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Separation and investigation of structure–mobility relationship of gonadotropin-releasing hormones by capillary zone electrophoresis in conventional and isoelectric acidic background electrolytes

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Abstract

Capillary zone electrophoresis (CZE) has been applied to qualitative and quantitative analysis, separation and physicochemical characterization of synthetic gonadotropin-releasing hormones (GnRHs) and their analogs and fragments. Structurally related peptides were separated in conventional and isoelectric acidic background electrolytes (BGEs), pH 2.18–2.50. Best separation was achieved in isoelectric BGE composed of 200 mM iminodiacetic acid, pH 2.32. The effective electrophoretic mobilities, m_{ep}, of GnRHs in five BGEs were determined and four semiempirical models correlating effective mobility with charge, q, and relative molecular mass, M_r, (m_{ep} versus q/M_r^k, where k is related to the molecular shape) were tested to describe the migration behavior of GnRHs in CZE. None of the models was found to be quite definitively applicable for the whole set of 10 GnRHs differing in size (tetrapeptide–decapeptide) and positive charge (0.91–3.00 elementary charges). Nevertheless, for the dependence of m_{ep} on q/M_r^k, the highest coefficient of correlation, $R = 0.995$–0.999, was obtained for k close to the value 0.5 in all five acidic BGEs. This indicates that the most probable structure of GnRHs in these BGEs can be predicted as a random coil.

Keywords: Capillary electrophoresis; GnRH; LHRH; Peptides; Effective electrophoretic mobility; Structure–mobility relationship

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a peptide neurohormone responsible for the release of follicle stimulating hormone and luteinizing hormone from the anterior pituitary (adenohypophysis). It is present in different biological species such as human, pig, lamb, chicken, sea bream and salmon with small modifications in its peptide sequence. Human GnRH, hGnRH, alternatively termed luteinizing hormone-releasing hormone (LHRH), is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), which is synthesized in neurosecretory cells within the hypothalamus and regulates reproductive functions and maintenance of secondary sex characteristics in males and females. LHRH agonists are used for the treatment of hormone-dependent breast and prostate cancers [1]. In reproductive medicine, they serve to prevent a premature LH surge prior to stimulation of ovulation. Synthetic hGnRH is produced under the name gonadorelin. Several commercially available analogs are used as drugs. Buserelin, [Des-Gly^{10},D-Ser(*t*Bu)^6,Pro-NHEt^9]hGnRH, and triptorelin, [D-Trp^6]hGnRH, are used in treatment of prostate cancer, deslorelin, [Des-Gly^{10},D-Trp^6,Pro-NHEt^9]hGnRH, for treatment of true precocious puberty, goserelin, [D-Ser(*t*Bu)^6,Azagly^{10}]hGnRH for treatment of advanced breast cancer and nafarelin, [D-2-Nal^6]hGnRH, in treatment of endometriosis.

Synthetic GnRHs and their analogs and fragments are frequently used as model analytes, usually in mixtures with other peptides, to demonstrate new methodology and/or instrumentation developments and optimization of separation conditions for analysis and separation of peptides by capillary electromigration methods [2]. Successful combination of capillary electrophoresis (CE) with mass spectrometry (MS) detection without make-up flow or nebulizing gas was shown by analysis of gonadorelin with sensitivity on the level of immunoassay [3]. Home-made on-line preconcentration CE based on the coupling of capillary packed with C18 sorbent to the fused

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silica capillary was optimized for the purity control of synthetic biologically active peptides including GnRH in BGE composed of 25 mM K_2HPO_4, pH 3.5 [4]. Membrane preconcentration CE-MS/MS was appropriately constructed for sequencing biologically active peptides at the sub-100 fmol level [5]. The analysis of nine model peptides was performed in 2 mM ammonium acetate and 1% (v/v) acetic acid. Successful application of fused silica (FS) capillary modified by positively charged alkylaminosilyl monomer to capillary zone electrophoresis (CZE) and capillary electrochromatographic (CEC) separations of the mixture of peptides and proteins was presented using UV-absorption and MS detection [6]. Rapid separation of five peptides including GnRH was achieved in 5 mM acetic acid in 50% (v/v) MeCN. A new CE-nanoflow electrospray ionization (ESI) interface, where separation column, an electrical porous junction and spray tip were integrated on single FS capillary, was found as a suitable device for analysis and separation of nine peptides including GnRH and proteins in 1 M acetic acid, pH 2.4 [7]. Human GnRH and its four analogs, [D-Ala^6]hGnRH, [D-Lys^6]hGnRH, [D-Phe^2,D-Ala^6]hGnRH and [Gly-OH^{10}]hGnRH were analyzed in capillary derivatized with 3-(aminopropyl)trimethoxysilane in 0.01 M acetic acid, pH 3.5, by CE with ESI-MS detection [8]. For determination of side products of buserelin synthesis, CE with field-amplified sample injection was used [9] and for determination of purity degree and counter-ion content in lecirelin, [D-Tle^6,Pro-NHEt^9]GnRH, CZE and capillary isotachophoresis were applied [10]. Optimal separation conditions were found in acidic BGEs with pH less than 3.5. An off-line coupling of CE and MALDI-MS was successfully applied for the analysis of four standard peptides, proteins and real tear fluid in 50 mM ammonium acetate buffer, pH 7.4 [11]. According to the known or established characteristics, such as dissociation constants of ionogenic groups of therapeutic peptides optimal separation conditions were determined for set of seven peptides including buserelin and triptorelin for CE [12]. The conditions were tested in a wide pH range 2–12; the best separation was achieved at pH 2.85, in BGE composed of 50 mM acetic acid and 50 mM formic acid, pH adjusted by NH_4OH. The same set of peptides was successfully applied for separation and characterization in hydroorganic mixture of formic acid, acetic acid and 2-propanol by CE-ESI-MS with commercial [13] and home-made graphite coated sheath-flow interface [14] and as well for comparison of predicted and experimentally obtained resolution, electrophoretic mobility and retention factor in CE and HPLC [15]. Complete separation of gonadorelin and its five analogs was provided by RP-HPLC in mobile phase consisting of MeCN and phosphate buffer, pH 2.5, by CE in phosphate or borate buffer and as well by micellar electrokinetic chromatography (MEKC) with the same BGE with addition of cetyltrimethylammonium bromide (CTAB), 3-[(3-choloamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Triton X-100 as micellar constituents [16]. Alternative detection technique to UV-absorption, contactless conductivity detection was employed for the analysis of mixture of nine peptides including GnRH in phosphate buffer, pH 2.5 [17]. Detection limits at the μM level in combination with good resolution were shown. Native GnRHs are generally used to study biological regulatory processes such as kinetics and mechanism of their action. A rapid CE assay for measuring the stability of human and salmon GnRH in the presence of intestinal enzymes was developed and validated [18]. The analysis was performed in acetic acid-based BGE, pH 4.0, and applied to the stability of GnRH analogs in salmon intestinal digest. Determination of gonadorelin in plasma by on-capillary preconcentration CE system was demonstrated [19]. For suppressing adsorption on the wall, the capillary had cationic coating and for increasing sensitivity and selectivity MS detection was used.

The aim of this work was to perform qualitative and quantitative analysis of synthetic preparations of human, salmon and chicken GnRHs, and their analogs and fragments, by CZE in acidic conventional and isoelectric BGEs. Suitable experimental conditions should be developed for CZE separation of the mixtures of these structurally related peptides. In addition to the purity degree also some physicochemical characteristics of the analyzed peptides, such as effective electrophoretic mobilities of GnRHs at standard temperature, 25 °C, should be determined, and different models of the dependence of mobility of GnRHs on their charge and size should be tested in order to predict the probable structure of GnRHs in solution.

2. Theory

2.1. Models of correlations between mobility of peptides and their charge and size

Several semiempirical models correlating effective electrophoretic mobilities of peptides, m_{ep}, with their effective charge, q, and molecular size expressed as relative molecular mass, M_r, or number of amino acids in polypeptide chain, n, respectively, have been developed. The models are based on Stokes law, describing the motion of a particle in liquid medium, and on the action of electric field force on charged molecule.

Offord [20] has firstly quantitatively described this relationship for the series of oligo- and polypeptides separated by paper electrophoresis:

$$m_{ep} = \frac{k_1.q}{M_r^{2/3}} \quad (1)$$

where k_1 is a constant of proportionality. This relationship was found to be applicable also for peptides separated by CZE in a free solution [21–24].

For rigid spherically shaped molecules in low ionic strength buffers the following relationship was gained by Grossman [25]:

$$m_{ep} = \frac{k_1.q}{M_r^{1/3}} \quad (2)$$

Another semiempirical model was suggested for the synthetic polymers with cylindrical or rod-shaped molecules [26]:

$$m_{ep} = \frac{k_1.q}{M_r^{1/2}} \quad (3)$$

Cross and Garnham [27] adapted the above relations to the logarithmic form:

$$\log\left(\frac{m_{ep}}{q}\right) = k\ \log M_r \quad (4)$$

where k is the exponent of M_r in non-logarithmic relation, which can be determined as a slope of this dependence and which is related to the shape of peptide molecule.

For some other models and formulas see our recent paper [28] where application of these semiempirical models for prediction of conformation (shape) of peptide molecules in solution is discussed and demonstrated on the example of insect oostatic peptides. The structure of peptide molecule is derived from the relationship between frictional coefficient or electrophoretic mobility of the peptide, respectively, and the exponent k of its relative molecular mass or of the number of amino acid residues in its molecule. Each type of the correlation (particular value of exponent k) corresponds to the specific shape of molecule in a free solution. As summarized in Ref. [25], $k \sim 1/3$ corresponds to solid sphere, k close to 1/2 refers to random coil and $k \sim 2/3$ is related to the shape of wide thin disc. For determination of molecular conformations it is very important to find the best way, in which electrophoretic mobility in a free solution is related to molecular size. Peptides form more organized secondary structures, which are strongly dependent on the medium, particularly on solvent, ionic strength and pH of BGE [29–31]. At the high molecular mass range as long-chain polymers, polypeptides may form different types of ordered structures such as α-helical or β-sheet conformation [29,32]. The polypeptide structures help to predict shapes of proteins in a free solution and their knowledge is helpful in peptide mapping of proteins [33]. The data on secondary peptide structures derived from relation between electrophoretic mobility and charge to size ratio, can be advantageously utilized also in the structure–activity studies of peptide hormones.

3. Experimental

3.1. Chemicals

All chemicals used were of analytical reagent grade. Iminodiacetic acid (IDAA) was obtained from Bachem (Bubendorf, Switzerland), phosphoric and acetic acids were obtained from Lachema (Brno, Czech Republic) and Tris (tris (hydroxymethyl)aminomethane) was supplied by Serva (Heidelberg, Germany). Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) was supplied by Fluka (Buchs, Switzerland).

3.2. Peptides

The list of analyzed peptides and their abbreviations, sequences and relative molecular masses, M_r, are presented in Table 1. The oligopeptide fragments of human GnRH, YGLRPG-NH_2, LRPG-NH_2, (pGlu)HWSTGLRPG-NH_2 and (pGlu)HWS, were purchased from Sigma (St. Louis, MO, USA). The other analogs and fragments of GnRHs were prepared by solid phase synthesis in the Institute of Organic Chemistry and Biochemistry.

3.3. Instrumentation

The capillary electrophoretic experiments were carried out in commercial P/ACE MDQ System (Beckman-Coulter, Fullerton, CA, USA), data acquisition and evaluation were performed using the software P/ACE System MDQ, version Karat supplied by Beckman.

The apparatus was equipped with the internally non-coated FS capillary with outer polyimide coating, total/effective length 39.0/28.8 cm, I.D./O.D. 50/375 μm (Polymicro Technologies, Phoenix, AR, USA). The analytes were detected by UV–vis absorption spectrophotometric photodiode array detector (190–600 nm) set at constant wavelength 206 nm. The temperature of capillary liquid coolant was set at 25 °C.

The new capillary was gradually flushed with water, 1 M NaOH, water and BGE, each wash for 10 min. Finally, the capillary was conditioned by a 20 min application of the high voltage to equilibrate the inner surface with BGE and to stabilize electroosmotic flow. Between runs under the same conditions, the capillary was rinsed with the BGE for 2 min. Before a change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The samples were injected with pressure 6.9–13.8 mbar for 5–15 s. The samples were dissolved in deionized water and their concentrations were in the

Table 1
Sequences of analyzed peptides and their relative molecular masses (M_r)

Peptide	Sequence in three-letter code	M_r
Human GnRH (hGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	1181.5
[βAla^6-ProNHEt^{10}]hGnRH	pGlu-His-Trp-Ser-Tyr-β-Ala-Leu-Arg-Pro-Pro-NHEt	1262.5
[Des-Pro-Gly-NH_2]hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-OH	1028.3
[Des-Arg-Pro-Gly-NH_2]hGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-OH	872.1
Fragment of hGnRH 1-4	pGlu-His-Trp-Ser-OH	538.6
Fragment of hGnRH 4-10	H-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2	766.0
Fragment of hGnRH 7-10	H-Leu-Arg-Pro-Gly-NH_2	440.6
Salmon GnRH (sGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2	1211.5
[Des-Gly-NH_2]sGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-OH	1155.4
[Des-pGlu^1-D-Orn^6]sGnRH	H-His-Trp-Ser-Tyr-D-Orn-Trp-Leu-Pro-Gly-NH_2	1058.3
Chicken GnRH I (cGnRH)	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH_2	1180.4
[Des-Gly-NH_2]cGnRH II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-OH	1179.4

Table 2
Composition and pH of the BGEs applied for CZE analyses and separations of GnRHs and their analogs and fragments, separation voltage, U, and electric current, I

BGE no.	BGE constituents	pH	U (kV)	I (μA)
I	2 M acetic acid	2.18	20	19.9
II	100 mM H_3PO_4, 50 mM Tris	2.25	15	43.2
III	100 mM iminodiacetic acid	2.30	20	33.0
IV	200 mM iminodiacetic acid	2.32	15	35.0
V	500 mM acetic acid	2.50	25	15.6

range 0.6–1.3 mg/ml. The BGEs were filtered through a 0.45-μm syringe filter (Millipore, Bedford, MA, USA) before use. The list of composition and pH of BGEs, separation voltage and electric current are presented in Table 2.

4. Results and discussion

4.1. Selection of separation conditions and determination of peptide charge

The strategy for the rational selection of experimental conditions for CZE analysis and separations of GnRHs and their analogs and fragments followed the general rules of selection of suitable CZE separation conditions [34] and took into account the specific properties of these peptides resulting from their structure. The selection of the composition of the BGEs includes the type and concentration of buffer components and pH, and it also takes into account the requests for solubility and chemical stability of analyzed peptides [35,36]. Effective charges of peptides are strongly dependent on pH and pK_a of ionogenic groups of amino acid residues present in peptide chain. The analyzed peptides, see Table 1, contain several types of ionogenic groups; all peptides, except fragments of hGnRH 4-10 and 7-10, possess imidazolyl group of the histidine (average pK_a 6.3), and all peptides, except fragments of hGnRH 1-4 and 7-10, contain phenol group of tyrosine (average pK_a 10.4), all hGnRHs except [Des-Arg-Pro-Gly-NH_2]hGnRH and fragment of hGnRH 1-4, possess guanidinyl group of arginine (average pK_a 11.3), three peptides (fragments of hGnRH 4-10 and 7-10 and [Des-pGlu^1-D-Orn^6]sGnRH) possess α-amino group of the N-terminus of the peptide chain (average pK_a 8.1), five peptides ([Des-Arg-Pro-Gly-NH_2]hGnRH, [Des-Pro-Gly-NH_2]hGnRH, fragment of hGnRH 1-4, [Des-Gly-NH_2]sGnRH and [Des-Gly-NH_2]cGnRH II) contain α-carboxyl group of the C-terminus of the peptide chain (average pK_a 3.5) and salmon fragment of its analog [Des-pGlu^1-D-Orn^6]sGnRH contains amino group of ornithine (average pK_a 9.9). The relative molecular mass of analyzed peptides was in the range 440.6–1262.5 (see Table 1).

One of the most important parameters for selection of suitable experimental conditions for CZE analysis and separation of peptides is the pH dependence of their effective and specific charges, since the electrophoretic mobility of peptides is directly proportional to their effective charge. For that reason the dependence of effective charge and specific charge (effective charges divided by relative molecular mass) of all peptides to be analyzed has been calculated by the earlier developed computer program Nabamfo [37] using the above given values of pK_a of ionogenic groups, which were obtained as average values from their ranges presented in Ref. [36]. From the course of the pH dependence of the specific charge of peptides to be analyzed and separated (see Fig. 1) it follows that these peptides can be analyzed as cations at pH < 7 and as anions mostly at pH > 10. Consequently, the strongly acidic conventional and isoelectric BGEs (pH 2.18–2.50) were selected for CZE analyses and separations of the above peptides, since at this pH the dissociation of the silanol groups is suppressed and the electrostatic interactions of positively charged peptides and inner capillary surface are reduced. Full composition and pH of the used BGEs together with the separation voltage and electric currents are presented in Table 2. In all used BGEs, the GnRHs had positive charges in the range from 0.91 to 3.00 of elementary charge. The calculated values of effective charge at five selected pHs of the BGEs used are given in Table 3. With respect to their relatively hydrophilic character all analyzed peptides were dissolved in deionized water, which brought an advantage that the same sample solution could be applied to CZE analyses in different BGEs, and in addition the electric-field-enhanced concentrating effect was utilized to concentrate the diluted peptide solutions.

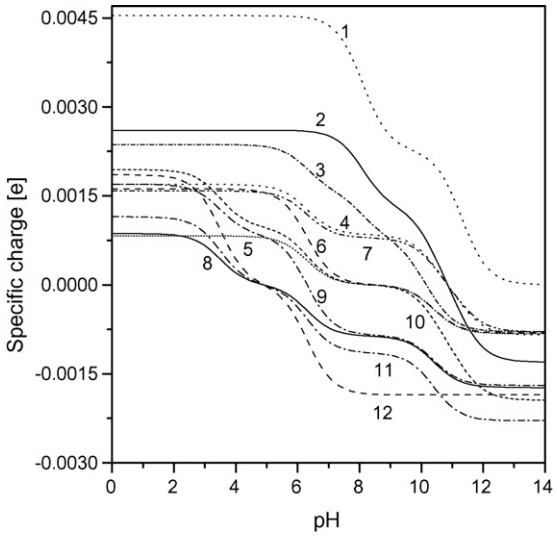

Fig. 1. The pH dependence of specific charge (effective charge divided by relative molecular mass) of analyzed peptides, (1) fragment of hGnRH 7-10, (2) fragment of hGnRH 4-10, (3) [Des-pGlu^1-D-Orn^6]sGnRH, (4) hGnRH, (5) sGnRH, (6) cGnRH, (7) [βAla^6-ProNHEt^{10}]hGnRH, (8) [Des-Gly-NH_2]sGnRH, (9) [Des-Gly-NH_2]cGnRH, (10) [Des-Pro-Gly-NH_2]hGnRH, (11) [Des-Arg-Pro-Gly-NH_2]hGnRH, (12) fragment of hGnRH 1-4.

4.2. Qualitative analysis of GnRHs and determination of purity degree

For full characterization of peptide preparations, especially pharmaceuticals and peptides used in biological tests it is important to know the content of admixtures, originating from peptide synthesis and purification procedures. The purity degrees of GnRHs were determined as the ratio of the corrected peak area of peptide itself to the sum of corrected areas of all peaks present

Table 3
Calculated effective charges, q, and CZE determined effective electrophoretic mobilities, m_{ep}, corrected to standard temperature, 25 °C, of analyzed peptides in five different BGEs

Peptide	q (e)					m_{ep} (10^{-9} m^2 V^{-1} s^{-1})				
	BGE I	BGE II	BGE III	BGE IV	BGE V	BGE I	BGE II	BGE III	BGE IV	BGE V
Human GnRH (hGnRH)	2.00	2.00	2.00	2.00	2.00	14.26	11.69	14.60	12.87	16.85
[βAla^6-ProNHEt^{10}]hGnRH	2.00	2.00	2.00	2.00	2.00	14.68	12.05	15.22	13.47	17.90
[Des-Pro-Gly-NH_2]hGnRH	2.00	2.00	2.00	2.00	2.00	14.97	12.27	15.37	13.52	17.29
[Des-Arg-Pro-Gly-NH_2]hGnRH	0.95	0.94	0.93	0.92	0.91	8.33	7.15	8.54	7.66	9.55
Fragment of hGnRH 1-4	0.95	0.94	0.93	0.92	0.91	10.81	9.44	11.18	10.13	11.94
Fragment of hGnRH 4-10	2.00	2.00	2.00	2.00	2.00	18.21	15.22	18.96	16.86	21.99
Salmon GnRH (sGnRH)	1.00	1.00	1.00	1.00	1.00	7.63	6.38	7.98	7.01	9.42
[Des-Gly-NH_2]sGnRH	0.95	0.94	0.93	0.92	0.91	7.43	6.37	7.68	6.85	8.78
[Des-pGlu^1-D-Orn^6]sGnRH	3.00	3.00	3.00	3.00	3.00	22.16	17.24	22.47	20.95	27.20
Chicken GnRH I (cGnRH)	1.00	1.00	1.00	1.00	1.00	7.63	6.38	7.98	7.01	9.42

Full composition of BGEs is given in Table 2.

on electrophoregrams, corrected peak area is peak area corrected with respect to migration velocity of the given peak, it was calculated as the ratio of peak area and migration time of this peak. The qualitative analyses of GnRHs were carried out in BGE II, 100 mM H_3PO_4 and 50 mM Tris, pH 2.25; peptides with purity degree lower than 75% were not used for further experiments. The analyzed peptides were mostly well purified by HPLC, the values of purity degrees reached 88–99%. The CZE analysis of highly pure C-terminal tetrapeptide fragment of hGnRH, hGnRH 7-10, with purity degree 96.2% is depicted in Fig. 2A. CZE analysis of the nonapeptide [Des-Gly-NH_2]cGnRH II (see Fig. 2B) demonstrates example of impure peptide with four major and several minor admixtures, which could not be used for further experiments. The purity degree in this case was only 55.1%.

4.3. Separation of structurally related peptides and determination of peptide mobilities

In addition to qualitative analysis of individual synthetic peptide preparations, the separation of closely related GnRHs and their analogs and fragments was tested. These separations are important from the point of view of simultaneous analysis of the whole molecules of GnRHs and their degradation products. The suitable separation conditions for the separations were derived from the course of the pH dependence of specific charge of these peptides (see Fig. 1), and from the experience obtained in the analysis of individual peptides by CZE in different BGEs. Three mixtures of four GnRHs were separated in five BGEs in pH range 2.18–2.50. The mixture I contained hGnRH and its fragments, mixture II contained GnRH decapeptides of three species and in mixture III sGnRH, its two fragments and hGnRH fragment were present. The efficiency and resolution of separation in conventional and isoelectric BGEs as well as composition of mixtures are given in Table 4. Acetic acid based BGEs, BGE I and BGE V, were the best ones from the standpoint of the speed of analysis, but separation efficiency and resolution in these BGEs were lower than those in the other BGEs. The shortest times of analyses, 3.4–4.5 min, were achieved in BGE V, 500 mM acetic acid, pH 2.5, see the electrophoregrams in Fig. 3C for mixture I and in Fig. 3F for mixture III, respectively, but the separation efficiency was up to 10 times lower than in the BGEs II and IV. The highest efficiency of separation was achieved in isoelectric BGE, BGE IV, 200 mM IDAA, pH 2.32, with theoretical plates number in

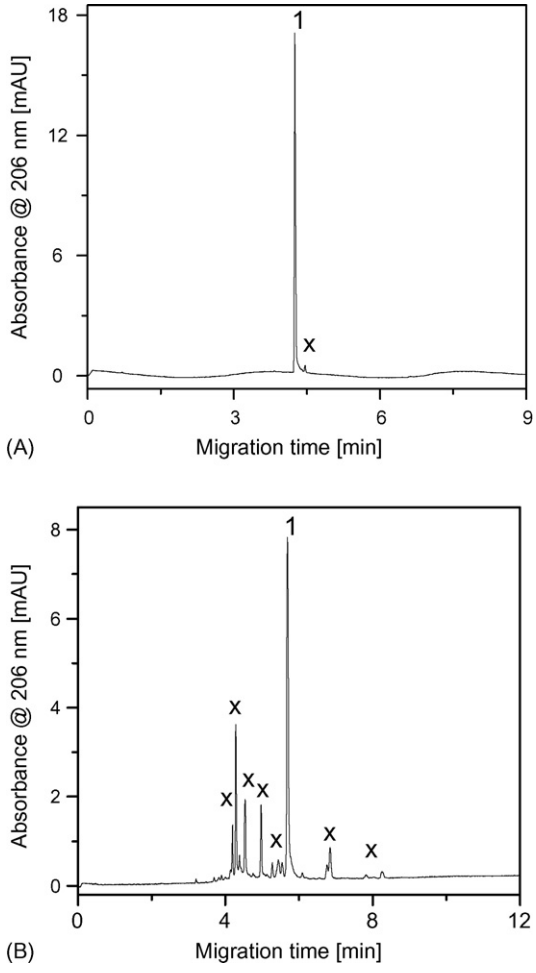

Fig. 2. CZE analyses of (A) HPLC purified fragment of hGnRH 7-10, 1.40 mg ml^{-1}, and (B) crude synthetic product of [Des-Gly-NH_2]cGnRH II, 1.10 mg.ml^{-1}, (1) main synthetic product, (x) non-identified impurities. Analyses were performed in BGE II (see Table 2). Other experimental conditions are given in the text (see Section 3.3).

Table 4
Separation efficiency (number of theoretical plates) and resolution of CZE separation of three mixtures of GnRHs and their fragments and analogs in five BGEs

Mixture no.	Peptide	Efficiency (number of theoretical plates)					Resolution				
		BGE I	BGE II	BGE III	BGE IV	BGE V	BGE I	BGE II	BGE III	BGE IV	BGE V
I	Fragment of hGnRH 4-10	21709	125188	60959	164189	14299	–	–	–	–	–
	hGnRH	18605	67104	43677	112854	12955	6.3	16.8	11.2	15.7	4.8
	Fragment of hGnRH 1-4	21967	56571	40334	106615	13811	6.7	10.9	9.6	11.5	5.4
	[Des-Arg-Pro-Gly-NH_2]hGnRH	17631	75473	47091	123868	12119	5.6	14.6	9.2	12.2	3.0
II	[βAla^6-ProNHEt^{10}]hGnRH	28602	104971	67695	152187	17714	–	–	–	–	–
	hGnRH	16941	58881	42961	100696	11929	0.8	2.1	1.8	2.5	1.2
	sGnRH	12807	36244	26417	55750	8739	6.9	24.9	17.9	23.6	8.0
	cGnRH I	12807	36244	26417	55750	8739	0	0	0	0	0
III	[Des-pGlu^1-D-Orn^6]sGnRH	27892	211526	74027	148832	14898	–	–	–	–	–
	[Des-Pro-Gly-NH_2]hGnRH	17129	106062	54877	120507	13414	10.7	25.8	18.3	24.7	9.2
	sGnRH	12985	36711	18131	59590	6529	13.4	29.3	18.2	29.6	10.6
	[Des-Gly-NH_2]sGnRH	12985	36711	13857	48050	4681	0	0	0.7	1.1	0.2

Composition of BGEs is given in Table 2.

the range 0.5–1.6 × 10^5 m^{-1}, CZE separation of mixture I in this BGE is shown in Fig. 3B and separation of mixture III in this BGE is presented in Fig. 3E. The resolution of separations in BGE IV and BGE II (100 mM H_3PO_4, 50 mM Tris, pH 2.25) was comparable as can be seen from CZE separations of mixture I in Fig. 3B and A. [Des-Gly-NH_2]sGnRH and sGnRH in mixture III comigrated in a single peak in BGE II (see Fig. 3D), but in BGE IV and BGE V the partial resolution of these two peptides was achieved, as shown in Fig. 3E and F. As follows from the above results it is obvious that in some cases better separation can be achieved with isoelectric buffer, iminodiacetic acid, than with classical phosphate buffer or acetic acid-based BGEs.

From CZE separations of GnRHs their effective electrophoretic mobilities were determined. The effective electrophoretic mobility, m_{ep}, of peptide in BGE was calculated from migration time of the peptide, t_{mig}, and migration time of neutral electroosmotic marker, t_{eo}, according to equation:

$$m_{ep} = \frac{l_t l_{ef}}{U}\left(\frac{1}{t_{mig}} - \frac{1}{t_{eo}}\right) \quad (5)$$

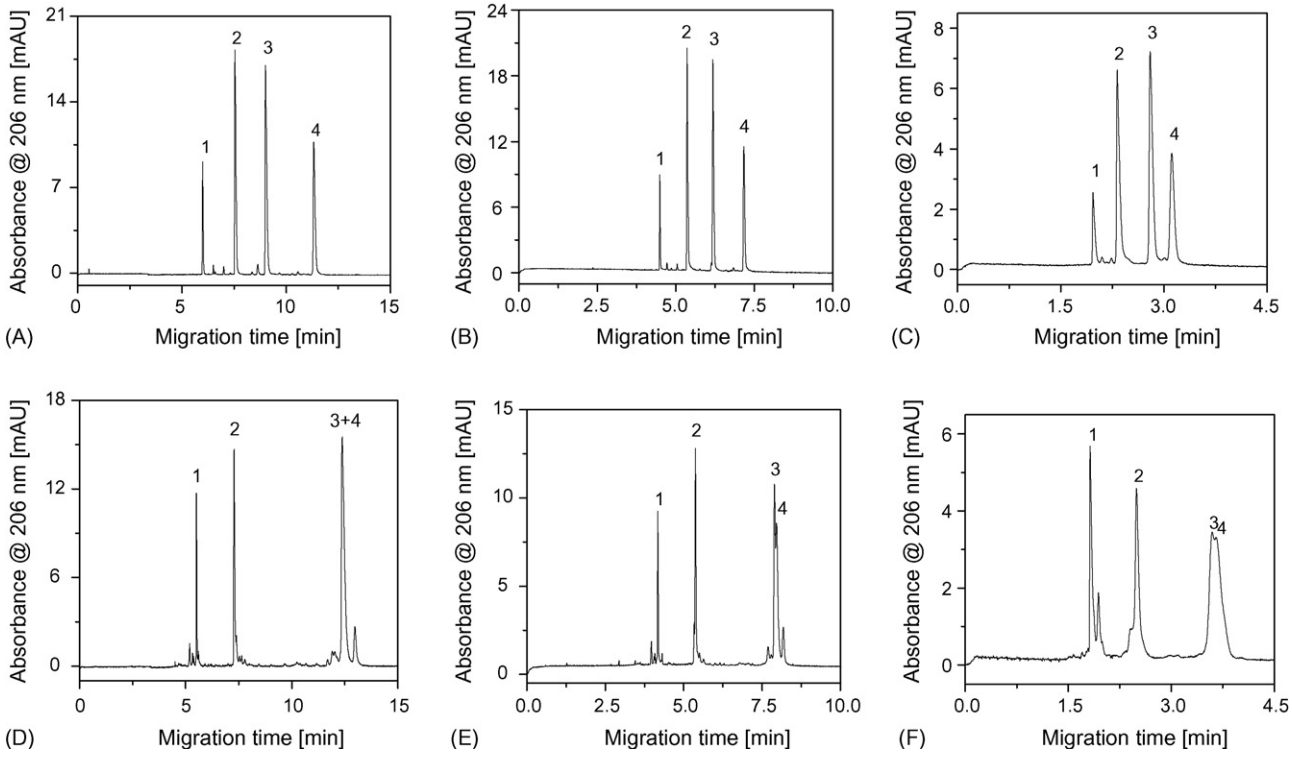

Fig. 3. CZE separations of structurally related GnRHs and their analogs and fragments. Mixture I in (A) BGE II, (B) BGE IV, (C) BGE V, and mixture III in (D) BGE II, (E) BGE IV, (F) BGE V. For composition of BGEs see Table 2. Mixture I, (1) fragment of hGnRH 4-10, (2) hGnRH, (3) fragment of hGnRH 1-4, (4) [Des-Arg-Pro-Gly-NH_2]hGnRH; mixture III, (1) [Des-pGlu^1-D-Orn^6]sGnRH, (2) [Des-Pro-Gly-NH_2]hGnRH, (3) sGnRH, (4) [Des-Gly-NH_2]sGnRH.

where l_t is total capillary length, l_{ef} the effective capillary length and U is the applied separation voltage. In CZE analyses in highly acidic BGEs with very low electroosmotic flow (EOF) due to the suppressed dissociation of silanol groups of FS capillary the effective electrophoretic mobility was determined using the pressure accelerated measurement of EOF according to Ref. [38]. The effective electrophoretic mobilities were determined as averages of two subsequent measurements, the values of which differed less than 1%.

The real average temperature inside the capillary was higher than the temperature of the capillary coolant due to Joule heating. The real temperature in the capillary for each BGE was obtained from the experimentally determined dependence of temperature increase inside the FS capillary on the input power per unit length of the capillary as described in Ref. [39]. The average temperature increase inside the capillary was 3.6 °C for BGEs I and V, 4.8 °C for BGE II, 4.5 °C for BGE III and 3.9 °C for BGE IV. The values of effective electrophoretic mobilities of GnRHs in different BGEs corrected to standard temperature, 25 °C, are presented in Table 3. The effective electrophoretic mobilities determined at real temperature inside the capillary differed about 8–11% in all BGEs in comparison with standard effective electrophoretic mobilities, at 25 °C. Relatively large differences of effective mobilities of GnRHs in different BGEs in spite narrow pH range (0.07–0.32 pH unit) are caused by different ionic strength of individual BGEs. For that reason the effective mobilities of GnRHs are minimal in BGE II, the ionic strength of which is maximal, and the mobilities are maximal in BGE V, the ionic strength of which is minimal. In addition, the different capabilities of the BGE counterions, acetate in BGEs I and V, phosphate in BGE II and iminodiacetate in BGEs III and IV, to form even very weak ionic pairs with cationic peptides may also contribute to the differences of effective mobilities of peptides in BGEs of similar pH and ionic strength.

4.4. Semiempirical models and estimation of probable peptide structure

In the current work we have tested and compared four semiempirical models of the correlation between effective electrophoretic mobility of GnRHs and their charge and size–rigid spherical model, Eq. (2) [25], classical linear polymer model, Eq. (3) [26], Offord's model, Eq. (1) [20], and Cross's model, Eq. (4) [27], which can be utilized for prediction of secondary or tertiary structure of peptides and proteins in solution [25]. The graphs of the correlations describing the above semiempirical models applied to 10 GnRHs are depicted in Fig. 4. Cross's plot seems to be the most suitable for predicting the peptide structure, because it directly provides the value of exponent k (related to the shape of the molecule in the m_{ep} versus q/M_r^k relation) as the slope of the line of the logarithmic plot expressed by Eq. (4). Parameters of the linear regression (slope of the line—value of exponent k, intercept, b, and coefficient of correlation, R_A) of the Cross's plot, $\log(m_{ep}/q)$ versus $\log M_r$, and coefficients of correlation of other three models for the GnRHs are presented in Table 5. None of the models was found to be

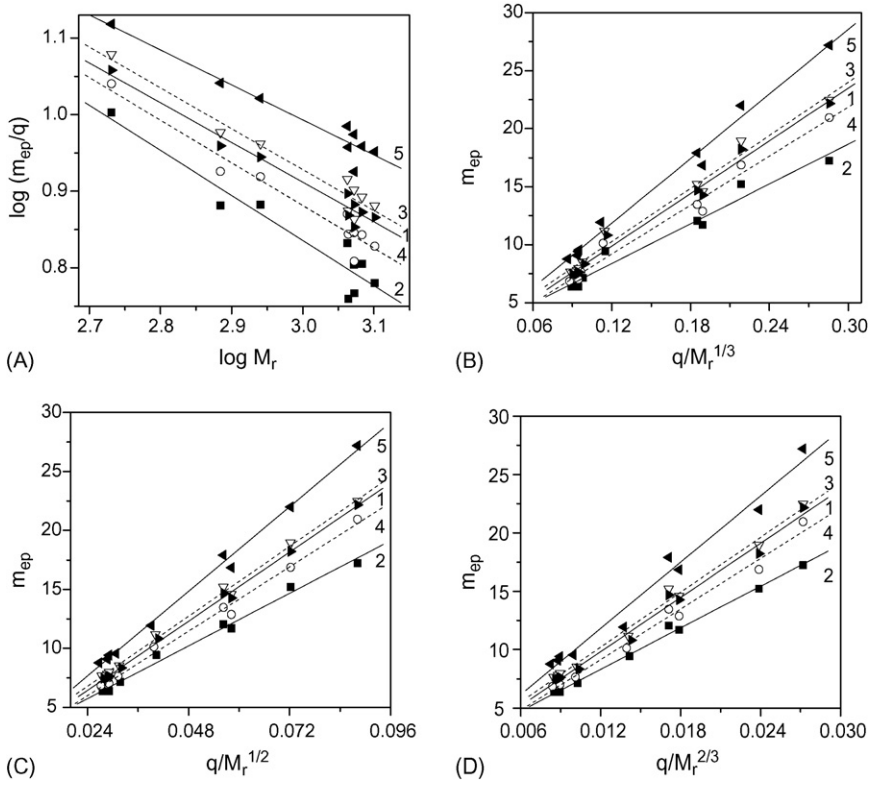

Fig. 4. Semiempirical models of the correlation between effective mobility, m_{ep}, and peptide charge, q, and size (expressed by relative molecular mass, M_r) applied to 10 peptides in five BGEs, (1) BGE I, (2) BGE II, (3) BGE III, (4) BGE IV, (5) BGE V, for BGEs composition see Table 2. (A) Cross's model, Eq. (4), (B) rigid spherical model, Eq. (2), (C) classical linear polymer model, Eq. (3), (D) Offord's model, Eq. (1).

Table 5
Parameters of linear regression of Cross's (k_A, b_A, R_A), rigid spherical (R_B), classical linear polymer (R_C) and Offord's (R_D) models for analyzed GnRHs in five BGEs

BGE no.	k_A	b_A	R_A	R_B	R_C	R_D
I	−0.519	2.468	0.9827	0.9926	0.9989	0.9943
II	−0.589	2.604	0.9520	0.9826	0.9952	0.9977
III	−0.534	2.531	0.9725	0.9902	0.9981	0.9956
IV	−0.557	2.552	0.9723	0.9893	0.9967	0.9940
V	−0.457	2.468	0.9654	0.9945	0.9975	0.9901

For BGEs composition see Table 2. k: Slope of line, b: intercept, R: coefficient of correlation.

quite definitively applicable for the whole set of 10 GnRHs differing in size (tetrapeptide–decapeptide) and positive charge (0.91–3.00 elementary charges) but, as follows from the data presented in Table 5 and from the graphs in Fig. 4, the best fitting of experimental data and the highest coefficient of correlation, $R = 0.995$–0.999, was obtained for k close to the value of 1/2 in all five acidic BGEs. This indicates that the electrophoretic migration of the set of GnRHs can be best described by classical linear polymer model (Fig. 4C) and the most probable structure of GnRHs in these BGEs can be predicted as a random coil. This is in agreement with the model predicted for the set of similar peptides containing triptorelin and buserelin in acidic solution at pH 2.85 [40] and for the set of insect oostatic peptides (tetrapeptide to decapeptide) in acidic BGEs at pH 2.30 and 2.40, respectively, [28]. This conclusion is supported also by the fact that oligopeptides of this size are too large to behave as solid spheres and too short to form ordered structures and usually they are present in a solution in the form of a random coil [29]. However, since relatively high coefficients of correlation were obtained also for other models, see Table 5, one has to have in mind that the structure derived from these measurements can be considered as approximated only.

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