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Analysis and separation of enkephalin and dalargin analogues and fragments by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) has been applied to qualitative and quantitative analysis and separation of synthetic analogues and fragments of enkephalins ([Leu^5]enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH, [Met^5]enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH), and dalargin (H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH), biologically active peptides with morphin-like effects acting as ligands for the opiate receptors in the brain. These oligopeptides (dipeptides to hexapeptides) were analyzed as cations in two acidic background electrolytes (BGEs), BGE I (100 mM H_3PO_4, 50 mM Tris, pH 2.25), BGE II (100 mM iminodiacetic acid, pH 2.30), and both as cations and anions in alkaline BGE IV (40 mM Tris, 40 mM Tricine, pH 8.10). Purity degrees of peptides, expressed in three different ways (relative peak height, relative peak area and relative corrected peak area), were determined by their CZE analyses in the above BGEs, and their values were compared with respect to the peak shapes and migration times of the main synthetic products and their admixtures. Selected analogues and fragments of enkephalins and dalargin were successfully separated by CZE in acidic isoelectric buffers, 100 and 200 mM iminodiacetic acid, pH 2.30 and 2.32, respectively. The effective electrophoretic mobilities at standard temperature 25 °C, and effective and specific charges of all analyzed peptides in the above three BGEs were determined. Correlation between effective electrophoretic mobility of the analyzed peptides and their charge and size (relative molecular mass) was investigated, which revealed different molecular shape of analyzed peptides in acidic and alkaline BGEs. In addition, the selected characteristics of the UV-absorption detector (noise, signal to noise ratio, sensitivity, and limits of detection and quantification) were determined.

Keywords: Capillary zone electrophoresis; Peptides; Enkephalins; Dalargin

1. Introduction

Capillary zone electrophoresis (CZE), one of the high-performance capillary electromigration methods, providing fast (few minutes) and high-efficient separation (10^5–10^6 theoretical plates per meter) of picomole to attomole amounts of analytes in the nanolitre sample volume, possesses a high application potential in the field of separations of peptides: it is broadly utilized for analysis, preparation and physicochemical and biochemical characterization of peptides both in the research and in practical applications in chemistry, biochemistry, biomedicine, biotechnology, pharmaceutical industry, food and feed industry and fishing farming, as documented in several recent reviews [1–7].

Enkephalins (ENKs), ([Leu^5]enkephalin [Leu^5]ENK), pentapeptide H-Tyr-Gly-Gly-Phe-Leu-OH, and [Met^5]enkephalin ([Met^5]ENK), pentapeptide H-Tyr-Gly-Gly-Phe-Met-OH, and dalargin (DLR), hexapeptide H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH, are biologically active peptides with morphin-like effects acting as ligands for the opiate receptors in the brain [8]. Enkephalins are fragments of nature opioid hormones, dynorphin A (17 amino acid residues) and dynorphin B (13 amino acid residues).

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Enkephalins and dynorphins originate from the two large precursor proteins, preproenkephalin and preprodynorphin [8]. They play significant roles in mediating stress and abatement of pain and are involved in temperature control, feeding behavior and respiration. These peptides and their analogues are used for the treatment of some mental illnesses (e.g. chronic schizophrenia, senile dementia of Alzheimer type).

CZE has been frequently used for analysis of enkephalins and dalargins, most often to check the purity of synthetic preparations of these peptides but also for their determination in biological fluids (serum, plasma, cerebrospinal fluids). Rational approach to selection and optimization of important experimental parameters (composition and pH of the background electrolyte (BGE), loading limit, capillary diameter and fraction collection) in analytical and preparative CZE of opioid peptides including enkephalin and dynorphin analogues with off-line mass spectrometric (MS) detection was reviewed by Lee and Desiderio [9]. [Leu^5]ENK, [Met^5]ENK and [desTyr^1-Leu^5]ENK were analyzed with high sensitivity (with limits of detection as low as 3–11 attomole) by CZE in citrate and phosphate BGEs using electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) [10]. Analogues of enkephalins and dalargin and other biopeptides, such as e.g. vasopressin, desmopressin, insulin-like growth factors, have been separated by CZE in strongly acidic BGE, 150 mM phosphoric acid, pH 1.8 [11]. These analogues were also analyzed and separated by capillary micellar electrokinetic chromatography (CMEKC) with micellar pseudophase formed by anionic detergent sodium dodecylsulfate (SDS) in alkaline BGEs (20 mM tetraborate, pH 9.2, and 20 mM phosphate, pH 8.8) or by cationic detergent cetyltrimethylammonium bromide (CTAB) in acidic BGE (50 mM phosphate, pH 4.1) [11–13]. Analogue of dalargin, [D-Tle^{2,5}]dalargin, was analyzed by CZE in 0.5 M acetic acid, pH 2.5, and then purified in preparative scale by free-flow zone electrophoresis [14]. Six analogues and fragments of enkephalins were separated and determined in biological matrices by multidimensional separation system consisting of on-line coupling of size-exclusion chromatography (SEC), reverse-phase C18 trapping column and CZE [15,16] and by liquid secondary ion mass spectrometry and tandem mass spectrometry (LSI-MS–MS–MS) [17]. Dynorphin peptide analogues were separated by CMEKC employing anionic (SDS), cationic (CTAB) and zwitterionic (CHAPS) surfactants [18]. Enkephalin-related peptides were derivatized by fluorescein isothiocyanate and then separated by CMEKC in borate BGE with SDS micelles and detected with laser-induced fluorescence detector [19]. [D-Pen^{2,5}]ENK (D-Pen is D-penicillamine or D-3-mercaptovaline) and [D-Ser^2,Thr^6]DLR were analyzed in rat serum by CZE in phosphate (pH 2.4) and borate (pH 8.3) buffers [20,21]. ENKs and their fragments were electrochemically detected as in-capillary formed copper complexes after their CZE separation [22]. ENK analogues were separated in tris-phosphate and sodium phosphate BGEs, pH 2.5, in capillary noncovalently coated with two layers of oppositely charged polymers [23]. [Leu^5]ENK, [Met^5]ENK and other peptide hormones were separated in three BGEs, pH 2.61, 2.85 and 10.0, and their pK_a were determined from the pH dependence of their electrophoretic mobilities in the broad pH range, 2–12, [24], and subsequently analyzed with CE-ESI-MS in 50 mM acetic acid and 50 mM formic acid, pH 2.85 [25].

The aim of this work was to perform qualitative and quantitative analysis of synthetic preparations of opioid peptide hormones, enkephalins and dalargin, and their analogs and fragments, by CZE both in acidic and alkaline BGEs. Suitable experimental conditions should be found 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, effective and specific charges should be determined, and the correlation between effective electrophoretic mobility of analyzed peptides and their charge and size (relative molecular mass) should be investigated.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. Iminodiacetic acid (IDAA) was obtained from Bachem (Bubendorf, Switzerland), Tris (tris(hydroxymethyl)aminomethane) was supplied by Serva (Heidelberg, Germany), phosphoric acid and dimethyl sulfoxide (DMSO) were obtained from Lachema (Brno, Czech Republic) and Tricine ([tris(hydroxymethyl)-methyl]-glycine) was from Merck (Darmstadt, Germany). Isophorone (3,5,5-trimethyl-2-cyclohexen-1-one) was supplied by Fluka (Buchs, Switzerland).

2.2. 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 ENKs, Tyr-Gly (YG) and Tyr-Gly-Gly (YGG), were obtained from Bachem (Bubendorf, Switzerland), the di- and tripeptide fragments of DLR, Tyr-Ala (YA) and Tyr-D-Ala-Gly (YaG), were purchased from Sigma (St. Louis, MO, USA). The analogs and derivatives of ENKs and DLR were prepared by solid phase synthesis [26]. The structure of DLR was altered by the substitution of leucine in position 5 by bulky amino acid such as D-tertiary leucine [27], by glycosylation [28] or iodination of tyrosine in position 1, by derivatization of the C-terminal arginine carboxyl group and by combination of these modifications.

2.3. Instrumentation

The capillary electrophoretic experiments were carried out in commercial P/ACE MDQ System (Beckman-Coulter,

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

Peptide	Abbreviation	Sequence in three-letters code	M_r
[Leu^5]enkephalin	[Leu^5]ENK	H-Tyr-Gly-Gly-Phe-Leu-OH	555.8
[Met^5]enkephalin	[Met^5]ENK	H-Tyr-Gly-Gly-Phe-Met-OH	573.8
ENK dipeptide fragment	YG	H-Tyr-Gly-OH	238.3
ENK tripeptide fragment	YGG	H-Tyr-Gly-Gly-OH	295.4
Dalargin	DLR	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	726.1
DLR dipeptide fragment	YA	H-Tyr-Ala-OH	252.3
DLR tripeptide fragment	YaG	H-Tyr-D-Ala-Gly-OH	309.4
[D-Tle^5]dalargin	[D-Tle^5]DLR	H-Tyr-D-Ala-Gly-Phe-D-Tle-Arg-OH	726.1
Dalargin ethylamid	DLR-NHEt	H-Tyr-D-Ala-Gly-Phe-Leu-Arg-NH-Et	749.0
[Tyr(I_2)]dalargin	I_2DLR	H-(3,5-di-I)-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	960.8
[D-Ser^2,Thr^6]dalargin	[D-Ser^{2}Thr^6]DLR	H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH	689.9
N-Phenylacetyl-dalargin	PacDLR	C_6H_5-Ac-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH	859.1

Fullerton, CA, USA), data acquisition and evaluation were performed using the software P/ACE System MDQ, version 2.3 supplied by Beckman.

The apparatus was equipped with the internally non-coated fused silica capillary with outer polyimide coating, total/effective length 39.4/29.2 cm, I.D./O.D. 75/360 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 was set at 25 °C with liquid coolant continuously circulating around the capillary.

The new capillary was gradually flushed with water, 0.1 M NaOH, water and BGE, each wash for 5 min. Finally, the capillary was conditioned by a 20 min application of the high voltage to equilibrate the inner surface and to stabilize electroosmotic flow. Between runs under the same conditions, the capillary was rinsed with the BGE for 2 min. Prior to any change of the BGE the capillary was rinsed with 0.1 M NaOH for 5 min and then repeatedly stabilized. The separation voltage was 10 kV. The samples were injected with pressure 5–10 mbar for 5–15 s. The samples were dissolved in deionized water or in BGE and their concentrations were in the range 0.1–1.5 mg/ml. The BGEs were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA) before use.

3. Results and discussion

3.1. Selection of separation conditions

The strategy for the rational selection of experimental conditions for CZE analysis and separations of ENKs, DLR and their derivatives and fragments followed the general rules of selection of suitable CZE separation conditions [29] and took into account the specific properties of these peptides resulting from their structure—number and sequence of the linked amino acid residues, i.e. their amphoteric character and strong pH dependence of effective electrophoretic mobilities, which are governed by the effective electric charge, size (relative molecular mass) and shape (conformation) of these peptides.

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 preserving chemical and temperature stability and biological activity of analyzed peptides [30,31]. 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 different types of ionogenic groups—all peptides, except Pac-DLR, possess α-amino group of the N-terminus of the peptide chain (average pK_a 8.0), and all peptides, except DLR-NHEt, contain α-carboxyl group of the C-terminus of the peptide chain (average pK_a 2.6), dalargin analogues (except [D-Ser^{2}Thr^6]DLR) possess guanidium group of arginine (average pK_a 11.3) and all of them hydroxy group of tyrosine (average pK_a 10.4). The relative molecular mass of analyzed peptides was in the range 238.3–1065, 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 [32] using the above given average values of pK_a of ionogenic groups. 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 both as cations (preferably in acid pH range) and as anions (preferably in alkaline pH range). Consequently, the two strongly acidic BGEs (pH 2.25–2.30) and one weakly alkaline BGE (pH 8.1) were selected for CZE analyses and separations of the above peptides. Full composition of the used BGEs together with the separation voltage and electric currents are presented in Table 2. Separation conditions are significantly influenced by solubility of peptides. With respect to the relatively hydrophilic character of the analyzed

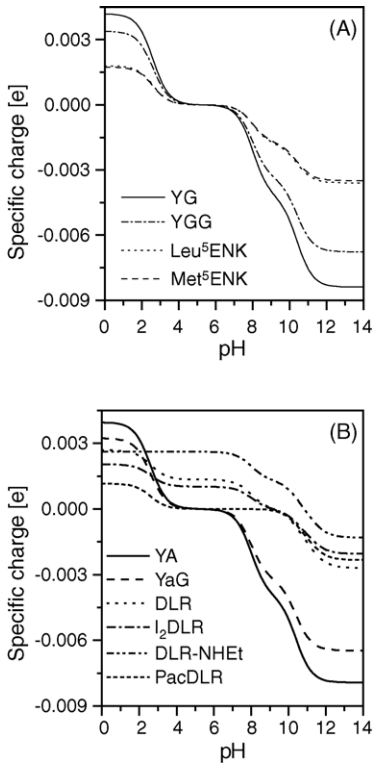

Fig. 1. The pH dependence of specific charge (effective charge divided by relative molecular mass) of selected peptides. (A) Enkephalin analogues and fragments and (B) dalargin analogues and fragments.

peptides most of them could be dissolved in deionized water, which brings an advantage that the same sample solution can be applied to CZE analyses in different BGEs, and in addition the electric-field-enhanced concentrating effect is utilized to concentrate the diluted peptide solutions. On the other hand using BGE as sample solvent ensures the constant separation conditions (movement in the BGE of the same composition) in the whole CZE experiment, which is important when CZE is applied for the physicochemical measurements.

3.2. Qualitative and quantitative analysis

The selected BGEs were used for qualitative and quantitative analysis of synthetic preparations of ENKs, DLR and their new analogues and fragments. For full characterization of these peptides before their application in the biological tests it is necessary to find out the content of their all admixtures, originating from the synthesis of peptides and/or from their subsequent purification procedures. Consequently, the purity degrees of these peptides, both crude synthetic products and peptides purified by chromatographic methods, size-exclusion chromatography and reverse phase high-performance liquid chromatography (RP-HPLC), were determined. Peptides were analyzed as cations in acidic BGEs and as anions or cations in weakly alkaline BGE. The examples of CZE analyses of some peptides are shown in Fig. 2. Standards of synthesized peptides were not available, therefore only relative degrees of purity could be determined. Peptide purity and peptide content in the sample was quantified by three ways: (i) relative peak height, $P_h(i)$ (1), (ii) relative peak area, $P_A(i)$ (2), and (iii) relative corrected peak area, $P_{CA}(i)$ (3), of the UV-positive peaks for the ith component of the peptide preparation:

$$P_h(i) = \frac{h(i)}{\sum h(i)}, \quad i = 1, \ldots, n \tag{1}$$

$$P_A(i) = \frac{A(i)}{\sum A(i)}, \quad i = 1, \ldots, n \tag{2}$$

$$P_{CA}(i) = \frac{A_c(i)}{\sum A_c(i)}, \quad i = 1, \ldots, n \tag{3}$$

where $h(i)$ is height of the ith peak, $A(i)$ is area of the ith peak and $A_c(i)$ is corrected area of the ith peak, corrected peak area is peak area corrected with respect to migration velocity of the given peak, which is obtained as peak area divided by the migration time of the given peak, n is the number of sample components.

The values of differently expressed purity degrees of analyzed peptides are presented in Table 3. The values of purity degrees were determined as averages of values obtained in two subsequent analyses, which differed less than 1%. The synthetic peptides exhibited rather different degree of purity. Analyses of peptides with high purity degree, [Leu^5]ENK and DLR-NHEt, are demonstrated in Fig. 2A and F, respectively. On the other hand analysis of peptide with low purity degree, with one major and several minor impurities, is shown in electrophoregram of [D-Tle^5]DLR in Fig. 2D. Analysis of peptide with some minor impurities is demonstrated in electrophoregram of I_2DLR in Fig. 2C. As follows from the above definitions, the most exact characteristic of peptide purity is the relative corrected peak area. Sometimes other, the simpler ways of purity evaluation, relative peak height or relative peak area, can be used, particularly in the cases when the peaks of sample components are uniformly dispersed and their migration times (velocities) are not too different. The great difference in differently expressed purity degree was found, e.g. in the CZE analysis of DLR presented in Fig. 2B. Relative peak height of DLR was about 10–15% lower than its relative peak area and relative peak corrected area in acidic BGEs I and II due to the relatively high height and small area of the sharp peak of the main admixture X. On the other hand relative peak height of tripeptide fragment of ENK, YGG, was about 6–7% higher than relative peak area

Table 2
Composition and pH of the BGEs applied for CZE analyses and separations of ENKs and DLR analogues and fragments, and electric current, I, in these BGEs at constant voltage 10 kV

BGE no.	BGE constituents	pH	I (μA)
I	50 mM Tris, 100 mM H_3PO_4	2.25	58–65
II	100 mM iminodiacetic acid	2.30	36.7–37
III	200 mM iminodiacetic acid	2.32	52.3–52.8
IV	40 mM Tris, 40 mM Tricine	8.10	13.2–14.2

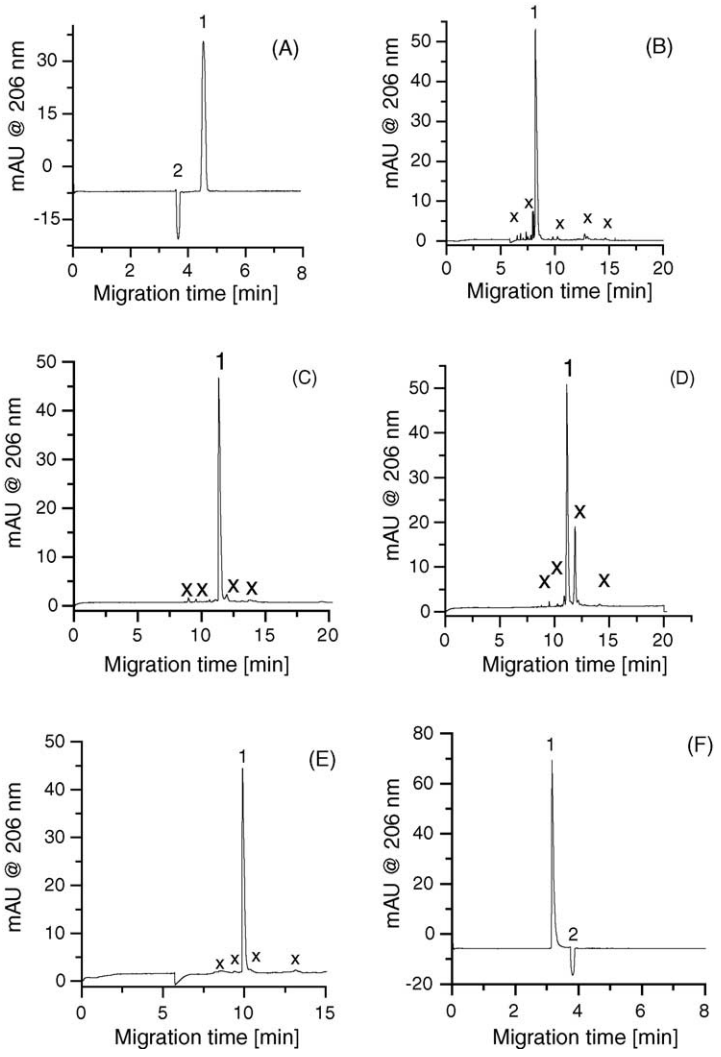

Fig. 2. CZE analyses of selected synthetic peptides. Experiments were carried out in uncoated fused-silica capillary: total/effective length 394/292 mm, I.D. 75 μm, O.D. 360 μm, constant voltage $U = 10$ kV, UV-absorption detection at 206 nm. (A) [Leu^5]ENK, 1.25 mg ml^{-1}, in BGE IV; (B) DLR, 1.25 mg ml^{-1}, in BGE II; (C) I_2DLR, 1.00 mg ml^{-1}, in BGE II; (D) [D-Tle^5]DLR, 1.00 mg ml^{-1}, in BGE I; (E) YGG, 1.10 mg ml^{-1}, in BGE II; (F) DLR-NHEt, 1.15 mg ml^{-1}, in BGE IV. (1) Main synthetic product; (2) neutral electroosmotic flow marker; and (X) non-identified impurities.

Table 3
Purity degrees of analyzed peptides, expressed alternatively as relative peak height, P_h, relative peak area, P_A, and relative peak corrected area, P_{CA}, determined by CZE in three BGEs

Peptide	BGE I, pH 2.25			BGE II, pH 2.3			BGE IV, pH 8.1		
	P_h (%)	P_A (%)	P_{CA} (%)	P_h (%)	P_A (%)	P_{CA} (%)	P_h (%)	P_A (%)	P_{CA} (%)
[Leu^5]ENK	94.5	92.7	92.7	93.3	89.4	89.4	95.8	93.1	93.0
[Met^5]ENK	94.0	92.6	92.6	91.3	95.7	95.6	97.5	97.8	97.8
YG	98.3	98.8	98.9	97.4	98.5	98.7	98.5	99.0	99.0
YGG	92.5	86.0	85.7	95.8	89.6	89.6	96.2	91.0	91.8
DLR	75.4	84.6	84.6	65.8	80.7	81.9	91.5	89.2	90.3
YA	99.2	99.2	99.3	99.7	99.8	99.9	99.3	99.4	98.2
YaG	92.7	93.1	93.4	96.3	96.3	94.4	98.5	98.7	98.7
[D-Tle^5]DLR	66.6	69.8	70.9	50.8	58.8	58.8	91.3	92.1	92.1
DLR-NHEt	94.5	96.8	96.9	95.0	94.2	94.2	94.7	94.3	94.2
I_2DLR	91.5	90.5	90.6	87.5	82.8	82.8	90.2	89.9	90.5
[D-Ser^{2}Thr^6]DLR	87.7	88.3	87.3	92.5	94.2	94.2	97.9	98.7	98.8
PacDLR	81.0	78.1	81.8	94.9	95.0	89.7	n.d.	n.d.	n.d.

n.d.: not determined.

or relative corrected peak area in acidic BGEs I and II due to the relatively small height and broad width of the peaks of its admixtures (see Fig. 2E). Similar values of purity degrees expressed by relative peak height, relative peak area and corrected peak area were obtained in analysis of highly purified peptides, as e.g. in analysis of DLR-NHEt both in acidic and alkaline BGEs, see Table 3 and Fig. 2F. As follows from the presented data, the purity degree of given peptide depends on BGE used and on the way of purity degree evaluation. Consequently, purity degree of peptides should be tested in more than one BGE and only the purity degrees obtained under the same conditions and evaluated in the same way can be compared.

As can be seen from the analyses presented in Fig. 1A–F, in all BGEs used symmetrical peaks were obtained with high separation efficiency in short times, with migration times of the main component between 3 and 12 min. The best results were obtained in BGE II (100 mM iminodiacetic acid, pH 2.30) using the separation voltage 10 kV. The separation efficiency in BGE II was in range $(0.5–1.0) \times 10^6$ theoretical plates per meter, concentration limit of detection (LOD = three times noise) was $(0.60–2.26) \times 10^{-6}$ mol dm^{-3}, concentration limit of quantification (LOQ = 10 times noise) was $(2.01–7.54) \times 10^{-6}$ mol dm^{-3}, sensitivity of the UV-absorption detector was 46.5–114.4 AU mol^{-1} dm^3 and the noise of UV-detector signal was 0.023 mAU. These parameters for BGE I were almost the same as for BGE II, however the time of analysis was longer because of the very low electroosmotic flow (EOF) in this BGE. BGE IV was found less suitable for analysis of these series of peptides due to the basic character of some of the analyzed peptides, DLR and its derivatives, which are positively charged even at pH 8.1 and due to the fast EOF at this pH, which means that the electrophoretically migrated trajectory of these peptides is relatively short and they could not be well separated from their admixtures and other structurally related peptides. The characteristics of the UV-absorption detector for all BGEs are presented in Table 4.

3.3. Separation of structurally related peptides

In addition to qualitative and quantitative analysis of individual synthetic peptide preparations, the separation of closely related structures of ENKs and their fragments, and DLR and its analogues and fragments was tested. These separations are important from the point of view of separations of the whole molecules of ENKs and DLR from their degradation products. The suitable 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 BGEs I, II, and IV. As it was already mentioned above from the BGEs tested the BGE II, composed of isoelectric buffer, 100 mM iminodiacetic acid (IDAA), provided the best results. To achieve the best

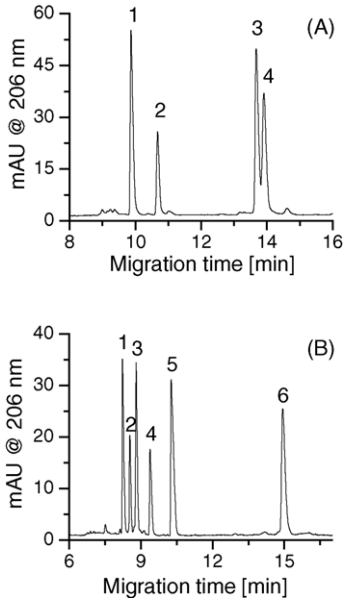

Fig. 3. CZE separations of structurally related peptides. Experiments were carried out in uncoated fused-silica capillary: total/effective length 394/292 mm, I.D. 75 μm, O.D. 360 μm, constant voltage U = 10 kV, UV-absorption detection at 206 nm. (A) Enkephalin analogues and fragments in BGE III: 1, YG; 2, YGG; 3, [Leu^5]ENK; 4, [Met^5]ENK. (B) Dalargin fragments and analogues in BGE II: 1, DLR-NHEt; 2, DLR; 3, I_2DLR; 4, YA; 5, YaG; and 6, PacDLR.

resolution different concentrations of IDAA and separation voltages were tested. Finally, 100 mM iminodiacetic acid, pH 2.30 and voltage 10 kV were selected as the best conditions for separation of DLR and its analogues and fragments. The doubled concentration of IDAA, i.e. 200 mM, pH 2.32, was found to be most suitable for separation of ENKs and their fragments, providing the best resolution at the same separation voltage, 10 kV. Separations of these two mixtures are shown in Fig. 3, mixture of [Leu^5]ENK, [Met^5]ENK and their fragments (YG, YGG) in Fig. 3A, and the mixture of DLR and its derivatives (DLR-NHEt, I_2DLR, PacDLR) and fragments (YA, YaG) in Fig. 3B. The resolution of any pair of peptides was higher than 2.1 with the exception of [Leu^5]ENK and [Met^5]ENK, which were separated with resolution 1.11. In the mixture of ENKs and their fragments the latter migrated faster, with migration times in the order YG < YGG < [Leu^5]ENK < [Met^5]ENK. In the mixture of DLR and their analogues and fragments the longer peptides with higher values of effective and specific charges migrated faster than the short fragments with lower specific charges.

3.4. Determination of effective mobility

From CZE analyses of ENKs, DLR and their analogues and fragments the effective electrophoretic mobilities of these peptides were determined. The effective electrophoretic mobility, m_{eff}, of peptide in the given BGE was calculated from the migration time of the peptide, t_{mig}, in this BGE, and from the migration time of the electroneutral marker of

Table 4
The characteristics of the UV-absorption photodiode array detector set at 206 nm

Peptide	BGE	c (mmol dm^{-3})	$R/n \times 10^{-3}$	S (AU mol^{-1} dm^3)	LOD (μmol dm^{-3})	LOQ (μmol dm^{-3})
[Leu^5]ENK	I	2.08	4.02	42.6	1.55	5.17
	II	2.01	4.06	46.5	1.48	4.95
	IV	2.16	1.04	32.9	6.20	20.7
[Met^5]ENK	I	1.61	5.46	74.7	0.88	2.95
	II	1.49	7.41	114.4	0.60	2.01
	IV	1.70	1.49	59.7	3.42	11.4
YG	I	4.56	4.75	22.9	2.88	9.60
	II	5.38	7.14	30.5	2.26	7.54
	IV	5.61	1.20	14.5	14.1	46.9
YGG	I	3.19	2.55	17.6	3.75	12.5
	II	3.34	1.85	12.8	5.41	18.0
	IV	3.58	0.44	8.35	24.4	81.4
DLR	I	1.46	4.50	67.8	0.97	3.24
	II	1.41	5.74	93.6	0.74	2.46
	IV	1.55	1.07	46.7	4.37	14.6
YA	I	4.32	2.63	13.4	4.93	14.4
	II	4.35	1.73	9.13	7.56	25.2
	IV	4.33	0.49	7.67	26.6	88.7
YaG	I	3.46	5.23	33.2	1.99	6.62
	II	3.58	4.02	25.8	2.70	8.90
	IV	3.66	1.54	28.7	7.12	23.7
[D-Tle^5]DLR	I	0.98	3.76	84.4	0.78	2.61
	II	0.81	3.00	85.3	0.81	2.70
	IV	1.11	0.83	50.9	4.01	13.4
DLR-NHEt	I	1.48	7.57	112.6	0.59	1.95
	II	1.45	5.21	82.7	0.83	2.78
	IV	1.45	2.25	105.3	1.94	6.46
I_2DLR	I	0.94	4.16	97.4	0.68	2.26
	II	1.16	4.52	89.6	0.77	2.57
	IV	0.94	1.16	83.6	2.44	8.14
[D-Ser^{2}Thr^6]DLR	I	1.33	2.74	45.4	1.45	4.85
	II	1.44	2.96	47.3	1.46	4.86
	IV	1.51	0.86	38.6	5.29	17.6
PacDLR	I	0.91	2.56	62.0	1.07	3.55
	II	1.38	4.02	67.0	1.03	3.43
	IV	n.d.	n.d.	n.d.	n.d.	n.d.

R: detector response, n: noise, c: concentration, S: sensitivity of detector $S = R/c$, LOD: limit of detection LOD = $3n/S$, LOQ: limit of quantification LOQ = $10n/S$, n.d.: not determined. BGE I: 100 mM H_3PO_4, 50 mM Tris, pH 2.25, $n = 0.022$ mAU; BGE II: 100 mM iminodiacetic acid, pH 2.30, $n = 0.023$ mAU; BGE IV: 40 mM Tris, 40 mM Tricine, pH 8.1, $n = 0.068$ mAU.

EOF (DMSO or isophorone), t_{eo}, according to the following relation:

$$m_{eff} = \frac{l_t l_{ef}}{U}\left(\frac{1}{t_{mig}} - \frac{1}{t_{eo}}\right) \quad (4)$$

where l_t is total capillary length, l_{ef} is effective capillary length, U is the separation voltage.

In CZE analyses in highly acidic BGEs with very low EOF due to the suppressed dissociation of silanol groups the effective electrophoretic mobility was determined using the pressure accelerated measurement of EOF as described in [33]. Briefly the sample was injected along with zone A of the neutral EOF marker into the capillary filled with BGE. Then the separation voltage was applied, the analytes were separated and the neutral marker zone A migrated with the velocity of EOF. Analysis was stopped (separation voltage switched-off) at a specific time, t_u, just after the peptide zone passed the detector. Then the second zone (B) of the (same or different) EOF marker was injected and the injection pressure (15 mbar) was applied onto the pure BGE vessel. Both zones of the EOF marker, A and B, reached the detector at times, t_A and t_B, respectively, which were recorded in the usual manner. Then the electrophoretic mobility can be calculated from the following relation:

$$m_{eff} = \frac{l_t l_{ef}}{U}\left(\frac{1}{t_{mig}} - \frac{1}{t_u} + \frac{t_A}{t_u t_B}\right) \quad (5)$$

where the symbols have the same meanings as in Eq. (4). The values of effective electrophoretic mobility were determined as averages of two subsequent measurements, which differed less than 1%.

The real temperature inside the capillary was higher than the temperature of the coolant due to Joule heating. The real temperature in the capillary for each BGE was obtained from the dependence of temperature increase in fused silica capillary on the input power per unit length of the capillary, which was determined from the measurement of specific electric conductivity of standard solution of 0.01 mol dm^{-3} KCl in the capillary at different values of input power as described in [34]. The temperature increase in fused silica capillary was 4.5 °C for BGE I, 2.0 °C for BGE II, 3.2 °C for BGE III and 0.7 °C for BGE IV. The effective electrophoretic mobility corrected to standard temperature (25 °C), $m_{\text{eff},25}$, was calculated according to the equation:

$$m_{\text{eff},25} = m_{\text{eff},t}[1 - 0.022(t - 25)] \quad (6)$$

where $m_{\text{eff},25}$ is effective electrophoretic mobility at 25 °C, $m_{\text{eff},t}$, is effective electrophoretic mobility measured at real temperature, t, inside the capillary.

The values of effective electrophoretic mobilities of peptides in different BGEs corrected to standard temperature, 25 °C, are presented in Table 5. The effective electrophoretic mobilities measured at real temperature inside the capillary differed up to 10% in the most conductive BGE I in comparison with standard effective electrophoretic mobilities at 25 °C.

Generally the effective mobilities of analyzed peptides were in agreement with the values predicted from the course of the dependence of the specific charge on pH, i.e. peptides with higher specific charge exhibited higher effective mobilities than those with lower specific charge. Peptides containing guanidium group of arginine, particularly DLR and its analogues with the exception of [D-Ser^{2}Thr^6]DLR and PacDLR, migrated faster than the enkephalins, and due to their strongly basic character they migrated as cations even in the weak alkaline BGE IV, pH 8.10. Enkephalin dipeptide and tripeptide fragments (YG, YGG) migrated faster than enkephalins, all these peptides had almost the same charge (see Table 5). DLR analogs, [D-Ser^{2}Thr^6]DLR and PacDLR, lacking positive charged group of arginine behaved similarly as ENKs, however their mobilities were lower than those of ENKs due to their larger molecules.

3.5. Relationship between mobility of peptides and their charge and size

Several models correlating effective electrophoretic mobilities of peptides, m_{eff}, 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 [35–39]. The models are based on Stoke's law, describing the motion of a particle in liquid medium, and on the action of electric field force on charged molecule. The semiempirical models describe the electrophoretic behavior of various conformations of molecules, which can be utilized to predict secondary or tertiary structure of peptides and proteins in solution [40].

In the current work we have used Cross's model [41], due to its suitability for prediction of the most probable secondary structure of peptides:

$$\log\left(\frac{m_{\text{eff}}}{q}\right) = k \log M_r \quad (7)$$

where the constant of proportionality, k, is the exponent of M_r in the non-logarithmic expression of this relation, which can be determined as a slope of this dependence and which is related to the shape of the charged molecule. The graph of these dependences in three BGEs is shown in Fig. 4. Because not all of the analyzed peptides formed a homologous series, some points did not fit well in the linear part of this dependence. This concerns especially DLR, and its analogs [D-Tle^{2,5}]DLR and DLR-NHEt, analyzed at alkaline pH in BGE IV, where their effective mobilities were rather low with respect to their estimated effective charges (see Table 5). The best coefficient of the correlation of the dependence expressed by Eq. (7)

Table 5
Calculated effective charges, q, and CZE determined effective electrophoretic mobilities, m_{eff}, corrected to standard temperature, 25 °C, of analyzed peptides in three different BGEs

Peptide	q (e)			m_{eff} ($\times 10^{-9}$ m^2 V^{-1}s^{-1})		
	BGE I, pH 2.25	BGE II, pH 2.30	BGE IV, pH 8.1	BGE I, pH 2.25	BGE II, pH 2.30	BGE IV, pH 8.1
[Leu^5]ENK	0.72	0.62	−0.57	9.44	11.4	−9.99
[Met^5]ENK	0.71	0.61	−0.56	9.12	11.3	−10.0
YG	0.69	0.69	−0.57	14.3	17.6	−13.9
YGG	0.69	0.69	−0.56	12.8	15.3	−12.8
DLR	1.71	1.63	0.42	15.7	19.0	1.91
YA	0.69	0.69	−0.57	14.5	16.7	−13.3
YaG	0.69	0.69	−0.55	12.3	15.2	−12.7
[D-Tle^5]DLR	1.63	1.55	0.42	15.7	18.7	2.13
DLR-NHEt	2.00	2.00	1.42	15.9	20.0	10.3
I$_2$DLR	1.75	1.59	0.45	15.4	18.1	7.58
[D-Ser^{2}Thr^6]DLR	0.68	0.64	−0.56	7.83	9.78	−8.91
PacDLR	0.71	0.68	0.00	7.49	9.32	0.00

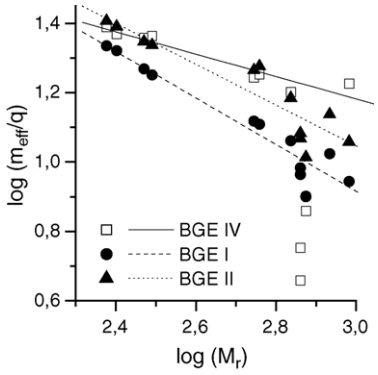

Fig. 4. Dependence of the decadic logarithm of the ratio of the effective electrophoretic mobility and effective charge on the decadic logarithm of relative molecular mass of analyzed enkephalin and dalargin analogues and fragments.

was obtained in BGE I, pH 2.25, $R = 0.9602$. When "outlier" points of DLR and its derivatives in BGE IV were omitted, the high coefficient of correlation was obtained also for BGE IV, reaching the value 0.9566. From the graph in Fig. 4 it is obvious, that the slopes of these dependences, k, are similar for two acidic BGEs, $k = -0.67386$ for BGE I, and $k = -0.5958$ for BGE II, whereas the slope of this dependence in alkaline BGE IV, is significantly different, $k = -0.3233$. The values of k in acidic BGEs are close to the value 2/3, which indicates that at this pH the peptides molecules have the shape of a wide thin disk, whereas the value of k in alkaline BGE is close to 1/3, which corresponds to the spherical shape of the molecule [40].

4. Conclusions

CZE proved to be powerful and useful tool for the fast and sensitive analyses and highly efficient separations of opioid peptide hormones, enkephalins, dalargins, and their analogues and fragments, in picomole to femtomole level in the nanolitre applied sample volume. The purity degree of analyzed peptides was best evaluated by relative corrected peak area. From the CZE analyses of peptides also their important physicochemical characteristics, particularly effective electrophoretic mobilities at both acidic and alkaline pH, could be determined. From the investigated correlation between effective electrophoretic mobility of analyzed peptides and their charge and size (relative molecular mass) the probable secondary structure of peptides could be predicted.

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