the separation must be also reversed in order to ensure that the positively charged analytes migrate toward the detector [6-7].

In this work, the electrophoretic behaviour of a series of polyprotic amphoteric peptide hormones was modelled as a function of pH of the background electrolytes between pH 2 and 12 in PB-coated capillaries. The considered peptide hormones were compounds with a widespread therapeutic interest namely oxytocin, eledoisin, bradykinin, Met-enkephalin (Met=Methionine), Leu-enkephalin (Leu=Leucine), triptorelin and buserelin. The models have been used to obtain ionization constants and to select the optimum pH values allowing the separation of a mixture of the studied peptides.

2. Experimental

Chemicals and reagents

All chemicals used in the preparation of buffers and other solutions were analytical reagent grade. Hexadimethrin bromide (Polybrene, PB, M = 15000) was purchased from Aldrich (Madrid, Spain). Water with a conductivity lower than 0.05 $\mu S\ cm^{-1}$ was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Eledoisin (ele), bradykinin (bra), Met-enkephalin (met) and Leu-enkephalin (leu) were purchased from Sigma (St.

ISSN-1223-7221

Louis, MO, USA), and buserelin (bus), oxytocin (oxy) and triptorelin (tri) were purchased from Neo MPS (Strasbourg, France). Structures are shown in Table 1.

Background electrolyte (BGE).

BGE solutions covering the pH range 2-12 were prepared at the following concentrations and adjusted to the appropriate pH values using NaOH 1 M or HCl 1 M: 20 mM H_3PO_4 (pH 2), 50 mM acetic acid: 50 mM formic acid (pH 2.5-5), 20 mM diethylmalonic acid (pH 5.5-6.5), 50 mM TRIS (pH 7-9), 50 mM H_3BO_3 (pH 9.5-10.5) and 10 mM H_3PO_4 (pH 11-12). Solutions of each peptide hormone (250 $\mu g \cdot mL^{-1}$) were separately prepared in water, containing acetone at 3% (v/v) as the EOF marker. A mixture containing all the peptides at 250 $\mu g \cdot mL^{-1}$ was prepared.

All samples and solutions were passed through a 0.45-µm nylon filter (MSI, Westborough, MA), and they were stored at 4 °C when not in use.

Instrumental parameters

An HP ^{3D}CE capillary electrophoresis instrument (Agilent Technologies, Inc., Wilmington, DE) equipped with a diode array detector was used for CE analysis. Instrument control, data acquisition, and data processing were performed using ChemStation software (Agilent Technologies). 75 µm i.d. × 360 o.d. (total length, 57 cm; effective length, 48.5 cm) bare fused-silica capillary columns, purchased from Polymicro Techonologies (Phoenix, AZ, USA) were inserted in a capillary cartridge, thermostated to 25 ºC (± 0.1 ºC). All capillary rinses were performed at 930 mbar. Samples were injected hydrodynamically at 35 mbar for 5 s. Separation voltage was 25 kV. The detection window was placed at 8.5 cm from the outlet of the capillary (λ=195 nm).

pH measurements were performed with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain), equipped with a ROSS electrode 8102 (Orion Research, Boston, MA, USA).

Bare fused-silica capillary (FS capillary)

All experiments were carried out under normal polarity (with the anode at the injection end of the capillary). Capillaries were activated, conditioned or reequilibrated for the first use, between injections or after a change of BGE as indicated in a previous work [1]. Capillary was stored overnight filled with BGE.

Polybrene-coated capillary (PB-coated capillary)

Separations were carried out under reversed polarity (with the cathode at the injection end of the capillary). A solution of 5% (w/v) PB and 2% (v/v) ethylene glycol was used to coat a FS capillary. Capillaries were coated, activated, conditioned or reequilibrated for the first use, between injections or after a change of BGE [6]. The PB-coated capillary was stored overnight filled with BGE.

Theoretical approach

The dissociation equilibria for a generic fully protonated polyprotic species H_nX^z, are according to the Eq. 1:

$$\begin{aligned} &H_nX^z \Leftrightarrow H_{n-1}X^{z-1} + H^+ && K_1 \\ &H_{n-(i-1)}X^{z-(i-1)} \Leftrightarrow H_{n-i}X^{z-i} + H^+ && K_i \\ &H_{n-(n-1)}X^{z-(n-1)} \Leftrightarrow X^{z-n} + H^+ && K_n \end{aligned} \quad (1)$$

where n is the total number of ionogenic groups, z the maximum positive charge, given by the protonated basic groups, and K_i is the dissociation equilibrium constant of the ith dissociation step, that is given by the Eq. (2):

$$K_i = \frac{\left[H_{n-i}X^{z-i}\right] y^{z-i} a_{H^+}}{\left[H_{n-(i-1)}X^{z-(i-1)}\right] y^{z-(i-1)}} \quad (2)$$

where a_{H^+} is the activity of protons.

The effect of the ionic strength upon the dissociation constants has been taken into account, considering the activity coefficients of the solutes, *y*. The activity coefficients are obtained, according to IUPAC rules, from the Debye-Hückel equation [1]. Apparent dissociation constants can be defined as in the Eq. (3),

$$K_i^{'} = \frac{y^{z-(i-1)}}{y^{z-i}} K_i \quad (3)$$

The effective electrophoretic mobility, m_e, of a polyprotic compound, H_nX^z, coexisting in the form of various species at a given pH, is a function of the mobility and the molar fraction, x_i, of the individual species [1-4], as given by Eq. (4):

$$m_e = \sum_{i=0}^{n} x_{H_{n-i}X^{z-i}} m_{H_{n-i}X^{z-i}} \quad (4)$$

Replacing the molar fraction by its expression, it is obtained the Eq. (5),

$$m_e = \sum_{i=0}^{n} \frac{\left[H_{n-i}X^{z-i}\right]}{\sum_{i=0}^{n}\left[H_{n-i}X^{z-i}\right]} m_{H_{n-i}X^{z-i}} \qquad (5)$$

Eq. (5) can be rewritten as a function of dissociation constants, dividing the numerator and the denominator by an appropriate concentration $\left[H_{n-r}X^{z-r}\right]$, where 0<r<n, that is, r=0 for the fully protonated form, r=n for the fully deprotonated species and r=z if we refer to the zwitterionic form (Eq. 6):

$$m_e = \frac{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j^{'}} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j^{'}}{a_{H^+}^{i-r}} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} \frac{a_{H^+}^{r-i}}{\prod_{j=i+1}^{r} K_j^{'}} + 1 + \sum_{i=r+1}^{n} \frac{\prod_{j=r+1}^{i} K_j^{'}}{a_{H^+}^{i-r}}} \qquad (6)$$

Furthermore, considering that $a_{H^+}^{n} = 10^{-npH}$ and that $K_i^{'} = 10^{-pK_i^{'}}$, Eq. (6) can be rearranged more conveniently as a function of pH to give Eq. (7).

$$m_e = \frac{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j^{'}\right]} m_{H_{n-i}X^{z-i}} + m_{H_{n-r}X^{z-r}} + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j^{'}\right]} m_{H_{n-i}X^{z-i}}}{\sum_{i=0}^{r-1} 10^{\left[-(r-i)pH + \sum_{j=i+1}^{r} pK_j^{'}\right]} + 1 + \sum_{i=r+1}^{n} 10^{\left[(i-r)pH - \sum_{j=r+1}^{i} pK_j^{'}\right]}} \qquad (7)$$

Procedures

To obtain m_e, individual solutions of peptide hormones were injected at each pH until the m_e was constant. BGEs indicated in section 2 were run in sequence from low to high pH. m_e values were calculated as the difference between the apparent mobility of each peptide, m_{app}, and the mobility of acetone used as neutral marker, m_{EOF}, [1-5]: $m_e = m_{app} - m_{EOF} = L_C L_D / V\left(1/t_{app} - 1/t_{EOF}\right)$, where L_D is the distance from the injection point to the detector, L_C is the capillary length, and t_{app} and t_{EOF} are the migration time of the peptide hormone and the neutral marker, respectively. In order to determine the apparent pK values (pK') of the studied peptides, data pairs of m_e-pH where fitted to Eq. (7) using nonlinear regression analysis. Later, pK_i values was easily calculated by using Eq. (3) and the Debye-Hückel expression, using Eq. (8).

$$pK_i = pK_i^{'} + log\frac{y^{z-(i-1)}}{y^{z-i}} \qquad (8)$$

3. Results and discussions

In this work, Eq. (7) is applied to study the electrophoretic behaviour of a series of peptide hormones in PB-coated capillaries. Peptides are polyprotic amphoteric compounds with a variable number of acidic (C-terminus, tyrosine, cysteine, aspartic and glutamic acid) and basic groups (N-terminus, histidine, lysine, arginine). The amino acidic structure, molecular mass and number and type of ionizable groups present in the studied peptide hormones are given in Table 1, together with the generic formula that represents the fully protonated amphoteric compound H_nX^z. Table 1 summarizes the particular equations that can be obtained from general Eq. (7), taking into account only the relevant ionization constants over the pH range studied. Experimental pairs m_e-pH for each peptide have been fitted to the corresponding equation shown in Table 1. Fig. 1 shows the plot of the experimental and predicted m_e values of all the studied peptide hormones over the studied pH range.

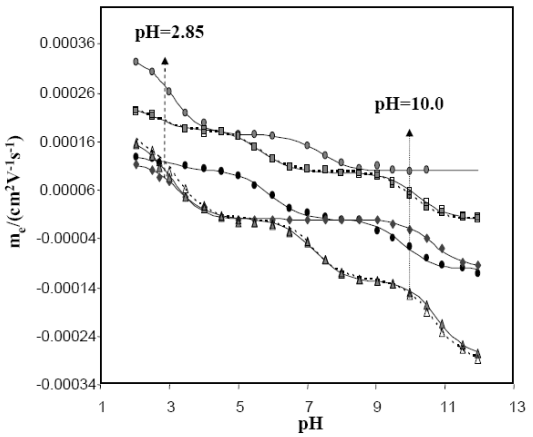

Fig. 1. Experimental (points) and predicted (lines) m_e vs pH of the BGE for bradykinin (●), triptorelin (■), buserelin (□), Met-enkephalin (▲), Leu-enkephalin (△), oxytocin (●) and eledoisin (◆).

Table 1 Structures, polyprotic formulation and electrophoretic models of the studied peptide hormones.

Peptide	M (Da)	Structure	Ionizable groups		Formula	Equation 7
			Acidic (A)	Basic (B)	H_nX^z	
Oxytocin	1006.2	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 (*)	1 phenol (-Tyr-)	1 amino group (Cys-)	H_3X^{2+}	$m_e = \frac{10^{(pK'_1+pK'_2-2pH)} m_{H_3X^{2+}} + 10^{(pK'_2-pH)} m_{H_2X^+} + 10^{(pH-pK'_3)} m_{X^-}}{10^{(pK'_1+pK'_2-2pH)} + 10^{(pK'_2-pH)} + 1 + 10^{(pH-pK'_3)}}$
Eledoisin	1187.6	Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	1 carboxylic acid (-Asp-)	1 amino group (-Lys-)	H_2X^+	$m_e = \frac{10^{(pK'_1-pH)} m_{H_2X^+} + 10^{(pH-pK'_2)} m_{X^-}}{10^{(pK'_1-pH)} + 1 + 10^{(pH-pK'_2)}}$
Bradykinin	1059.6	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	1 carboxylic acid (-Arg)	1 amino group (Arg-) 2 guanidin groups (Arg-/-Arg)	H_4X^{3+}	$m_e = \frac{10^{(pK'_1-pH)} m_{H_4X^{3+}} + m_{H_3X^{2+}} + 10^{(pH-pK'_2)} m_{H_2X^+}}{10^{(pK'_1-pH)} + 1 + 10^{(pH-pK'_2)}}$
Met-enkephalin	573.2	Tyr-Gly-Gly-Phe-Met	1 carboxylic acid (-Met) 1 phenol (Tyr-)	1 amino group (Tyr-)	H_3X^+	$m_e = \frac{10^{(pK'_1-pH)} m_{H_2X^+} + 10^{(pH-pK'_3)} m_{HX^-} + 10^{(2pH-pK'_2-pK'_3)} m_{X^{2-}}}{10^{(pK'_1-pH)} + 1 + 10^{(pH-pK'_3)} + 10^{(2pH-pK'_2-pK'_3)}}$
Leu-enkephalin	555.6	Tyr-Gly-Gly-Phe-Leu	1 carboxylic acid (-Leu) 1 phenol (Tyr-)	1 amino group (Tyr-)		
Triptorelin	1310.6	Pyr-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2 (*)	1 phenol (-Tyr-)	1 imidazol group (-His-) 1 guanidin group (Arg)	H_4X^{2+}	$m_e = \frac{10^{(pK'_1+pK'_2+pK'_3-3pH)} m_{H_4X^{2+}} + 10^{(pK'_2+pK'_3-2pH)} m_{H_3X^{2+}} + 10^{(pK'_3-pH)} m_{H_2X^+}}{10^{(pK'_1+pK'_2+pK'_3-3pH)} + 10^{(pK'_2+pK'_3-2pH)} + 10^{(pK'_3-pH)} + 1}$
Buserelin	1238.7	Pyr-Pro-His-Trp-Ser-Tyr-D-Ser (^{t}Bu)-Leu-Arg-Pro-NHC_2H_5 (*)	1 phenol (-Tyr-)	1 imidazol group (-His-) 1 guanidin group (Arg)		

(*) One extra basic group was considered in addition to marked groups. Explanation is given in the text.

The symbols stand for the experimental data and the solid lines indicate the predicted m_e values. As can be observed, the concordance between experimental and predicted curves is excellent, as in FS capillaries [1].

Special attention must be paid to oxytocin, triptorelin and buserelin. In oxytocin only two ionizable groups are theoretically present and in triptorelin and buserelin there are three. However, the plot of m_e vs. pH for these peptides shows an unexpected increase at highly acidic pH value as can be seen in Fig. 1. That was also observed before in FS capillaries [1], discarding now in PB-coated capillaries the undesired interactions of these peptides with the capillary wall in this pH range. The simplest interpretation for this abnormal behavior is the presence of an additional basic ionizable group, as was suggested to explain a similar behaviour by Castagnola et al. in water-trifluoroethanol buffers [8].

Table 2 summarizes the pK' and pK values obtained for the studied peptide hormones in PB-coated capillaries, together with the values obtained in a previous work in FS capillaries [1]. The pK values obtained in PB-coated capillaries are similar to those reported before in FS capillaries [1].

Table 2 pK values of the studied peptide hormones.

Peptide	PB-coated		FS [1]
	pK'	pK	pK
Eledoisin	3.28 (0.03)	3.22	3.48
	10.65 (0.04)	10.73	10.54
Oxytocin	3.01 (0.46)	3.01	2.98
	5.95 (0.08)	5.88	6.04
	9.81 (0.07)	9.89	9.84
Bradykinin	3.05 (0.01)	2.87	2.68
	7.38 (0.02)	7.23	6.66
Triptorelin	2.60 (0.16)	2.39	2.80
	5.65 (0.03)	5.44	5.93
	9.99 (0.03)	9.91	9.63
Buserelin	2.62 (0.27)	2.44	2.66
	5.65 (0.07)	5.45	5.92
	10.21 (0.04)	10.13	9.76
Met-enkephalin	3.02 (0.04)	2.97	3.17
	7.19 (0.04)	7.23	7.30
	10.81 (0.05)	11.05	10.30
Leu-enkephalin	3.22 (0.05)	3.17	3.31
	7.21 (0.05)	7.25	7.29
	10.68 (0.05)	10.93	10.34

PB-coated capillaries exhibit a pH-independent anodic EOF (data not shown). A great advantage of PB-coated versus FS capillaries, is that at pH lower than 4.5, the anodic EOF in PB-coated capillaries is significantly higher than the cathodic EOF in FS capillaries, allowing a significant decrease of the analysis times in that acidic pH range. This last feature of PB-coated capillaries may be detrimental to achieve optimum separation resolution under those pH conditions.

The optimum pH for separation of a mixture of the studied peptide hormones can be simply selected from Fig. 1, by looking for the greatest differences between the m_e values of adjacent peaks (see the insets of Fig. 1) [1].

As resolution is not fully achieved at a single pH value, two pH ranges are selected. One very narrow, around pH 2.85 and another wider around pH 10.0, where almost all the most critical pairs are separated.

Fig. 2-a, 2-b and 2-c show the electropherograms of the mixture at pH 2.60, 2.85 and 10.0, respectively. Fig. 2-a shows five electrophoretic peaks corresponding to the peptides, because at pH 2.6 the pairs oxy-met and tri-bus comigrate due to the slight differences in their m_e values at this pH value (Fig. 1).

pH selection in this acidic region is crucial as can be seen comparing the separation selectivity and migration order of Fig. 2-a and 2-b, where the pH differs only in 0.25 units. Thus, at pH 2.85 (Fig. 2-b), the pairs leu-oxy and tri-bus comigrate. On the other hand, at pH 10.0 tri and bus are partially resolved, while the pair leu-met comigrates. As expected, the migration order is reversed with respect to the order observed with FS capillaries [1].

4. Conclusions

The study demonstrated that the electrophoretic behaviour of low molecular weight peptide hormones can be modeled as a function of pH in PB-coated capillaries using equations that have been traditionally used to investigate migration of polyprotic compounds in FS capillaries.

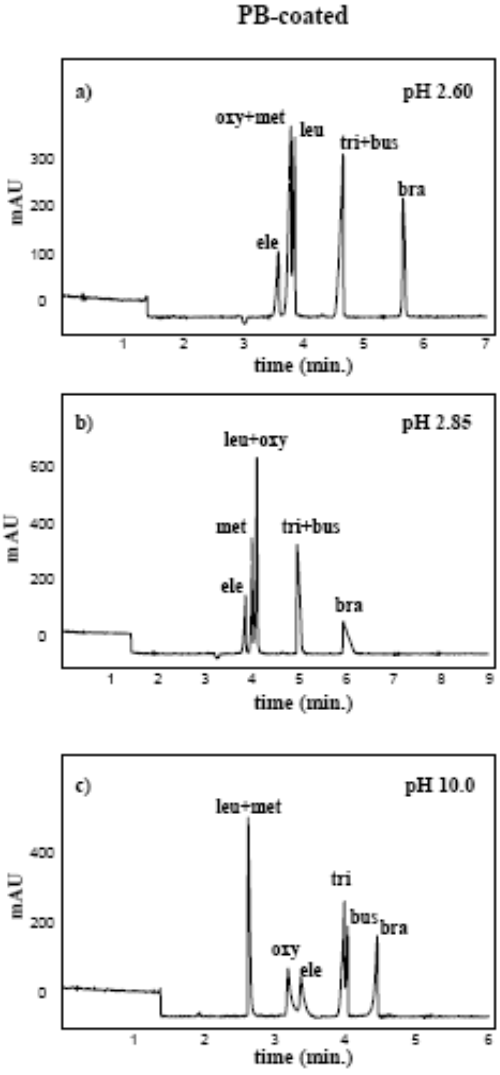

Fig. 2. Electropherograms of a mixture of the studied peptides in PB-coated capillaries at pH a) 2.60, b) 2.85 and c) 10.0.

The pK values obtained in PB-coated capillaries are similar to those previously reported in FS capillaries. PB-coated capillaries may be helpful for determination of ionization constants of compounds which adsorb to the inner wall of FS capillaries. Furthermore, may be useful to speed-up these determinations in the acidic pH range, due to the increased EOF values.

5. Acknowledgments

This study was supported by a grant from the Spanish government (CTQ2008-00507/BQU).

6. References

* E-mail address: fbenavente@ub.edu

[1]. V. Sanz-Nebot, F. Benavente, I. Toro and J. Barbosa, Electrophoresis **22**, 4333-4340 (2001).

[2]. J. M. Herrero-Martinez, M. Sanmartin, M. Roses, E. Bosch and C. Rafols, Electrophoresis **26**, 1886-1895 (2005).

[3]. J. Barbosa, D. Barrón and E. Jiménez-Lozano, J. Chromatogr. A. **839**, 183-192 (1999).

[4]. F. Benavente, B. Andón, E. Giménez, J. Barbosa and V. Sanz-Nebot, Electrophoresis **29**, 2790-2800 (2008).

[5]. J. P. Landers, Handbook of Capillary Electrophoresis, 2nd Edn., CRC Press, Boca Raton, FL, 1997.

[6]. V. Sanz-Nebot, F. Benavente, A. Vallverdú, N. A. Guzman and J. Barbosa, Anal. Chem. **75**, 5220-5229 (2003).

[7]. J. E. Wiktorowicz and J. E. Colburn, Electrophoresis **11**, 769-773 (1990).

[8]. M. Castagnola, D. V. Rosetti, F. Misiti, L. Casiano, B. Giardina and I. Messana, J. Chromatogr. A **792**, 57–65 (1997).